EVALUATION OF INTEGRATED CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT USING YEAST, POTASSIUM SILICATE AND HOT WATER TREATMENTS

By

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ABSTRACT

The public concern over synthetic pesticides in foods and the environment has created an interest to find effective and safe non-fungicide means of controlling postharvest pathogens. The overall objective of this thesis was to evaluate the effect of potassium silicate, yeast antagonists and hot water dip treatment to control postharvest grey mould and blue mould of pome fruits, caused by *Botrytis cinerea* and *Penicillium expansum*, respectively.

Botrytis cinerea and *Penicillium expansum* were isolated from infected strawberry and pear fruits, respectively. These isolates were found to be non-resistant to YieldPlus® (Anchor yeast, Cape Town, South Africa), a biofungicide containing a yeast *Cryptococcus albidus*. A total of 100 epiphytic yeast isolates were obtained from the fruit surface of “Golden Delicious” apples and “Packham’s Triumph” pears, and screened against *B. cinerea* and *P. expansum*. Fifteen yeast isolates reduced grey mould incidence by > 50%, when applied four hours before inoculation with *B. cinerea*. Similarly, seven yeast isolates reduced blue mould incidence by > 50%, when applied four hours before inoculation with *P. expansum*. YieldPlus® and yeast Isolate YP25 provided the best control of *B. cinerea*, while Isolate YP60 and YieldPlus® provided the best control of *P. expansum* on “Golden Delicious” apples. A mixture of YP25 and YP60 provided complete control of both *B. cinerea* and *P. expansum,* when applied to “Golden Delicious” apples before inoculation with either *B. cinerea* or *P. expansum*. Electron microscopy studies showed that yeast Isolates YP25 and YP60 inhibited the mycelial growth of *B. cinerea* and *P. expansum*, respectively.

Preventative and curative application of potassium silicate resulted in reduced incidence of *B. cinerea* or *P. expansum* of “Golden Delicious” apples. Electron microscopy studies indicated that potassium silicate inhibited the growth of *B. cinerea* and *P. expansum*. Furthermore, treatment of “Golden Delicious” apples with either potassium chloride or potassium hydroxide resulted in reduced incidence of both *B. cinerea* and *P. expansum*. *In vivo* tests showed that the disease incidence of *P. expansum* and *B. cinerea* on “Golden Delicious” apples was reduced by hot water dip treatments at 58-60°C for 60 to

120 seconds, compared with the control fruit treated with sterile distilled water, without causing skin damage. The use of potassium silicate, yeasts (Isolates YP25 and YP60), YieldPlus® and the antagonists mixture (YP25+YP60) in combination, resulted in the control of *B. cinerea* and *P. expansum* of “Golden Delicious” apples compared with Imazalil® treated fruit.

DECLARATION

I, NOKWAZI CAROL MBILI, declare that

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DEDICATION

To my mother Bonani, my sister Nokuphila and my brother, Celani for their love, support, understanding, and encouragement.

INTRODUCTION

Losses in fruit quality, such as loss in consumer acceptance or nutritional value, and quantity, usually happen in the period between harvest and consumption (Kader, 2005). The loss quantity ranges between 5% and 25% in developed countries and between 20% and 50% in developing countries (Janisiewicz and Korsten, 2002). Greater losses occur in developing countries due to non-availability of proper storage and transportation facilities and improper handling methods, resulting in greater levels of injuries or wounds during harvesting and transit (Narayanasamy, 2006). Blue mould caused by *Penicillium expansum* (Link) Thom. and grey mould caused by *Botrytis cinerea* Pers.:Fr. are the major postharvest diseases of pome fruits, causing substantial economic losses during a long-term storage of harvested fruits (Sholberg and Conway, 2004).

The use of fungicides is the primary method of control of postharvest fungal decay of pome fruit (Kovach *et al.*, 2000). However, several reasons, such as the public's growing concern for human health conditions and environmental pollution associated with pesticide usage in orchards, persistence of residues on treated fruits, development of resistance of fungal pathogens to fungicides and high development costs of new chemicals (Eckert, 1990; Eckert *et al.*, 1994; Wilson and Wisniewski, 1994; Kovach *et al.*, 2000) have motivated the search for alternative approaches. Biological control using antagonistic yeasts is one of the most promising non-fungicidal means, especially for control of wound-invading pathogens (Janisiewicz and Korsten, 2002). Use of physical control measures (Lurie, 1998) and materials that are generally regarded as safe (GRAS), (Larrigaudiere *et al.*, 2002), also show promise as alternatives for postharvest control of fruit infection.

The overall objective of this study was to investigate the integration of biocontrol agents (yeasts) with other control methods, such as the postharvest application of potassium silicate and a hot water treatment, in order to control grey mould (*Botrytis cinerea* Pers.: Fr.) and blue mould (*Penicillium expansum* (Link) Thom.) of pome fruits.

The detailed objectives of this study were:

- To determine the influence of conidial concentration of *B. cinerea* and *P. expansum* on their pathogenicity on pears (*Pyrus communis* L.*)*.
- To determine the sensitivity of *B. cinerea* and *P. expansum* to the biofungicide YieldPlus® (*Cryptococcus albidus*).
- To isolate antagonistic yeasts originating from the surface of apple (*Malus domestica* Borkh.) and pear fruits and evaluate their efficacy for the control of *B. cinerea* and *P. expansum*.
- To evaluate the effect of applying potassium silicate as a postharvest treatment to control *B. cinerea* and *P. expansum* on pome fruit.
- To evaluate the use of hot water treatment for the control of *B. cinerea* and *P. expansum* on pome fruit.
- To determine possible modes of action of antagonists in the control of *B. cinerea* and *P. expansum* by observing interactions in apple fruit wounds.
- To determine independent and combined effects of potassium silicate, yeast Isolates YP25 and YP60, and the biofungicide YieldPlus® treatments for the control of *B. cinerea* and *P. expansum* of apples.

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TABLE OF CONTENT

ABSTRACT i
DECLARATION iii
ACKNOWLEDGEMENTS iv
DEDICATION v
INTRODUCTION vi
TABLE OF CONTENT ix
CHAPTER 1 1
INTEGRATED CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT: A LITERATURE REVIEW 1
1.1 Introduction 1
1.1.1 Origin and history of cultivation of apples and pears 2
1.2 GREY MOULD 2
1.2.1 Causal organism 2
1.2.2 Symptoms 3
1.2.3 Economic importance and distribution 4
1.2.4 Disease cycle and epidemiology 5
1.3 BLUE MOULD 6
1.3.1 Causal organism 6
1.3.2 Symptoms 7
1.3.3 Economic importance 8
1.3.4 Distribution 8
1.3.5 Disease cycle and epidemiology 8
1.4 CONTROL OF GREY MOULD AND BLUE MOULD OF POME FRUIT USING HYGIENIC PROCESSING PRACTICES 9
1.5 POSTHARVEST CONTROL OF GREY MOULD AND BLUE MOULD OF POME USING BIOCONTROL AGENT 10
1.5.1 Characteristics of a successful biological control agent 11
1.5.2 Mechanisms of action of biocontrol agents 11
1.5.3 Yeasts as biological control agents of postharvest grey mould and blue mould of pome fruit 12

1.6 HOT WATER TREATMENTS FOR THE CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT ... 14
1.7 CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT USING SALT ADDITIVES ... 15
1.8 INTEGRATION OF BIOCONTROL AGENTS WITH OTHER CONTROL PRACTICES ... 16
1.9 REFERENCES ... 18
CHAPTER 2 ... 31
THE PATHOGENICITY OF *BOTRYTIS CINEREA* AND *PENICILLIUM EXPANSUM* AND THEIR SENSITIVITY TO THE BIOFUNGICIDE YIELDPLUS® ... 31
ABSTRACT ... 31
2.1 INTRODUCTION ... 31
2.2 MATERIALS AND METHODS ... 33
2.2.1 Isolation of *Botrytis cinerea* and *Penicillium expansum* from infected plant material ... 33
2.2.2 Pathogenicity testing of *Botrytis cinerea* and *Penicillium expansum* on 'Packham Triumph' pears ... 33
2.2.3 Sensitivity of *Botrytis cinerea* and *Penicillium expansum* to YieldPlus® ... 34
2.2.4 Statistical analysis ... 34
2.3 RESULTS ... 34
2.3.1 Pathogenicity testing of *Botrytis cinerea* and *Penicillium expansum* on '"Packham's Triumph"' pears ... 34
2.3.2 Sensitivity of *Botrytis cinerea* and *Penicillium expansum* to YieldPlus® ... 36
2.4 DISCUSSION ... 39
2.5 CONCLUSION ... 40
2.6 REFERENCES ... 41
CHAPTER 3 ... 43
ISOLATION AND *IN VIVO* SCREENING OF YEAST ANTAGONISTS FOR THE CONTROL OF *BOTYRTIS CINEREA* AND *PENICILLIUM EXPANSUM* OF POME FRUIT ... 43
ABSTRACT ... 43

3.1 INTRODUCTION ... 43
3.2 MATERIALS AND METHODS ... 44
3.2.1 Fruit ... 44
3.2.2 Isolation of antagonistic yeasts ... 45
3.2.3 Screening of yeast isolates against *Botrytis cinerea* and *Penicillium expansum* ... 45
3.2.4 Preventative effect of selected yeast isolates antagonistic to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples ... 46
3.2.5 Dose effect of selected yeast isolates for the control of *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples, when applied preventatively 47
3.2.6 Effect of selected yeasts antagonists mixtures (YP25 + YP60) to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples ... 47
3.2.7 Statistical analysis ... 48
3.3 RESULTS ... 48
3.3.1 Preliminary screening of antagonistic yeast isolates to *Botrytis cinerea* and *Penicillium expansum* on pears ... 48
3.3.2 Further screening of selected yeast isolates antagonistic to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples ... 50
3.3.3 Preventative effect of selected yeast antagonists to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples ... 54
3.3.4 Dose effect of selected yeast isolates for the control of *Penicillium expansum* and *Botrytis cinerea* on "Golden Delicious" apples, when applied preventatively ... 56
3.3.5 Effect of a mixture of yeast antagonists (YP25 + YP60) on the control of *Botrytis cinerea* and *Penicillium expansum*, when applied preventatively on "Golden Delicious" apples ... 58
3.4 DISCUSSION ... 62
3.5 CONCLUSION ... 64
3.6 REFERENCES ... 64

CHAPTER 4 68
PREVENTATIVE EFFECT OF POTASSIUM SILICATE, POTASSIUM CHLORIDE AND POTASIUM HYDROXIDE POSTHARVEST TREATMENTS FOR THE CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* ON APPLES 68
ABSTRACT 68
4.1 INTRODUCTION 68
4.2 MATERIALS AND METHODS 71
4.2.1 Potassium solutions 71
4.2.2 Evaluation of the preventative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples 73
4.2.3 Evaluation of the curative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea* as a postharvest treatment on apples 73
4.2.4 Evaluation of the preventative activity of potassium chloride and potassium hydroxide against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples 74
4.2.5 Observations of *in vivo* interaction of potassium silicate with *Penicillium expansum* and *Botrytis cinerea* in apple wounds using scanning electron microscopy 74
4.2.6 Statistical analysis 75
4.3 RESULTS 75
4.3.1 Preventative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea*, when applied as a postharvest treatment on apples 75
4.3.2 Curative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea,* applied as a postharvest treatment on apples 79
4.3.3 Preventative activity of potassium chloride and potassium hydroxide against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples 24 hrs before pathogen inoculation 83
4.3.4 Visual observation of wounds of apples treated with potassium silicate and inoculated with either *Penicillium expansum* or *Botrytis cinerea* 91
4.4 DISCUSSION 94

4.5 CONCLUSION 95
4.6 REFERENCES 95
CHAPTER 5 101
EFFECT OF HOT WATER DIP TREATMENT ON POSTHARVEST CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* OF APPLES 101
ABSTRACT 101
5.1 INTRODUCTION 101
5.2 MATERIALS AND METHODS 103
5.2.1 Fruit 103
5.2.2 Observational Trial 1: hot water treatments to determine a safe temperature x exposure time for "Golden Delicious" apples 103
5.2.3 Observational Trial 2: hot water treatments followed by cold water dip for the control of skin injury of "Golden Delicious" apples 104
5.2.4 Replicated Trial 1: efficacy of hot water treatments for the control of *Penicillium expansum* and *Botrytis cinerea* infection of "Golden Delicious" apples 104
5.3 RESULTS 105
5.3.1 Observational Trial 1: effect of hot water dip treatments at different temperatures and exposure periods on the quality of "Golden Delicious" apples . 105
5.3.2 Observational Trial 2: effects of hot water dip treatments at different temperatures and exposure periods followed by a cold water dip for one minute, on skin quality of "Golden Delicious" apples 107
5.3.3 Replicated Trial 1: effectiveness of hot water dip treatments of "Golden Delicious" apples in the control of artificially inoculated *Penicillium expansum* and *Botrytis cinerea* 107
5.4 DISCUSSION 111
5.5 CONCLUSION 113
5.6 REFERENCES 113

CHAPTER 6 116
SCANNING ELECTRON MICROSCOPY STUDIES OF THE INTEARCTIONS OF YEAST ISOLATES YP60 AND YP25 WITH *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* IN APPLE WOUNDS 116
ABSTRACT 116
6.1 INTRODUCTION 116
6.2 MATERIALS AND METHODS 117
6.2.1 *In vitro* reaction of yeast Isolates YP60 and YP25 against *Penicillium expansum* and *Botrytis cinerea*..... 117
6.2.2 Preparation of the yeast Isolates YP25 and YP60 on agar using scanning electron microscopy 117
6.2.3 Preparation of the yeast Isolates YP25 and YP60 to examine their interactions in apple wounds, using scanning electron microscopy 118
6.2.4 Scanning electron microscopy studies of the growth of the yeast Isolates YP25 and YP60 on nutrient agar and their colonization ability in apple wounds... 118
6.2.5 Scanning electron microscopy studies of the interactions of yeast Isolate YP25 and *Botrytis cinerea*, and Isolate YP60 and *Penicillium expansum* in wounds of apple fruit..... 119
6.3 RESULTS 119
6.3.1 *In vitro* reaction of yeast Isolates YP60 and YP25 against *Penicillium expansum* and *Botrytis cinerea*..... 119
6.3.2 Scanning electron microscopy observations of the yeast Isolates YP25 and YP60 on nutrient agar..... 119
6.3.3 Visual observation of wounds of apples inoculated with either *Penicillium expansum* and *Botrytis cinerea*..... 121
6.3.4 Scanning electron microscopy studies of wounds of apple inoculated with either *Penicillium expansum* or *Botrytis cinerea* only 121
6.3.5 Visual observation of wounds of apples treated with the yeast Isolate YP25 and *Botrytis cinerea*, and YP60 and *Penicillium expansum*..... 124

6.3.6 Scanning electron microscopy observations of interaction of the yeast Isolates YP25 and YP60 on *Penicillium expansum* and *Botrytis cinerea*, respectively in apple wounds 124
6.4 DISCUSSION 126
6.5 REFERENCES 127
CHAPTER 7 130
INTEGRATED CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* OF APPLES USING POTASSIUM SILICATE, YEAST ANTAGONISTS AND YIELDPLUS® 130
ABSTRACT 130
7.1 INTRODUCTION 130
7.2 MATERIALS AND METHODS 131
7.2.1 Fruit 131
7.2.2 Yeast antagonists 132
7.2.3 Fungal pathogens 132
7.2.4 *In vitro* compatibility of yeast Isolates P60 and YP25 with potassium silicate (K_2SiO_3) 133
7.2.5 Assessment of potassium silicate, YieldPlus® and yeast Isolates YP25 and YP60 treatments, applied alone or in combination, for the control of *Penicillium expansum* and *Botrytis cinerea* of "Golden Delicious" apples 133
7.2.6 Statistical analysis 134
7.3 RESULTS 134
7.3.1 *In vitro* compatibility of yeast isolates (YP60 and YP25) with potassium silicate (K_2SiO_3) 134
7.3.2 Effects of potassium silicate, yeast Isolate YP25, yeast Isolate YP60 and YieldPlus® treatments, alone or in combination, on the control of *Botrytis cinerea* and *Penicillium expansum* of "Golden Delicious" apples 136
7.4 DISCUSSION 137
7.5 CONCLUSION 139
7.6 REFERENCES 139
THESIS OVERVIEW 144

CHAPTER 1
INTEGRATED CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT: A LITERATURE REVIEW

1.1 Introduction

Fruit are an important part of the human diet because they supply essential nutrients such as vitamins and minerals. The main concern of the modern world is “enough and nutritious food for all” (Salunkhe and Desai, 1984b). Despite the remarkable progress made in increasing food production at the global level, approximately half of the population in the Third World does not have access to adequate food supplies (FAO, 1989). There are many reasons for this, one of which is food loss occurring during the postharvest and marketing period (FAO, 1989). Generally, the supply of food can be enhanced by increasing production and reducing postharvest losses (Chakraverty and Singh, 2001).

Consumer satisfaction, which is the main objective of production, handling, storage and distribution of pome fruits, is mainly related to their quality (Hoehm *et al*., 2005). Definitions of pome fruit quality varies along the production chain, depending on the intended type of consumption (Kader, 2002). A lack of understanding of different perspectives may be the most limiting factor in improving the quality of fresh fruits and vegetables as delivered to the consumers (Hoehm *et al*., 2005). To be acceptable fruit must be free from mechanical damage, physiological disorders and pathological diseases (Perring, 1989; Baldwin, 2002; Ferguson and Boyd, 2002). However, non-visual characteristics such as flavour, texture and nutritional value on the acceptance of pome fruit in the market has increased in recent years (Awad and De Jager, 2003).

Apples and pears are pome fruits of great commercial importance (Salunkhe and Desai, 1984a), and are primarily grown for the fresh fruit market. Export of apples is very important for a number of national economies (Belrose, 1996). In South Africa, ±800,000 tons of apples and over 350,000 tons of pears are produced annually with export values of 29 billion and 1.4 billion, respectively

(www.hortgro.co.za/conent/view/151/lang,en/.). Apples and pears have many uses: fresh fruit juice, concentrated fruit juice, fresh fruit, vinegar, cider, brandy and various canned and dried fruit products (Jackson, 2003). Pome fruits are susceptible to a number of postharvest diseases that cause significant losses during the marketing of fresh fruit. Grey mould (*Botrytis cinerea* Pers.:Fr.) and blue mould (*Penicillium expansum* (Link) Thom.) are two of the major diseases of apples and pears (Sholberg and Conway, 2004). This review will focus only on grey mould and blue mould.

1.1.1 Origin and history of cultivation of apples and pears

Apples (*Malus domestica* Borkh.) and pears (*Pyrus communis* L.) are placed in the Rosaceae family, subfamily Pomoideae which constitute pome fruit (Jackson, 2003). Apples and pears are among the oldest of the world's fruit crops. The centre of origin of the genus *Malus* is southern Caucasus. It was found in Mesopotamia, Iran, and Asia many thousand years ago (Jackson, 2003). Apples spread to Europe where their cultivation was well known by the Ancient Greeks and Romans (Janick *et al*., 1996; Juniper *et al*., 2001). As a consequence of colonization, apples were brought to North America, South America, Australia and Africa by the English, the French, and the Spaniards (Feree and Warrington, 2003). Cultivated pears appear to have arisen from three centres of diversity: a Chinese centre where forms of *Pyrus pyryfolia* and *P. ussuriensis* are grown, a centre in the Caucasus Mountains, and a Central Asian centre where *P. communis* and its hybrids occur (Bell *et al*., 1996).

1.2 GREY MOULD

1.2.1 Causal organism

Grey mould is caused by *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). *Botrytis cinerea* has a white cottony appearance, which turns light grey with age on potato dextrose agar (PDA) (Xiao, 2006). The young hyphae are thin, hyaline, and 8-16 µm wide, and become brown and septate with age (Xiao, 2006). Conidiophores are light brown, septate with slightly enlarged tips bearing small pointed sterigmata bearing 1-2 celled, hyaline, oval conidia forming clusters. Characteristics and

sporulation of *B. cinerea* depend on, and vary with, nutrient medium, temperature, and ecological factors (Xiao, 2006).

1.2.2 Symptoms

Grey mould originates from infection of wounds such as bruises and punctures that are created at harvest and during postharvest handling (Figures 1.1A and B) (Xiao, 2006). Rotted fruit has a pleasant fermented odour (Beattie *et al.,* 1989). Stem-end grey mould is also common on pears (Figures 1.1C and 1.2A). *Botrytis cinerea* also invades floral parts of fruit and causes calyx-end rot (Figure 1.2B). Under high relative humidity, grey spore masses and/or fluffy white to grey mycelia may appear on the decayed area (Figures 1.1B and 1.2A) (Xiao, 2006). Sclerotia may form on the lesion surface of an advanced decayed fruit (Figure 1.3C). Fruit-to-fruit spread of grey mould also results in nesting of decayed fruit in storage containers (Figure 1.3A). The internal decayed flesh appears light brown to brown at the margin area (Figure 1.3B) (Xiao, 2006).

Figure 1.1 Grey mould originating from infection of wounds on pears created during harvest and postharvest handling (A); grey spore masses of *Botrytis cinerea* on grey mould decayed fruit under high relative humidity (B) and grey mould originating from infection of the stem, resulting in stem-end rot (C) (Xiao, 2006).

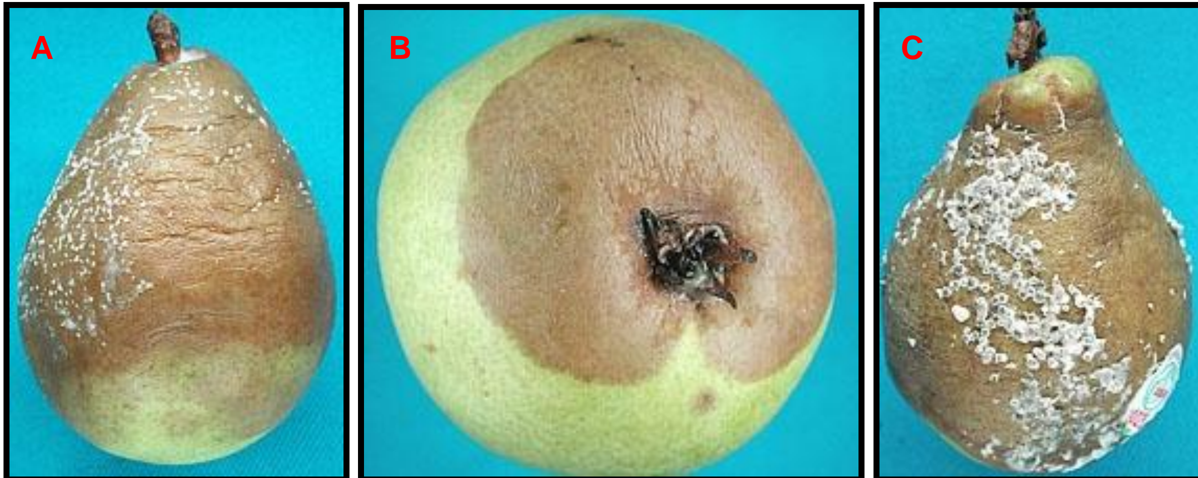

Figure 1.2 Advanced stages of grey mould showing a white to grey mycelium on the surface of decayed fruit (A); grey mould can also originate from calyx infection (B); sclerotia of *B. cinerea* on the surface of decayed fruit at an advanced stage (C) (Xiao, 2006).

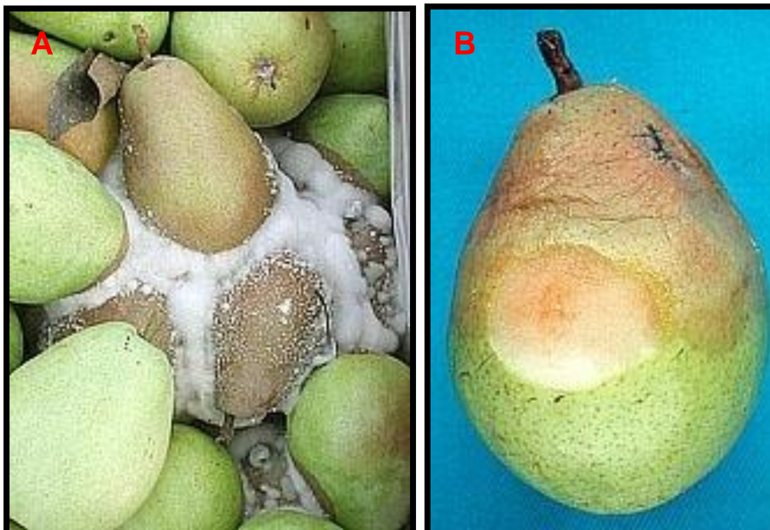

Figure 1.3 Nesting of grey mould due to fruit-to-fruit spread during storage (A); internal decayed flesh of grey mould of decayed fruit is light brown to brown at the margin of decayed area (B) (Xiao, 2006).

1.2.3 Economic importance and distribution

Grey mould is a disease of mature fruit which is of minor importance in well managed orchards. *Botrytis* diseases are probably the most common and widely distributed

diseases of vegetables, ornamentals, fruit, and even field crops throughout the world (Agrios, 1997). According to Rosslenbroich *et al.* (1998), the economic importance is higher when considering the fact that these cash crops are not only endangered in the field but also during transport and storage. *Botrytis* diseases commonly appear as blossom blights and fruit rots. Other diseases caused by this fungus are damping off, stem cankers and rots, leaf spots, and tuber, corm, bulb, and root rots (Gonsalves and Ferreira, 1994).

1.2.4 Disease cycle and epidemiology

The life cycle of *B. cinerea* is illustrated in Figure 1.4 (Agrios, 1997). The major source of inoculum is in the orchard where the fungus lives on plant material. Infection occurs mainly through wounds and skin injuries. The fungus develops faster at cool storage temperatures than blue mould (Beattie *et al.,* 1989). Spores of *B. cinerea* are carried in orchard soil and produced on decaying plant material brought into storage in bulk bins and other containers (Beattie *et al.,* 1989). Additional inoculum is provided by fruit that decays during storage (Beattie *et al.,* 1989). Infection spreads to adjacent fruit in cartons or bins causing nests or pockets of infection (Beattie *et al.,* 1989).

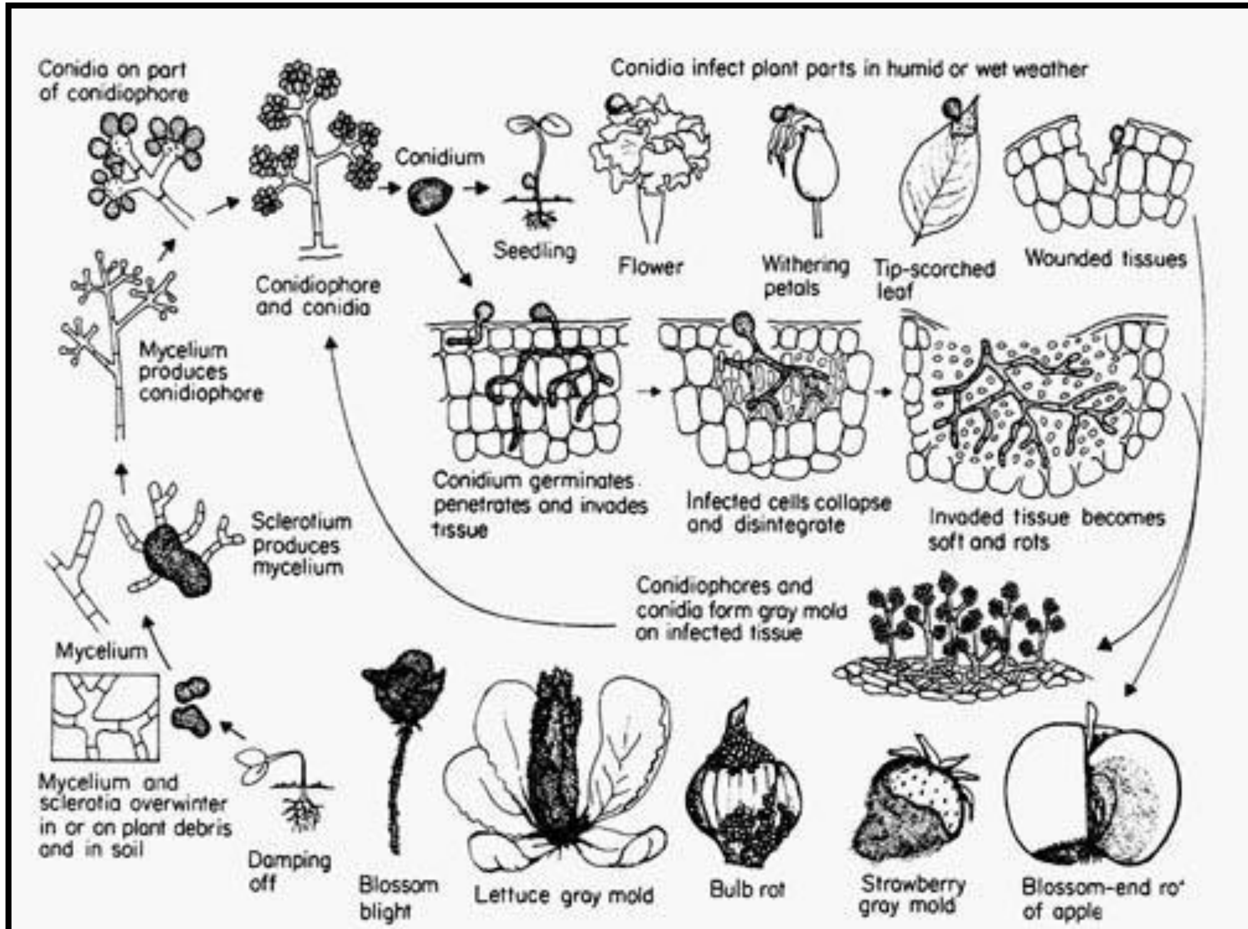

Figure 1.4 Life cycle of *Botrytis cinerea* on fruit and vegetables (Agrios, 1997).

1.3 BLUE MOULD

1.3.1 Causal organism

Penicillium expansum (Link) Thom. is the primary cause of blue mould of apples and pears. Several other *Penicillium* species, including *P. chrysogenum, P. commune*, *P. regulosum*, *P. solitum* and *P. verrucosum*, have also been reported to cause decay on apples and pears (Rosenberger and Sugar, 1990). *Penicillium expansum* can grow at temperatures as low as -3ºC and conidia can germinate at 0ºC (Rosenberger and Sugar, 1990).

1.3.2 Symptoms

Blue mould originates from infection of wounds such as punctures and bruises on fruit (Figures 1.5 A, B and C) (Rosenberger and Sugar, 1990). The fruit at first shows soft, pale-brown, watery spots with the decayed portions completely separable from healthy tissue (Figure 1.6B) (Beattie *et al.,* 1989). The decayed tissue is soft and watery and the lesion has a very sharp margin between diseased and healthy tissues (Rosenberger and Sugar, 1990). Spots enlarge rapidly and under favourable conditions may completely envelope the fruit. Under warm, moist conditions, the fungal growth is at first white, then pale blue and finally blue-green powdery clumps of fungal spores develop on the surface of the lesions (Persley, 1993).The fruit eventually becomes a soft, watery mass with a characteristic musty smell. Spread from fruit to fruit infection results in nests of infection (Figure 1.6A) (Beattie *et al.*, 1989).

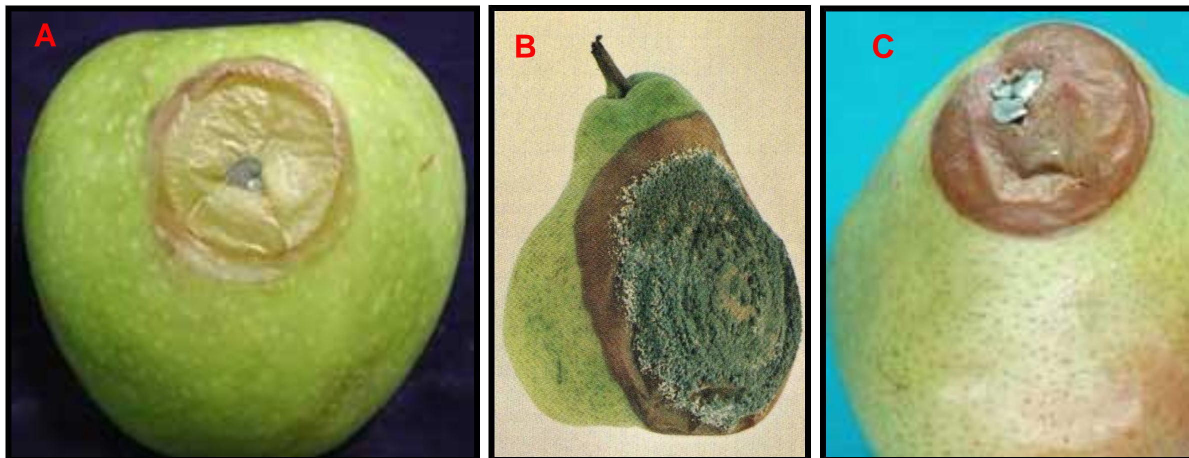

Figure 1.5 Blue mould originating from infection of wound on Granny Smith apples; spore masses formed at the infection site (A); advanced lesion showing brown margin, white mycelium and blue-green spore masses on “Packham’s Triumph” pear (B); stem-end blue mould commonly seen on d'Anjou pears, particularly after an extended period of storage (C) (Beattie *et al.*, 1989).

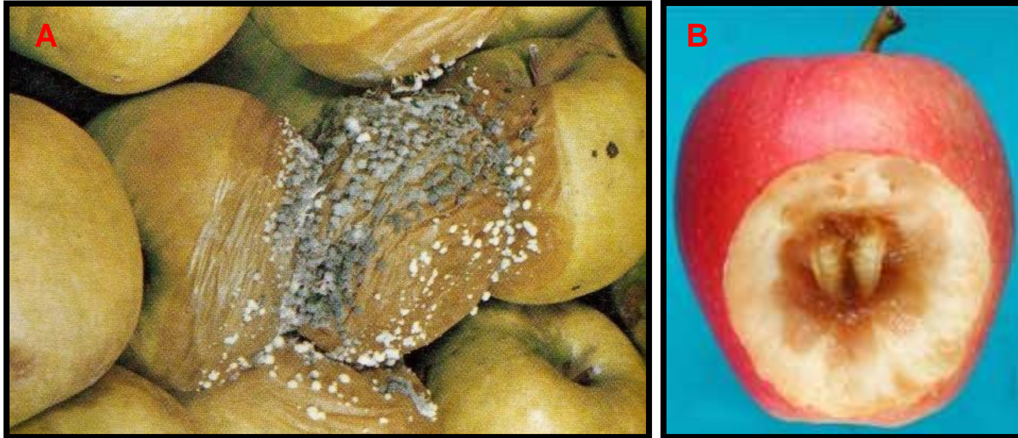

Figure 1.6 A nest of infection showing soft, watery symptoms, white mycelia and grey-blue spore masses on apples (A); decayed tissue completely separable from healthy tissue (B) (Beattie *et al.*, 1989).

1.3.3 Economic importance

Blue mould is the most important postharvest disease of pome fruit, occurring wherever the crops are grown. The disease can be controlled by fungicide dips and careful attention to hygiene during handling and storage (Persley, 1993).

1.3.4 Distribution

Blue mould is a common postharvest disease on apples and pears worldwide. According to Rosenberger and Sugar (1990), this disease is an economic concern not only to the fresh fruit industry, but also to the fruit processing industry because some strains of *Penicillium expansum* produce the mycotoxin patulin, which can rise to unacceptable levels and thus affect the quality of apple juice (Rosenberger and Sugar, 1990).

1.3.5 Disease cycle and epidemiology

Blue mould is the most common and destructive rot of all varieties of apples and pears (Persley, 1993). It can occur at any stage of the marketing chain after harvest (Persley, 1993). The fungus enters the fruit through a wound. Blue mould develops slowly at cool storage temperatures and in cool, dry conditions the spore masses usually do not appear. However, warm humid conditions favour blue mould development (Persley,

1993). Susceptibility to mould attack increases with maturity and is favoured by high humidities and blue mould is therefore more of a problem on fruit stored or shipped in plastic film liners (Persley, 1993).

Fruit, soil and bulk bins are the major sources of blue mould spores (Beattie *et al.*, 1989). Blue mould fungi infect damaged fruit on the orchard floor. Spores develop on the surfaces of these fruit, contaminate the soil and are blown by wind throughout the orchard to fruit on the trees (Beattie *et al.*, 1989). During harvest, this contaminated soil is transported with the fruit in bulk bins to packaging sheds and loaded into fruit dumps, dips or recirculating drench solutions. Rejected fruit in the packing sheds produce blue mould spores which are spread with dust onto the grading machinery surfaces (Beattie *et al.*, 1989).

Bulk bins are an important source of blue mould spores during the season and are a major source of carry-over between seasons, particularly of spores with resistance to fungicides. Immersing many bulk bins of fruit in the same dump, dip or drench solution results in the accumulation of blue mould spores. The contaminated dips can then act as a source of infection for fruit dipped subsequently (Beattie *et al.*, 1989).

Fruit rot can develop during grading and packing operations as a result of infection through damaged skin or an open calyx cavity (Beattie *et al.*, 1989). Fruit dumps, dips and drenches provide ideal situations for infection. As a result, fruit rot can occur even in apparently healthy fruit during storage and marketing (Beattie *et al.*, 1989).

1.4 CONTROL OF GREY MOULD AND BLUE MOULD OF POME FRUIT USING HYGIENIC PROCESSING PRACTICES

Maintenance of hygiene at all stages during production and postharvest handling is critical in minimizing sources of inoculum for postharvest diseases. To most effectively reduce inoculum, a good knowledge of the life cycle of the pathogen is essential

(Gonsalves and Ferreira, 1994). Sources of inoculum for postharvest diseases depend largely on the pathogen and when infection occurs (Beattie *et al.,* 1989).

The general aim of hygienic processing practices is to reduce the available supply of fungal spores to the lowest possible point for any given environment. This includes reducing contamination of bins with orchard soil, which is a reservoir for the spores, sterilization of contaminated bins and packing machinery, and frequent changes of solutions and water used for drenching and handling fruit (Beattie *et al.*, 1989). Fruit should be picked at the correct maturity and placed in cold storage as soon as possible. Picking wet fruit should be avoided. Bins containing harvested fruit in an orchard should be protected from rain to prevent fruit wetness (Beattie *et al.*, 1989).

Gentle handling of fruit by pickers during harvesting and care during the transportation of fruit from the orchard to the packing house may prevent injuries (Beattie *et al.*, 1989). Attention should be given to mechanical features of the handling machinery in packing houses to eliminate sources of injury from rough corners, unnecessary drops, or unprotected receiving surfaces. Sanitizing dump-tanks and flume water is an essential practice to reduce infection of fruit by *Penicillium expansum* and *Botrytis cinerea* during the packing process (Beattie *et al.*, 1989).

1.5 POSTHARVEST CONTROL OF GREY MOULD AND BLUE MOULD OF POME USING BIOCONTROL AGENT

Biological control as a crop protection strategy system emerged as a response to the search for a safe, environmentally friendly and effective approach to supplement the use of chemical pesticides (Campbell, 1989). Biological control as defined by Cook and Baker (1983) is the reduction in the amount of inoculum or disease producing activity of a pathogen accomplished by, or through, one or more organisms other than man. It is seen as a favourable choice because alternative methods of disease control, such as the development of resistant plant cultivars, has often been too slow, and economic

pressure on land use has limited some of the traditional cultural techniques of control (Burger, 1988).

Biocontrol agents have been widely investigated and promising results have been achieved (Janisiewicz and Korsten, 2002). Microbial antagonists such as bacteria, yeast, and filamentous fungi play an important role in the natural control of numerous postharvest pathogens of fruits (Janisiewicz, 1987; Wilson and Chalutz, 1989; Roberts, 1990; Lima *et al.*, 1997).

1.5.1 Characteristics of a successful biological control agent

Wilson and Wisniewski (1989) listed the desirable characteristics of a potential antagonist: (a) genetically stable; (b) effective at low concentrations; (c) simple nutrient requirements; (d) capable of surviving adverse environmental conditions; (e) effective against a wide range of pathogens and on various fruits and vegetables; (f) resistant to pesticides; (g) a non-producer of metabolites harmful and deleterious to human health; and (h) nonpathogenic to the host. In addition, a microbial antagonist should have an adaptive advantage over specific pathogens. For satisfactory control of postharvest pathogens of pome cultivars, the antagonist should be able to function under cold storage conditions (Barkai-Golan, 2001).

1.5.2 Mechanisms of action of biocontrol agents

Several modes of action have been suggested to explain the biocontrol activity of microbial antagonists. Competition for nutrients and space between the pathogen and the antagonist is considered the major mode of action by which microbial agents control pathogens causing postharvest decay (Droby *et al.,* 1992; Wilson *et al.*, 1993; Filonow, 1998; Ippolito *et al.,* 2000; Jijakli *et al.*, 2001). In addition, production of antibiotics (antibiosis), direct parasitism, and possible induced resistance are other modes of action of the microbial antagonists by which they suppress the activity of postharvest pathogens on fruits and vegetables (Janisiewicz *et al.*, 2000; Barkai-Golan, 2001; El-Ghaouth *et al.*, 2004).

1.5.3 Yeasts as biological control agents of postharvest grey mould and blue mould of pome fruit

Yeasts are fungi that grow as single cells, producing daughter cells either by budding or by binary fission. Yeasts belong in the kingdom Fungi and are a form of eukaryotic microorganisms. Most yeasts are unicellular, although some species may also become multicellular through the formation of a string of connected budding cells (Jones *et al.,* 1992).

The mode of action of yeast biocontrol agents include: 1) colonizing the fungal hyphae and a direct effect of live yeast cells on germination of pathogen conidia (Janisiewicz and Korsten, 2002); 2) competing for space and nutrients (Arras *et al*., 1998; Lima *et al*., 1999); 3) eliciting host resistance (Arras, 1996; Arras *et al*., 1998) and 4) extracellular lytic enzymes, such as glucanase and chitinase (Janisiewicz and Korsten, 2002), and. Tables 1.1 and 1.2 summarize some of the successful biocontrol agents that have been studied for their biological control potentials for pome postharvest *Botrytis* and *Penicillium* control, and other postharvest diseases of other crops.

Table 1.1 Yeast antagonists used for the successful control of postharvest grey mould and blue mould of pome fruit.

Antagonist	**Crop**	**Pathogen**	**Reference**
Candida saitoana	Apple	*Botrytis cinerea*	El-Ghaouth *et al.* (2000a, 2000b, 2000c)
Candida sake	Pear	*Botrytis cinerea*	Nunes *et al.* (2002)
	Apple	*Botrytis cinerea*	Viňas *et al.* (1998)
Candida sake (CPA-1)	Apple	*Penicillium expansum*	Usall *et al.* (2001); Morales *et al.* (2008)
	Pear	*Penicillium expansum*	Torres *et al.* (2006)
Candida spp.	Apple	*Botrytis cinerea*	McLaughlin *et al.* (1990)
Cryptococcus albidus	Apple	*Penicillium expansum*	Fan and Tian (2001)
Cryptococcus laurentii	Pear	*Botrytis cinerea*	Zhang *et al.* (2005)
Rhodoturula glutinis	Pear	*Penicillium expansum*	Zhang *et al.* (2008)
	Apple	*Botrytis cinerea*	Zhang *et al.* (2009)
Trichosporon sp.	Apple	*Botrytis cinerea*	Tian *et al.* (2002)

Table 1.2 Yeast antagonists used for the successful control of postharvest diseases of other crops.

Antagonist	Crop	Pathogen	Reference
Candida oleophila	Citrus	*Penicillium digitatum, Penicillium expansum*	Lahlali *et al*. (2004, 2005)
	Banana	*Colletotrichum musae*	Lassois *et al*. (2008)
Candida saitoana	Oranges	*Penicillium digitatum*	El-Ghaouth *et al.* (2000a, 2000b, 2000c)
Candida spp.	Nectarines Peach	*Botrytis cinerea, Penicillium expansum*	Karabulut *et al.,* (2002)
	Citrus	*Geotrichum candidum*	Chalutz and Wilson (1990)
Cryptococcus laurentii	Sweet cherry	*Penicillium expansum, Monilinia fructicola*	Qin and Tian (2005)
Debaryomyces hansenii	Citrus	*Penicillium digitatum, Penicillium italicum*	Singh (2002)
Pichia guilliermondii	Grape	*Rhizopus stolonifer*	Chalutz *et al.* (1988)
	Tomato	*Botrytis cinerea*	Chalutz *et al.*(1988)
	Chillies	*Colletotrichum capsici*	Chancaichaovivat *et al.* (2007)
Rhodoturula glutinis	Strawberry	*Botrytis cinerea*	Zhang *et al.* (2007)
	Jujube	*Alternata alternata*	Tian *et al.* (2005)

1.6 HOT WATER TREATMENTS FOR THE CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT

Hot water treatments are non-damaging physical treatments that can be used to alleviate physiological diseases of various commodities. These treatments may be applied to the commodity by means of hot water dips and sprays, hot vapour or dry air (Couey, 1989). Janisiewicz *et al*. (2003) reported that heat treatment (38 °C for 4 days)

reduced the decay of apples caused by *Colletotrichum acutatum* (J.H. Simmonds) and *Penicillium expansum* (Link) Thom. by 4.5 log units. Fallik *et al*. (1993, 1995) showed that heating without forced air can reduce decay caused by *B. cinerea* Pers.:Fr. in tomatoes and *P. expansum* (Link) Thom. in apple fruit. Postharvest dips are applied for a few minutes at high temperatures because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit (Lurie, 1999).

1.7 CONTROL OF POSTHARVEST GREY MOULD AND BLUE MOULD OF POME FRUIT USING SALT ADDITIVES

Salt additives improve the bioefficacy of some microbial antagonists, including yeasts, in controlling postharvest decay of fruits and vegetables (El-Ghaouth *et al*., 2004). Wisniewski *et al*. (1995) and Droby *et al*. (1997) demonstrated that both calcium and magnesium ions can be toxic to postharvest decay fungi and that salt solutions containing these elements can be used to enhance biocontrol activity of the yeast, *Candida oleophila* Montrocher, the antagonist used in the commercial product, Aspire®.

Several researchers have reported that salt additives including calcium propionate, calcium chloride, sodium carbonate, ammonium molybdate and ethanol were found to be successful when used with microbial antagonists for the control of postharvest diseases of fruits and vegetables (Plaza *et al*., 2001; Teixido *et al*., 2001; Karabulut *et al*., 2005; Qin *et al*., 2006; and Janisiewicz *et al*., 2008).

Research by Palou *et al.* (2001) has shown that carbonic acid salts, sodium bicarbonate (baking powder) and sodium carbonate have a potential in controlling plant diseases. Several researchers have reported that silicon applications reduce plant diseases when applied as a fertilizer, using soil or foliar applications (Cherif *et al*., 1992; Menzies *et al*., 1992; Cherif *et al*., 1994; Belanger *et al*., 1995; and Rodrigues *et al*., 2003). Abraham (2010) showed that potassium silicate controlled germination of *P. digitatum* conidia on wounds of citrus. Soluble silicon applications as a pre- and postharvest treatment have

provided control of fungal pathogens of many crops (Menzies and Belanger, 1996). Some successful examples are given in the Tables 1.3 and 1.4 below.

Table 1.3 Examples of postharvest application of soluble silicon for the successful control of fungal pathogens of fruit

Crop	Disease/ Pathogen	Reference
Avocado	*Anthracnose*	Anderson *et al.* (2004)
Chinese cantaloupe	*Trichothecium roseum*	Guo *et al.* (2007)
	Fusarium spp.	Liu *et al.* (2009)
Hami lemons	*Alternaria alternata*	Bi *et al* (2006)
	Trichothecium roseum	Bi *et. al* (2006)
Peach	*Monilinia fructicola*	Biggs *et. al* (1997)

Table 1.4 Suppression of fungal diseases by silicate salts

Crop	Disease	Reference
Cucumber	Damping-of	Cherif and Bélanger (1992)
Potato	Dry rot	Li *et al.* (2009)
Rice	Brown spot	Datnoff *et al.* (1991)
Soybean	Rust	Rodrigues *et al.* (2009)
Wheat	Powdery mildew	Rémus-Borel *et al.* (2005)

1.8 INTEGRATION OF BIOCONTROL AGENTS WITH OTHER CONTROL PRACTICES

Biological control alone is often less effective compared with commercial fungicides or it provides inconsistent control (Janisiewicz *et al.*, 1992; El-Ghaouth *et al.*, 2002; Leverentz *et al.*, 2003). To achieve a similar level of efficacy provided by conventional chemicals, the use of microbial antagonists combined with commercial chemicals (Droby *et al.*, 1998), hot water (Pusey, 1994; Nunes *et al.*, 2002; Palou *et al.*, 2002;

Obagwu and Korsten, 2003), chloride salts (McLaughlin *et al*., 1990), carbonate salts (Smilanick *et al*., 1999; El-Ghaouth *et al*., 2000a; Palou *et al*., 2002; Obagwu and Korsten, 2003), and other physical treatment such as curing and heat treatments (Plaza *et al*., 2003) are used as part of integrated control strategies.

Integrated approaches have been successfully applied to apples to control *P. expansum* (Link) Thom. Optimum control of postharvest fungal infection has been achieved by combining heat treatment, calcium chloride and the antagonist *Pseudomonas syringae* Van Hall (Conway *et al.,* 1999) or a biocontrol mixture, applied with a heat treatment and/or sodium bicarbonate (Conway *et al.,* 2005). Errampalli and Brubacher (2006) and Sugar and Basile (2008) have shown that mixing *P. syringae* Van Hall with low doses of cypronidil brought effective control in decay caused by *P. expansum* (Link) Thom. on apples. Chand-Goyal and Spotts (1997) also reported control of blue mould on apple and brown rot on pear when yeasts were used with low doses of a fungicide.

Karabulut *et al.* (2002) have shown that a postharvest treatment combination of hot water brushing at 60°C followed by a yeast antagonist (*Candida* spp.) as a postharvest treatment was very successful in controlling natural infections of *Monilinia fructicola* (G. Winter) Honey. and *P. expansum* (Link) Thom. of nectarines and peaches, compared with the hot water treatment alone.

El-Ghaouth *et al*. (2000a, 2000b) have shown that the addition of glycochitin enhanced the biocontrol activity of *Candida saitoana* against decay of apples, lemons, and oranges, caused by *B. cinerea*, *P. expansum* and *P. digitatum* (Pers: Fr. Sacc.). Examples of some successful salt additives are given in Table 1.5 below.

Table 1.5 Examples of salt additives for enhancing the efficacy of microbial antagonists against grey mould and blue mould of pome fruit.

Fruit	Salt additive	Microbial agent	Disease	Reference
Apple	Calcium chloride	*Candida* spp.	Grey mould, Blue mould	Wisniewski *et al.* (1995)
Pear	Calcium chloride	*Cryptococcus laurentii*	Grey mould	Zhang *et al.* (2005)
Apple	Calcium propionate	*Candida oleophila*	Blue mould	Droby *et al.* 2003)
Apple	Sodium bicarbonate	*Candida oleophila*	Blue mould	Droby *et al*. (2003)
	Sodium carbonate	*Cryptococcus laurentii*	Blue mould	Yao *et al.* (2004

In conclusion, grey mould and blue mould are two of the most important postharvest infections of pome fruit. Integrated control of postharvest grey and blue moulds of pome fruit using biological control agents (yeasts); physical treatments (hot water) and inorganic salts (potassium silicate, potassium hydroxide and potassium chloride) can reduce postharvest losses of pome fruit.

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CHAPTER 2

THE PATHOGENICITY OF *BOTRYTIS CINEREA* AND *PENICILLIUM EXPANSUM* AND THEIR SENSITIVITY TO THE BIOFUNGICIDE YIELDPLUS®

ABSTRACT

The pathogenicity of *Botrytis cinerea* and *Penicillium expansum* was tested on "Packham's Triumph" pears. Wounded "Packham's Triumph" pears were inoculated with 20 µℓ suspensions at concentrations of 1×10^3, 1×10^4 and 1×10^5 conidia $m\ell^{-1}$. There was no significant dose effect on the incidence of grey and blue moulds at different conidial concentrations. Wounded "Packham's Triumph" pears were treated with 30 µℓ of YieldPlus® suspension at concentrations of 1×10^6, 1×10^7 and 1×10^8 spore $m\ell^{-1}$. After 24 hrs, wounds were inoculated with conidial suspension of either *B. cinerea* or *P. expansum* (at 1×10^4 conidia $m\ell^{-1}$). Both *B. cinerea* and *P. expansum* infections were significantly reduced (P<0.001) by the biofungicide treatment compared to the untreated control. Fruit preventatively treated with YieldPlus® at a concentration of 1×10^8 spore $m\ell^{-1}$ had the lowest incidence of grey mould (4.2%) and blue mould (8.3%), compared to control fruits. YieldPlus® is an effective biocontrol agent against grey and blue moulds of pears.

2.1 INTRODUCTION

Fresh fruits are stored after harvest to supply nutritious fruit throughout the year (He *et al.*, 2003). Pome fruits are attacked by a number of postharvest pathogens (Rosenberger and Sugar, 1990). Postharvest diseases can be a limiting factor for the long-term storage of pome fruit (He *et al.,* 2003). Grey mould (*Botrytis cinerea* Pers.: Fr.) and blue mould (*Penicillium expansum* (Link) Thom.) are the most destructive postharvest pathogen on apple and pear fruit (Spotts and Chen, 1987) attacking fruit through wounds.

Grey mould is a postharvest fungal disease of apples and pears that occurs throughout the marketing chain (Beattie *et al.,* 1989). Grey mould originates primarily from infection of wounds such as punctures and bruises that are created at harvest and during postharvest handling. Blue mould is a major important postharvest infection of pome fruit in South Africa (SA). *Penicillium expansum* can grow at temperatures as low as -3ºC and conidia can germinate at 0ºC (Rosenberger and Sugar, 1990). Blue mould infects pome fruit primarily via wounds (Rosenberger and Sugar, 1990).

Postharvest fungicidal treatment on apples is currently the main control measure applied to decrease losses caused by postharvest pathogens. Imazalil (Freshguard® and Fungaflor®) is one of the most commonly used systemic fungicides used to control postharvest fungal decay, especially on apples (Nunes *et al.,* 2001). The appearance of pathogen strains resistant to these fungicides (Spotts and Cervantes 1986; Viňas *et al.*, 1993) has increased the need to develop alternative methods of decay control.

Research on postharvest decay of pome fruit has led to the registration of YieldPlus®, the first South African produced and registered biological control agent for the control of postharvest decay on pome fruit (Vero *et al.*, 2002). YieldPlus® is a biocontrol product based on a yeast, *Cryptococcus albidus* (Saito) Skinner. The product was registered in 1997 for use on apples and pears, to combat *B. cinerea* and *P. expansum,* and is marketed by Anchor Bio-Technologies®.

The objectives of this research were: (1) to determine the influence of conidial concentration of *B. cinerea* and *P. expansum* pathogenicity on pears; and (2) to determine the sensitivity of *B. cinerea* and *P. expansum* to the biofungicide YieldPlus®.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of *Botrytis cinerea* and *Penicillium expansum* from infected plant material

Botrytis cinerea was isolated from infected strawberry fruits obtained from Checkers Supermarket, Scottsville, Pietermaritzburg, South Africa (SA). *Penicillium expansum* was isolated from infected pear fruit obtained from Checkers Supermarket, Scottsville, Pietermaritzburg, South Africa (SA). Spores were directly plated onto potato dextrose agar (PDA). Plates were incubated at 25°C for 7 days. Pure cultures were obtained by subculturing on fresh PDA plates. The cultures were maintained in McCartney bottles three-quarter filled with double sterilized water, and incubated at room temperature for long term storage. Inoculum was prepared from 7 day old culture dishes incubated at 25°C, by flooding with sterile distilled water, and passing the suspension through two layers of sterile cheese-cloth to remove hyphal fragments. The conidial concentration of *B. cinerea* and *P. expansum* was determined with a haemacytometer and adjusted by adding sterile distilled water.

2.2.2 Pathogenicity testing of *Botrytis cinerea* and *Penicillium expansum* on 'Packham Triumph' pears

Pears were disinfected with 70% alcohol for 1 min, rinsed three times in sterile distilled water and then air dried prior to wounding. The pears were wounded uniformly, approximately 3 mm deep and 3 mm wide, with a sterile needle at the equatorial side. Four wounds were made in each pear. The wounded fruit were inoculated with 20 µℓ of the conidial suspension of *B. cinerea* at concentrations of 1×10^3, 1×10^4 and 1×10^5 conidia $m\ell^{-1}$. Controls were inoculated with 20 µℓ of distilled water. Similar methods were also used for pathogenicity testing of *P. expansum.* Inoculated fruit were stored in enclosed plastic trays to maintain a high relative humidity (RH) of about 90% at 25°C. There were 3 fruits per replicate and 6 replicates per treatment. After seven days, wounds were examined and the percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The experiment was conducted twice.

2.2.3 Sensitivity of *Botrytis cinerea* and *Penicillium expansum* to YieldPlus®

To determine the effect of YieldPlus® on the development of *B. cinerea* and *P. expansum*, '"Packham's Triumph"' pears were similarly treated as described in Section 2.2.2. Wounded fruit was inoculated with 30 µℓ of YieldPlus® suspension at concentrations of 1×10^6, 1×10^7 and 1×10^8 spore mℓ$^{-1}$. After 4 hrs, each wound was inoculated with 20 µℓ of *B. cinerea* at a concentration of 1×10^4 conidia mℓ$^{-1}$. The control was inoculated with 20 µℓ of *B. cinerea* only. The sensitivity of *P. expansum* to YieldPlus® was determined by using the same procedure as for *B. cinerea*. The control was inoculated with 20 µℓ of *P. expansum* only. Inoculated fruit was stored in enclosed plastic trays to maintain a high relative humidity (RH) of about 90% at 25°C. There were 3 fruits per replicate and 6 replicates per treatment. After seven days, wounds were examined and the percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The experiment was conducted twice.

2.2.4 Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Genstat 14th edition. To determine differences between treatments, Fisher's Least Significant Difference test was used ($P = 0.05$).

2.3 RESULTS

2.3.1 Pathogenicity testing of *Botrytis cinerea* and *Penicillium expansum* on '"Packham's Triumph"' pears

There was no significant difference in grey mould incidence between pear wounds inoculated with *B. cinerea* at concentrations of 1×10^3, 1×10^4 and 1×10^5 conidia mℓ$^{-1}$ (Figure 2.1). The control treatment had the lowest incidence of grey mould (8.3%) and it was significantly different ($P \leq 0.001$) from fruit inoculated with 1×10^3, 1×10^4 and 1×10^5 conidia mℓ$^{-1}$ (Figure 2.1).

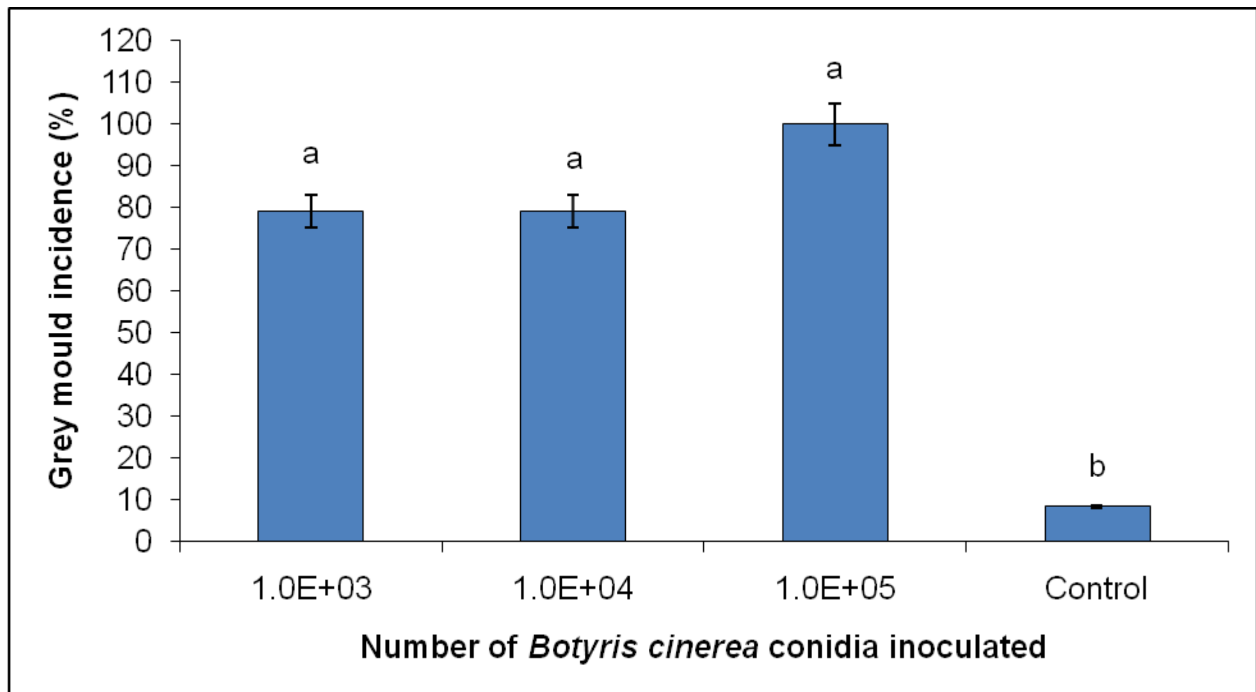

Figure 2.1 Disease incidence as a result of different inoculum doses applied as conidia of *Botrytis cinerea*. Different doses are treated as individual variables to allow graphing with the control; means followed by the same letter are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

There was no significant difference in blue mould incidence between pear wounds inoculated with *P. expansum* at concentrations of 1 x 10^3, 1 x 10^4 and 1 x 10^5 conidia mℓ^{-1} (Figure 2.2). The control treatment had the lowest incidence of blue mould (8.3%) and it was significantly different (P≤0.001) from fruit inoculated with 1 x 10^3, 1 x 10^4 and 1 x 10^5 conidia mℓ^{-1} (Figure 2.2).

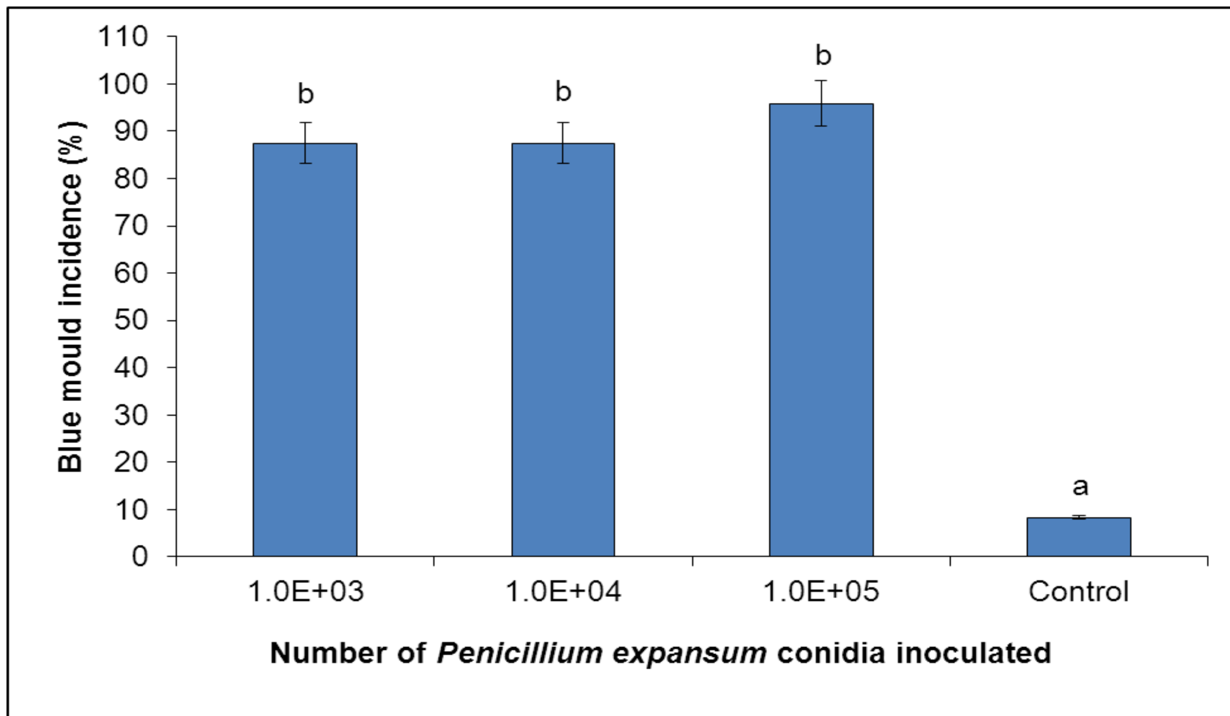

Figure 2.2 Disease incidence as a result of different inoculum doses applied, as conidia of *Penicillium expansum*. Different doses are treated as individual variables to allow graphing with the control; means followed by the same letter are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

2.3.2 Sensitivity of *Botrytis cinerea* and *Penicillium expansum* to YieldPlus®

There was a significant difference ($P \leq 0.001$) in grey mould incidence between the inoculated control and fruit treated with YieldPlus® suspensions (Figure 2.3). Control fruit exhibited 98.5% grey mould incidence compared to 4.2% in the best YieldPlus® application at a concentration of 1 x 10^8 spore $m\ell^{-1}$ (Figure 2.3). Grey mould incidence in pear wound sites at 25°C decreased as a function of YieldPlus® dose (R^2=0.964) (Figure 2.4).

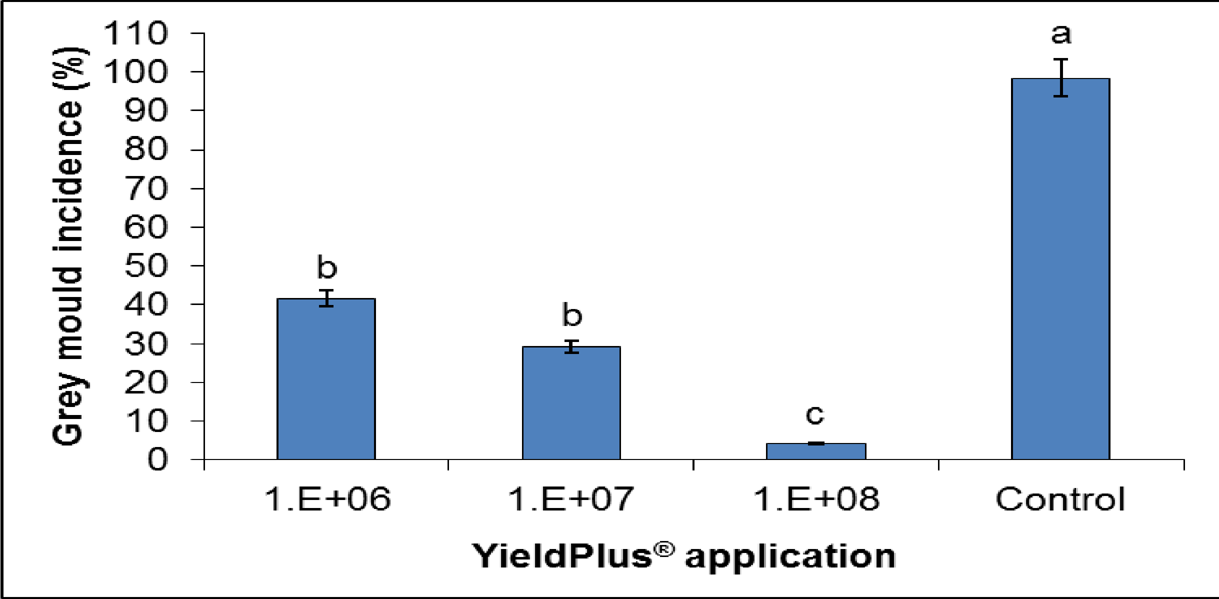

Figure 2.3 Sensitivity of *Botrytis cinerea* to YieldPlus® treatment on "Packham's Triumph" pears. Different doses are treated as individual variables to allow graphing with the control; means followed by the same letter are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

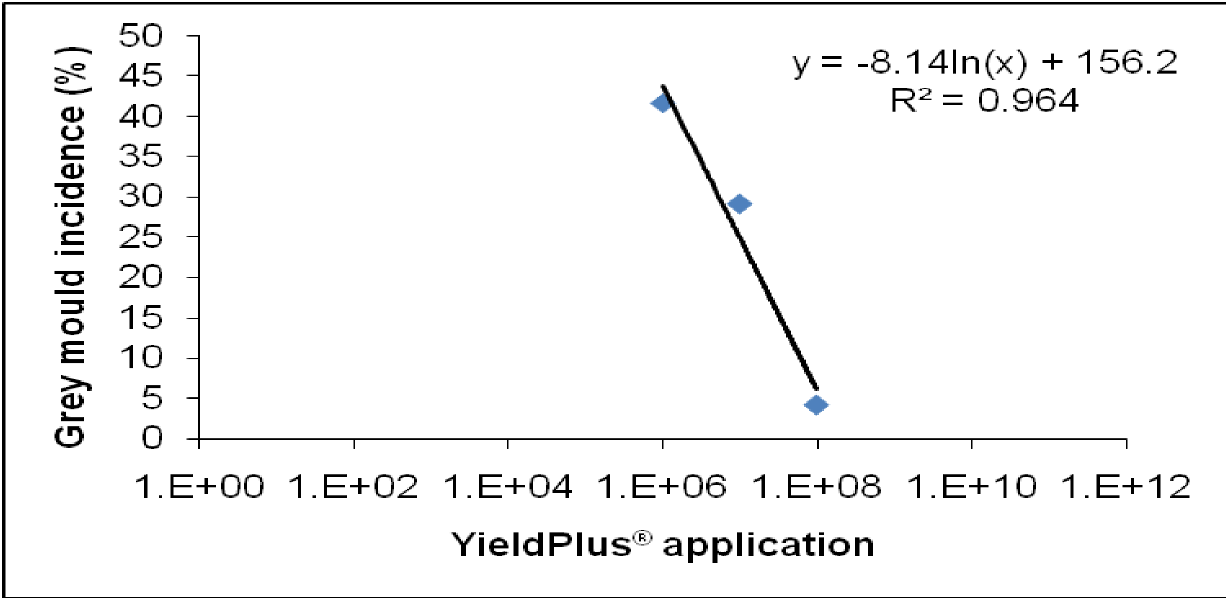

Figure 2.4 Logarithmic regression of dose of YieldPlus® on grey mould incidence.

There was a significant difference ($P \leq 0.001$) in blue mould incidence between the inoculated control and fruit treated with YieldPlus® suspensions (Figure 2.5). Control

fruit exhibited 98.5% grey mould incidence compared to 8.3% in the best YieldPlus® application at a concentration of 1 x 10^8 spore $m\ell^{-1}$ (Figure 2.5). Blue mould incidence in pear wound sites at 25°C decreased as a function of dose of YieldPlus® (R^2=0.993) (Figure 2.6).

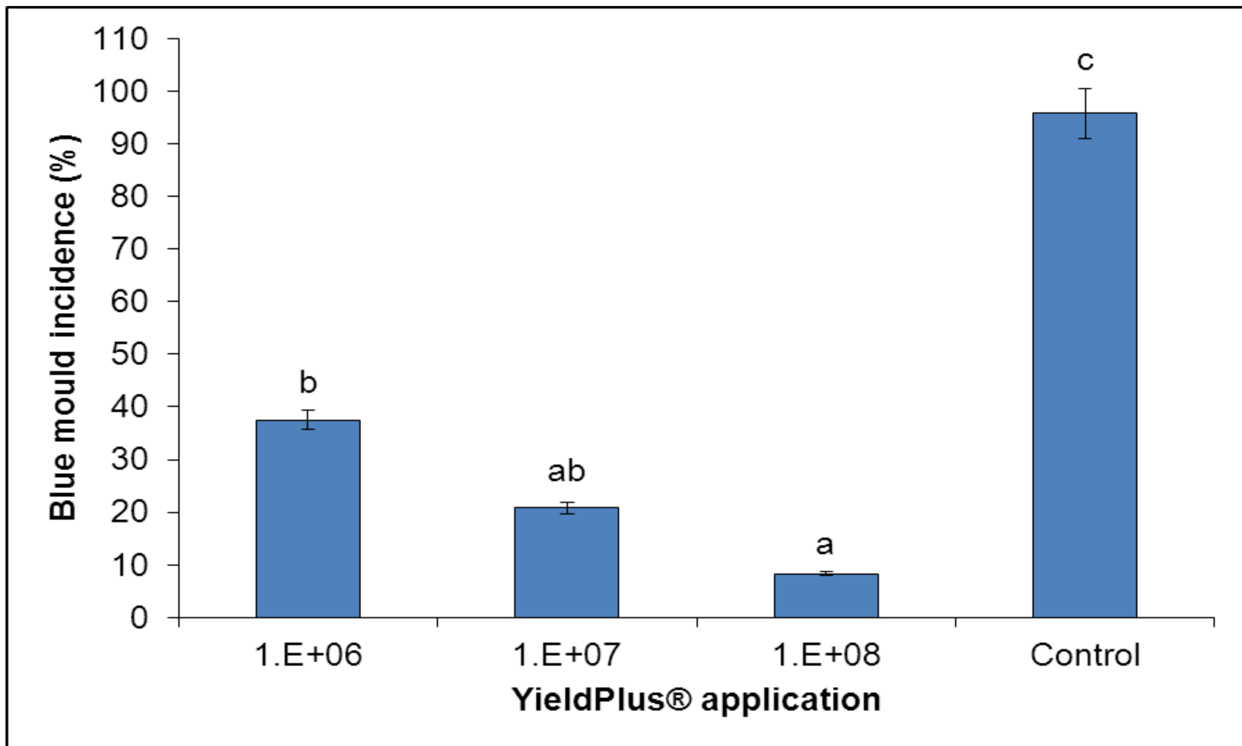

Figure 2.5 Sensitivity of *P. expansum* to YieldPlus® treatment on pears. Different doses are treated as individual variables to allow graphing with the control; means followed by the same letter designation are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

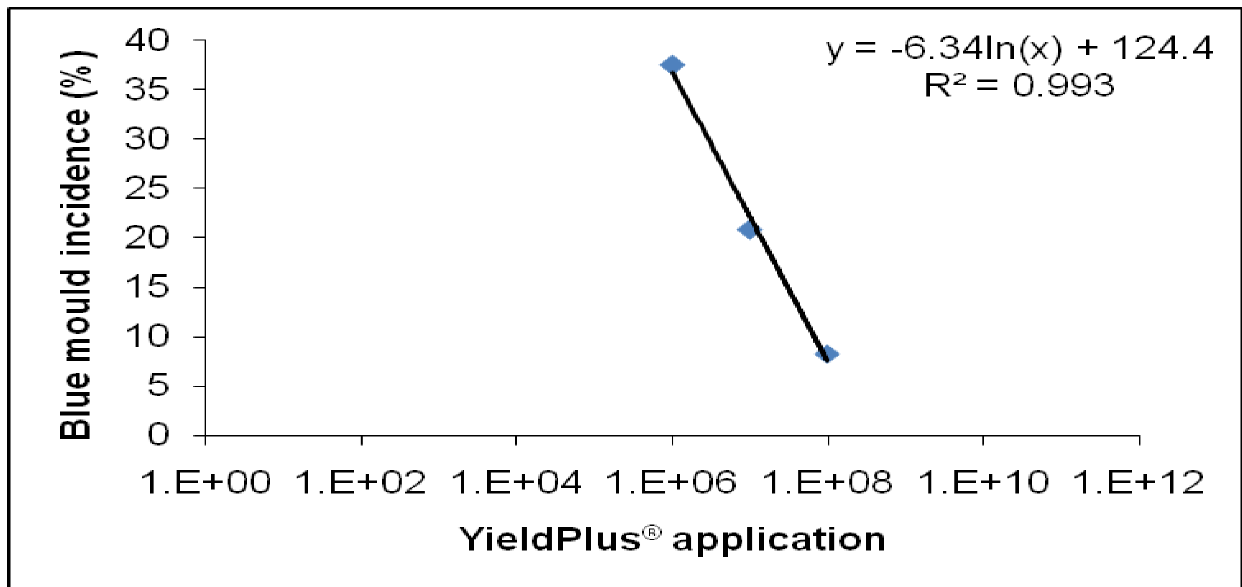

Figure 2.6 Logarithmic regression of dose effect of YieldPlus® on blue mould incidence.

2.4 DISCUSSION

The major objectives of this study were to determine the influence of conidial concentrations of *B. cinerea* and *P. expansum* on their pathogenicity on pears, and to determine the sensitivity of *B. cinerea* and *P. expansum* to the biofungicide YieldPlus®. All tested conidial concentrations of both pathogens caused infection. There was no significant effect on the incidence of disease as a result of the different conidial concentrations. This may have been due to competition between conidia, and disease development could have been limited by the availability of nutrients (Abraham, 2010). Therefore, we should have tested 10^0, $10^{1,}$ 10^2, 10^3, and 10^4 conidia $m\ell^{-1}$ as well.

Disease incidence of 8.3% was recorded on uninoculated “Packham’s Triumph” pears. This could have been caused by pre-existing infection of the pathogen on the fruit, which may have occurred during preharvest activities or transport. The wounds which occur during harvest, transport and handling do not only damage the fruit (Miller, 2003), but also provide pathways for pathogen invasion, especially for the wound-invading necrotrophic fungi (Janisiewicz and Korsten, 2002). Brown and Miller (1999) have shown that fruit infection by *P. digitatum* (Pers.Fr. Sacc.) arising from a single conidium

can produce about 100 million conidia on an infected fruit in seven days under optimum environmental conditions. Therefore, using high concentrations of conidial suspensions of *B. cinerea* or *P. expansum* for pathogenicity tests is not necessary.

Postharvest decay has been listed by the fruit industry as a major limiting factor in successful fruit sales. Both *Botrytis* and *Penicillium* infections were significantly reduced by the biofungicide treatment compared to the untreated controls. When wounds on pears were pretreated with YieldPlus®, development of *B. cinerea* and *P. expansum* was significantly reduced. YieldPlus® has the capacity to control postharvest incidence grey mould and blue mould caused by *B. cinerea* and *P. expansum*, respectively. It also shows that the pathogens used in this research are not resistant to YieldPlus®. When YieldPlus® was applied at a concentration of 1×10^8 spore $m\ell^{-1}$, the best control was obtained. Biocontrol strains of yeasts occupy the surface of fruit, and compete with the decay-causing pathogens for space and nutrients, so the pathogens are not able to penetrate the fruit and cause decay. Zhang *et al*. (2005) showed that a washed cell suspension of selected strains of *Cryptococcus laurentii* Kufferath C.E. Skinner at a concentration of 1×10^8 cells $m\ell^{-1}$ controlled grey mould infection on pears. Zheng *et al.* (2005) were able to control green mould of oranges with the yeast *Rhodoturula glutinis* (Harrison), applied at a concentration of 1×10^9 cells $m\ell^{-1}$. Zhang *et al.* (2009) reported that the application of *R. glutinis* at a concentration of 1×10^8 cells $m\ell^{-1}$ reduced the incidence of natural infections on Red Delicious apples.

2.5 CONCLUSION

YieldPlus® can be used as an effective treatment against postharvest diseases (grey mould and blue mould) of pears. Future research will compare the biocontrol activity of YieldPlus® and other yeast antagonists isolated from the surface of apples and pears.

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CHAPTER 3

ISOLATION AND *IN VIVO* SCREENING OF YEAST ANTAGONISTS FOR THE CONTROL OF *BOTYRTIS CINEREA* AND *PENICILLIUM EXPANSUM* OF POME FRUIT

ABSTRACT

A total of 100 epiphytic yeast isolates were obtained from the fruit surface of "Golden Delicious" apples and "Packham's Triumph" pears, and screened against *Botrytis cinerea* and *Penicillium expansum*, the causal agents of grey and blue moulds, respectively. Fifteen yeast isolates reduced grey mould incidence by > 50%, when applied 4 hrs before inoculation with *B. cinerea*. Similarly, seven yeast isolates reduced blue mould incidence by > 50%, when applied 4 hrs before inoculation with *P. expansum*. Yeast Isolates YP16, YP24, YP25, and YieldPlus®, a commercial biological control agent, provided the best control of grey mould on apples when applied 48 hrs prior to inoculation with *B. cinerea*. This reduction was significantly different ($P<0.001$) compared to the untreated control. Furthermore, YieldPlus® and yeast Isolates; YP28, YP53, YP60, YP43, YP5, YA33 and YP84, when applied 48 hrs prior to inoculation with *P. expansum*, significantly ($P<0.05$) reduced blue mould incidence compared to the untreated control. YieldPlus® and the yeast Isolate YP25 provided the best control of *B. cinerea*, while Isolate YP60 and YieldPlus® provided the best control of *P. expansum* on "Golden Delicious" apples. A mixture of YP25 and YP60 provided complete control of both *B. cinerea* and *P. expansum* when applied to "Golden Delicious" apples before inoculation. The benefits of using yeast antagonists as a measure to reduce the use of agrochemicals on postharvest fruit diseases will be discussed.

3.1 INTRODUCTION

Grey mould caused by *Botrytis cinerea* Pers.:Fr. and blue mould caused by *Penicillium expansum* (Link) Thom. are two important postharvest diseases of pome fruits (Saravanakumar *et al.*, 2008). Synthetic fungicides are widely used to control

postharvest fungal decay of pome fruits. However, several reasons, such as the persistence of residues on treated fruits, development of resistance to fungicides among fungal pathogens and the high development costs of new chemicals have motivated the search for alternative approaches (Wilson and Wisniewski, 1989; Eckert, 1990; Eckert *et al*., 1994) which are less harmful to human health and the environment (Holmes and Eckert, 1999).

Several researchers have achieved promising results on the use of biocontrol agents to control postharvest diseases on a number of commodities (Adeline and Sijam, 1999; Teixido *et al.* 1999; Janisiewicz and Korsten, 2002; Singh, 2002; Tian *et al*. 2002; Kota *et al*. 2006 and Batta, 2007). Postharvest infections have been controlled successfully on pome fruit using biological control agents (McLaughlin *et al.* 1990; Janisiewicz *et al.* 1998; Blum *et al.* 2004; Torres *et al*. 2006; Morales *et al*. 2008 and Zhang *et al.* 2009). The use of yeast antagonists as a biological control strategy is one of the most promising non-fungicidal means for the control of wound-invading pathogens (Janisiewicz and Korsten, 2002). Yeast antagonists are potential biological control agents because they proliferate rapidly by using available nutrients; they can produce extracellular polysaccharides that enhance their survival; they colonize the wound for long periods; and are tolerant of most agrochemicals (Janisiewicz, 1988; Richard and Prusky, 2002).

The objectives of this research were to isolate antagonistic yeasts and to evaluate their efficacy in controlling infections caused by *B. cinerea* and *P. expansum* on pome fruit.

3.2 MATERIALS AND METHODS

3.2.1 Fruit

Pears (*Pyrus communis* L. spp.) cultivar “Packham’s Triumph” and apples (*Malus domestica* Borkh.) cultivar “Golden Delicious” were collected from Pick ‘n Pay Supermarket in Pietermaritzburg, South Africa (SA). Only undamaged, healthy, mature fruit was used in the experiments.

3.2.2 Isolation of antagonistic yeasts

Yeasts, potentially antagonistic to *B. cinerea* and *P. expansum*, were isolated from the surfaces of apples and pears. The fruit peel was cut into small pieces weighing 80 g (Figure 3.1A) and placed in separate 250 mℓ Erlenmeyer flasks containing 100 mℓ sterile distilled water and shaken in a water bath at 130 rotations per minute (rpm) for 1 hr at 28°C. Fruit peel pieces were removed and the liquid suspension (Figure 3.1B) was used to make serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. An aliquot of 0.2 mℓ of each dilution was plated onto PDA plates and incubated at 25°C for 3 days. Pure cultures of yeast were made by sub-culturing colonies onto fresh PDA plates.

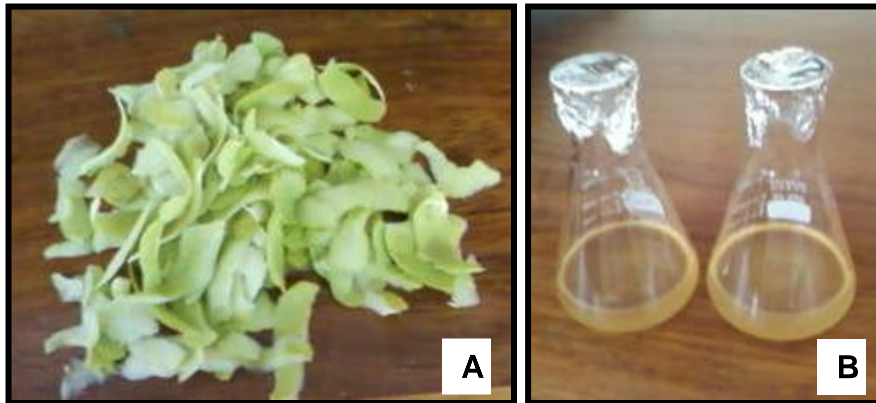

Figure 3.1 (A) Small pieces of fruit peel and (B) fruit peel wash in 250 ml Erlenmeyer flasks.

3.2.3 Screening of yeast isolates against *Botrytis cinerea* and *Penicillium expansum*

3.2.3.1 Preliminary screening

A total of 100 yeast isolates were tested on "Packham's Triumph" pears. Fruit was disinfected with 70% alcohol for 1 minute, rinsed three times in sterile distilled water and then air dried, prior to wounding. Each fruit was uniformly wounded, approximately 5 mm deep and 5 mm wide, with a sterile needle at the equatorial side. Four wounds were made in each pear. The wounds were inoculated with 30 µℓ of the yeast suspension (1×10^{8} cells mℓ$^{-1}$). Four hours after the wound site had dried; each wound was inoculated with a 30 µℓ conidial suspension of *B. cinerea* at 1×10^{4} conidia mℓ$^{-1}$, isolated from infected strawberries, from Checkers Supermarket, Scottsville, Pietermaritzburg,

SA. Control fruit were treated with sterile distilled water. Fruit were kept at 25°C and at a relative humidity (RH) of 95% for 7 days. After seven days, wounds were examined and the percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. Similar methods were also used to screen yeast isolates against *P. expansum* on pears. The experiment was repeated twice. There were 3 fruits per replicate and 3 replicates per treatment. The reduction of disease incidence to < 50%, compared to the control treatment, was adopted as a criterion for the selection of the best antagonistic yeasts. These promising yeast isolates were further tested to confirm their biocontrol activity against *B. cinerea* and *P. expansum*.

3.2.3.2 Secondary screening

The procedure described in Section 3.2.3.1 was used. "Packham's Triumph" pears were used for the secondary screening test. Apples were wounded uniformly, approximately 5 mm deep and 5 mm wide, with a sterile needle at four equatorial points. The wounds were then treated with a 30 μℓ of yeast suspension (1×10^8 cells $m\ell^{-1}$). Four hours after the wound site had dried each wound was inoculated with a 30 μℓ conidial suspension of *B. cinerea* at 1×10^4 conidia $m\ell^{-1}$. Control fruits were treated with the same concentration of *B. cinerea*, but no biocontrol pretreatment was made. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. Disease incidence (DI) of grey mould was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was also used to screen yeast isolates against *P. expansum* on pears. The experiment was repeated twice. There were 3 fruits per replicate and 3 replicates per treatment.

3.2.4 Preventative effect of selected yeast isolates antagonistic to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples

Selected yeast antagonists were tested for their preventative action against *B. cinerea* and *P. expansum* on "Golden Delicious" apples. The fruit were disinfected and wounded as described in Section 3.2.3. Wounds were inoculated with 30 μℓ of yeast suspension

(1×10^8 cells $m\ell^{-1}$). The fruit was kept for 2 days at 25°C after which they were inoculated with 30 µℓ of a conidial suspension (1×10^4 conidia $m\ell^{-1}$) of *B. cinerea*. Control fruit were inoculated with the same concentration of *B. cinerea*, but received no biocontrol pretreatment was made. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. The percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was also used to screen yeast isolates against *P. expansum* on apples. The experiment was conducted twice. There were 3 fruits per replicate and 3 replicates per treatment.

3.2.5 Dose effect of selected yeast isolates for the control of *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples, when applied preventatively

"Golden Delicious" apples were disinfected and wounded as described in Section 3.2.3. Wounded fruits were inoculated with 30 µℓ of selected antagonistic yeast suspensions at concentrations of 1×10^6, 1×10^7 and 1×10^8 cells $m\ell^{-1}$. The fruit was kept for 2 days at 25°C after which they were inoculated with 30 µℓ of a conidial suspension (1×10^4 conidia $m\ell^{-1}$) of *B. cinerea*. Control fruit were inoculated with the same amount of *B. cinerea*, but received no biocontrol pretreatment was applied. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. The percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was also used to determine the dose effect of selected yeast isolates against *P. expansum* on apples. The experiment was conducted twice. There were 3 fruits per replicate and 3 replicates per treatment.

3.2.6 Effect of selected yeasts antagonists mixtures (YP25 + YP60) to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples

The apples were disinfected, as described in Section 3.2.3 and then wounded approximately 5 mm deep and 5 mm wide, with a sterile needle at the equatorial side. Four wounds were made in each apple. Wounds were inoculated with 30 µℓ yeast

suspension mixture (YP25 + YP60) at concentrations of 1 × 10^6, 1 × 10^7 and 1 × 10^8 cells mℓ$^{-1}$. Apples were kept for 2 days at 25°C after which they were inoculated with 30 μℓ of conidial suspension (1 × 10^4 conidia mℓ$^{-1}$) of *B. cinerea*. Control fruit were inoculated with the same concentration of *B. cinerea*, but no biocontrol pretreatment was applied. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. The percentage of disease incidence (DI) was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was also used to determine the effect of yeasts antagonist mixtures against *P. expansum* on apples. The experiment was conducted twice. There were 3 fruits per replicate and 10 replicates per treatment.

3.2.7 Statistical analysis

With one exception, all data sets were subjected to analysis of variance (ANOVA) using Genstat 14th edition. Differences between treatment means were determined using Duncan's Multiple Range test (P = 0.05). The exception was in the case of determining dose effects of yeast isolates and a mixture of yeast antagonists (YP25+YP60), for the control of *B. cinerea* and *P. expansum* on "Golden Delicious" apples. In this case, the data was subjected to ANOVA using Genstat 14th edition. To determine differences between treatments, Fisher's Least Significant Difference Test was used (P = 0.05).

3.3 RESULTS

3.3.1 Preliminary screening of antagonistic yeast isolates to *Botrytis cinerea* and *Penicillium expansum* on pears

From a total of 100 yeast isolates that were used in the preliminary screening test, only 15 yeast isolates were able to reduce grey mould incidence by > 50%, compared to the untreated control, which developed 100% infection by *B. cinerea* (Table 3.1). Only 7 yeast isolates reduced blue mould incidence by > 50%, compared to the untreated control, which developed 100% infection by *P. expansum* (Table 3.2). The reduction of disease incidence to < 50%, compared to the untreated control, was adopted as a

criterion for selection for the best antagonistic yeasts. The promising yeast isolates were further tested to confirm their biocontrol activity against *B. cinerea* and *P. expansum*.

Table 3.1 Grey mould (*Botrytis cinerea*) incidence on "Packham's Triumph" pears reduced by antagonistic yeast isolates

Treatments	Isolate type	Grey mould incidence (%)
YP41	Yeast	8.33
YP46	Yeast	8.33
YP65	Yeast	8.33
YieldPlus®	Yeast	16.67
YP24	Yeast	25.00
YP78	Yeast	25.00
YP16	Yeast	25.00
YP49	Yeast	25.00
YP25	Yeast	33.33
YP45	Yeast	33.33
YP61	Yeast	33.33
YA55	Yeast	41.67
YP35	Yeast	41.67
YP82	Yeast	41.67
YP84	Yeast	41.67
YP85	Yeast	41.67
Control	*B. cinerea* only	100.00
Other isolates	Yeasts	≥ 50.00

Table 3.2 Blue mould (*Penicillium expansum*) incidence on "Packham's Triumph" pears reduced by antagonistic yeast isolates

Treatment	Isolate type	Blue mould incidence (%)
YieldPlus®	Yeast	16.67
YP53	Yeast	16.67
YA33	Yeast	33.33
YP84	Yeast	33.33
YP28	Yeast	41.67
YP43	Yeast	41.67
YP5	Yeast	41.67
YP60	Yeast	41.67
Control	*P. expansum* only	100.00
Other isolates	Yeasts	≥ 50.00

3.3.2 Further screening of selected yeast isolates antagonistic to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples

In the secondary biocontrol test, yeast isolates that were effective in the initial screening test were evaluated for their biocontrol activity against grey mould and blue mould infection. Yeast Isolates (YP25, YP16, YP41, YP78, YieldPlus®, YP61, YP24, YA55, YP46, YP49, YP65 and YP82) significantly ($P<0.003$) reduced grey mould incidence on "Golden Delicious" apples compared to the untreated control and other yeast Isolates (YP84, YP35, YP45, and YP85) (Table 3.3). Yeast Isolates YieldPlus®, YP28, YP53, YP60, YP5, YA33 and YP84 significantly ($P<0.002$) reduced blue mould incidence on "Golden Delicious" apples compared to the untreated control and Isolate YP43 (Table 3.4).

Table 3.3 Further screening of selected yeast antagonists to *Botrytis cinerea* on "Packham's Triumph" pear

Treatment	**Isolate type**	**Grey mould incidence (%)**
YP25	Yeast	8.3 a
YP16	Yeast	16.7 ab
YP41	Yeast	16.7 ab
YP78	Yeast	16.7 ab
YieldPlus®	Yeast	16.7 ab
YP24	Yeast	25.0 abc
YP46	Yeast	33.3 abc
YP61	Yeast	33.3 abc
YP49	Yeast	41.7 abc
YP65	Yeast	41.7 abc
YA55	Yeast	50.0 abc
YP82	Yeast	50.0 abc
YP84	Yeast	58.3 bcd
YP45	Yeast	58.3 bcd
YP35	Yeast	66.7 cd
YP85	Yeast	66.7 cd
Control	*B. cinerea* only	100.0 d
P-value		0.003
F-ratio		3.12
LSD		39.4
SED		19.4
CV%		57.7

Table 3.4 Further screening of selected yeast antagonists to *Penicillium expansum* on "Packham's Triumph" pears

Treatment	Isolate type	Blue mould incidence (%)
YieldPlus®	Yeast	16.7 a
YP60	Yeast	33.3 ab
YP84	Yeast	33.3 ab
YA33	Yeast	41.7 ab
YP28	Yeast	41.7 ab
YP53	Yeast	41.7 ab
YP5	Yeast	41.7 ab
YP43	Yeast	50.0 b
Control	*P. expansum* only	100.0 c
P-value		0.002
F-ratio		5.19
LSD		29.8
SED		14.2
CV%		39.0

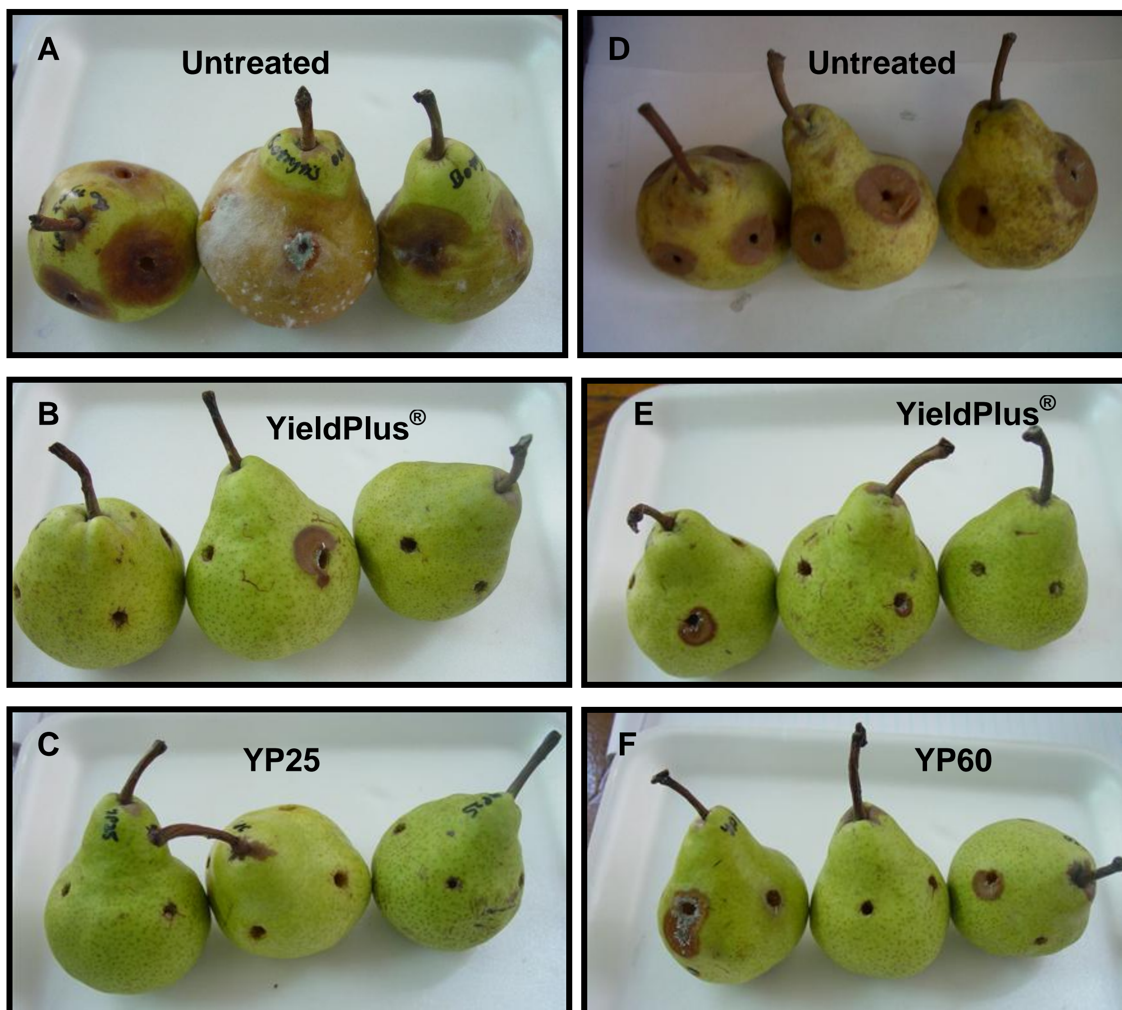

Figure 3.2 Control of grey mould and blue mould of "Golden Delicious" apples with yeast antagonists. Untreated fruit inoculated with *Botrytis cinerea* only (A); fruit treated with the biofungicide YieldPlus® (1×10^8 cells mℓ$^{-1}$) and inoculated with *B. cinerea* (1×10^4 conidia mℓ$^{-1}$) (B); fruit treated with yeast Isolate YP25 (1×10^8 cells mℓ$^{-1}$) and inoculated with *B. cinerea* (1×10^4 conidia mℓ$^{-1}$) (C); Untreated fruit inoculated with *Penicillium expansum* only (D), fruit treated with the biofungicide YieldPlus® (1×10^8 cells mℓ$^{-1}$) and inoculated with *B. cinerea* (1×10^4 conidia mℓ$^{-1}$) (E); fruit treated with yeast Isolate YP60 (1×10^8 cells mℓ$^{-1}$) and inoculated with *P. expansum* (1×10^4 conidia mℓ$^{-1}$) (F).

3.3.3 Preventative effect of selected yeast antagonists to *Botrytis cinerea* and *Penicillium expansum* on "Golden Delicious" apples

On "Golden Delicious" apples, all yeast isolates significantly reduced (P<0.001) grey mould. Yeast Isolates YP16, YP24, YP25, and the biofungicide YieldPlus® provided the best control of grey mould infection, and reduced grey mould incidence to 8.3% and 16.7%, respectively, compared to 100% incidence in control fruit. Yeast Isolates YP85 and YP84 were significantly less effective (P<0.001) than the other yeast isolates (Table 3.5). Although not significant, all yeast isolates and YieldPlus® reduced blue mould incidence, compared to the untreated control. YieldPlus® and YP60 provided the best control for blue mould infection with incidence levels of 16.7% and 25%, respectively, compared to 100% incidence in control fruit (Table 3.6).

Table 3.5 Preventative effect of selected yeast antagonists to *Botrytis cinerea* on "Golden Delicious" apples

Treatment	Isolate type	Grey mould incidence (%)
YP16	Yeast	8.3 a
YP24	Yeast	8.3 a
YP25	Yeast	8.3 a
YieldPlus®	Yeast	8.3 a
YA55	Yeast	16.7 a
YP61	Yeast	16.7 a
YP49	Yeast	25.0 ab
YP45	Yeast	25.0 ab
YP35	Yeast	33.3 abc
YP65	Yeast	33.3 abc
YP41	Yeast	33.3 abc
YP82	Yeast	33.3 abc
YP46	Yeast	41.7 abc
YP78	Yeast	41.7 abc
YP85	Yeast	50.0 bc
YP84	Yeast	66.7 c
Control	*B. cinerea* only	100.0 d
P-value		5.0
F-ratio		<0.001
LSD		30.7
SED		15.1
CV%		57.3

Table 3.6 Preventative effect of selected yeast antagonists to *Penicillium expansum* on "Golden Delicious" apples

Treatment	Isolate type	Blue mould incidence (%)
YieldPlus®	Yeast	16.7 a
YP60	Yeast	25.0 a
YP5	Yeast	33.3 a
YP28	Yeast	33.3 a
YP53	Yeast	33.3 a
YP43	Yeast	41.7 a
YP84	Yeast	41.7 a
YA33	Yeast	50.0 a
Control	*P. expansum* only	100.0 a
P value		0.05
F-ratio		2.48
LSD		45.2
SED		21.5
CV%		63.2

3.3.4 Dose effect of selected yeast isolates for the control of *Penicillium expansum* and *Botrytis cinerea* on "Golden Delicious" apples, when applied preventatively

Although not significant ($P \leq 0.95$), YieldPlus® and yeast Isolates YP16, YP24 and YP25, at a concentration 1×10^8 cells $m\ell^{-1}$, provided the best control of grey mould infection with incidence levels of 16.7%, 33.3%, 33.3% and 16.7%, respectively, compared to 100% incidence in control fruit (Figure 3.3). YieldPlus® and yeast Isolate YP60, at a concentration 1×10^8 cells $m\ell^{-1}$, provided the best control ($P \leq 0.78$) of blue mould infection with incidence levels of both 16.7%, compared to 100% incidence in control fruit (Figure 3.4). Reduced incidence of grey and blue moulds was observed with increasing concentration of yeast antagonist (Figure 3.3 and Figure 3.4, respectively).

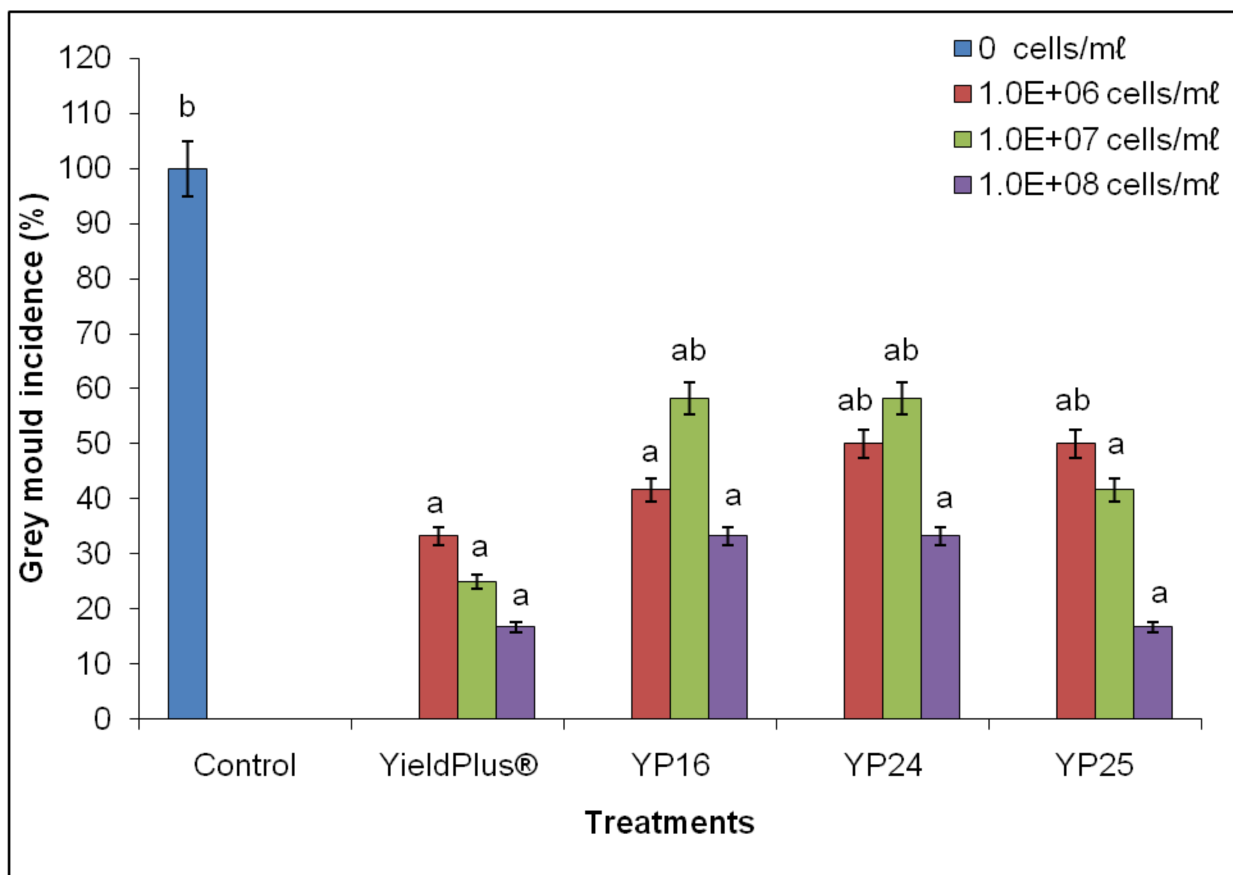

Figure 3.3 Dose effect of selected yeast antagonists on grey mould incidence, when applied 48 hrs before inoculation with *Botrytis cinerea* (1×10^4 conidia $mℓ^{-1}$) on "Golden Delicious" apples. Means followed by the same letter are not significantly different, according to the Fisher's Least Significant Difference Test (P = 0.05).

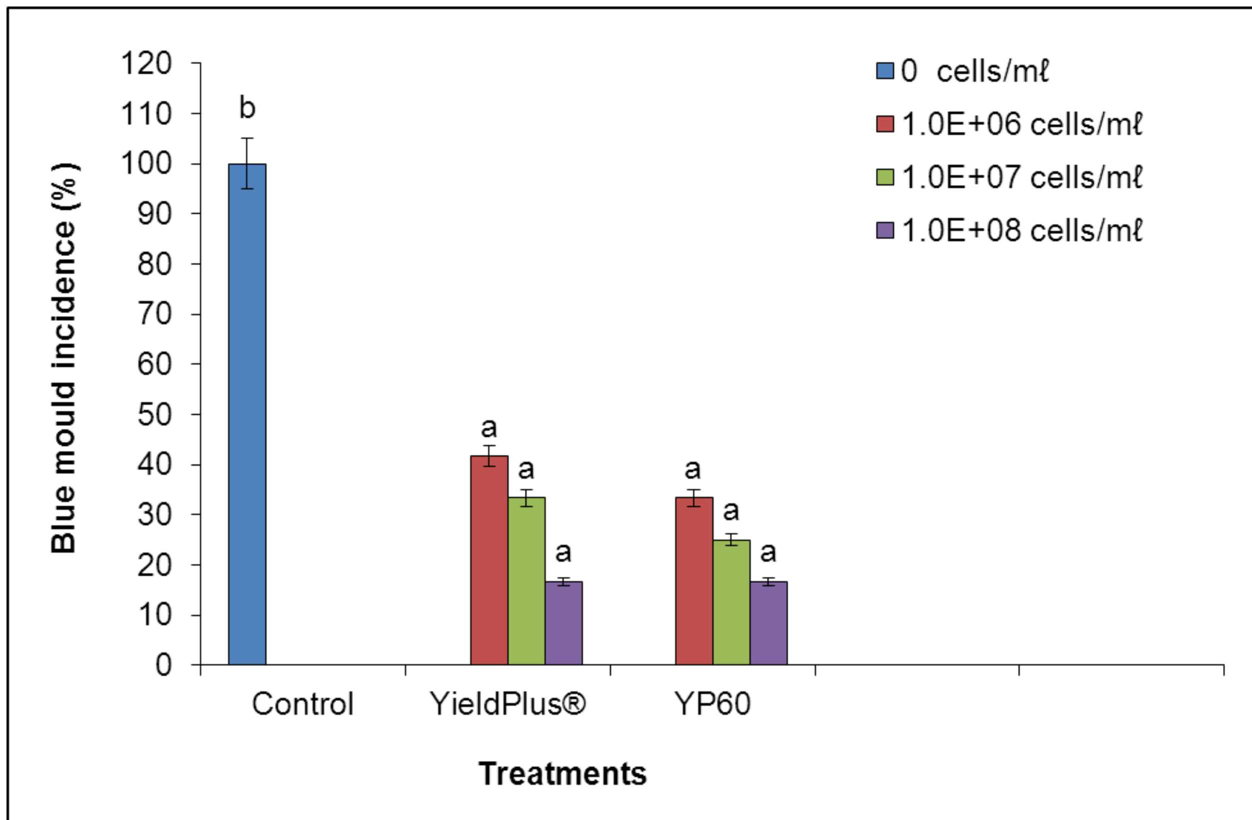

Figure 3.4 Dose effect of selected yeast antagonists on blue mould incidence, when applied 48 hrs before inoculation with *Penicillium expansum* (1×10^4 conidia $mℓ^{-1}$) on "Golden Delicious" apples. Means followed by the same letter are not significantly different, according to the Fisher's Least Significant Difference (Test P = 0.05).

3.3.5 Effect of a mixture of yeast antagonists (YP25 + YP60) on the control of *Botrytis cinerea* and *Penicillium expansum*, when applied preventatively on "Golden Delicious" apples

A mixture of yeast antagonists (YP25 + YP60) provided the best control of grey and blue moulds with incidence levels of 0%, respectively, compared to 100% incidence in control fruit (Figure 3.5 and Figure 3.6). Although not significant ($P<0.96$), a combination of the antagonists (YP25 + YP60) was more effective than YieldPlus®, yeast Isolate YP25 alone and yeast Isolate YP60 alone, in reducing grey mould incidence on "Golden Delicious" apples (Figure 3.5). Furthermore, a combination of the antagonists (YP25+YP60) was more effective than YieldPlus®, YP25 alone and YP60 alone, in reducing blue mould incidence on "Golden Delicious" apples (Figure 3.6).

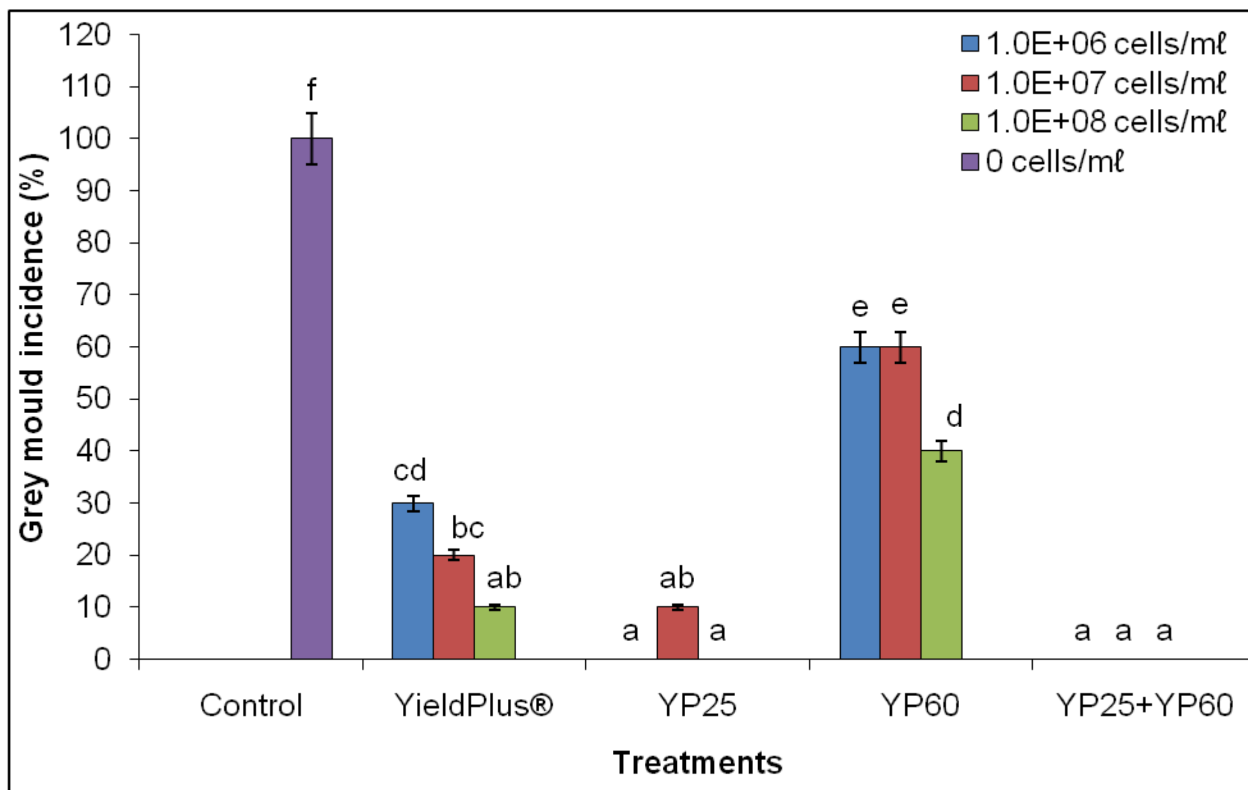

Figure 3.5 Effect of a yeast antagonist mixture (YP25+YP26) on grey mould incidence when applied 48 hrs before inoculation with *Botrytis cinerea* (1×10^4 conidia $mℓ^{-1}$) on "Golden Delicious" apples. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

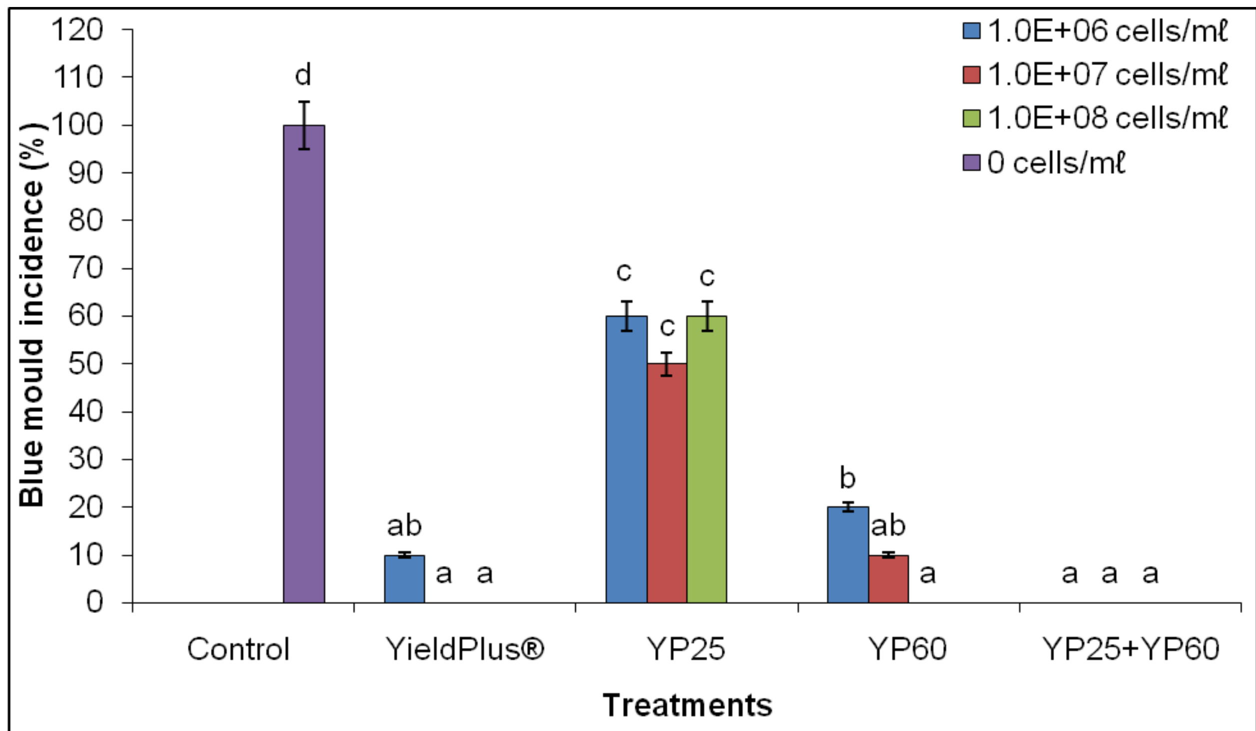

Figure 3.6 Effect of a yeast antagonist mixture (YP25+YP60) on blue mould incidence when applied 48 hrs before inoculation with *Penicillium expansum* (1×10^4 conidia $mℓ^{-1}$) on "Golden Delicious" apples. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

Figure 3.7 Control of grey mould and blue mould of "Golden Delicious" apples by a yeast antagonist mixture (YP25+YP60) applied preventatively (1×10^8 cells $m\ell^{-1}$), 48 hr before inoculation with *Botrytis cinerea* and *Penicillium expansum* (1×10^4 conidia $m\ell^{-1}$). Negative control inoculated with *B. cinerea* only (A); fruit treated with a mixture of yeast antagonist YP25+YP60 and inoculated with *B. cinerea* (B); Negative control inoculated with *Penicillium expansum* only (C); fruit treated with a mixture of yeast antagonist YP25+YP60 and inoculated with *P. expansum* (D); untreated fruits inoculated with distilled water only (E).

3.4 DISCUSSION

The objective of this research was to isolate antagonistic yeasts and to evaluate their efficacy in controlling infections caused by *B. cinerea* and *P. expansum* on pome fruit. The experimental data presented in this paper demonstrates the occurrence of antagonistic yeast isolates able to control grey mould and blue mould, caused by *B. cinerea and P. expansum*, respectively. A total of 100 yeast isolates were isolated from the surface of apples and pears and tested for *in vivo* antagonism against *B. cinerea and P. expansum*. Only 15 yeast isolates showed antagonistic activity against *B. cinerea* (Table 3.1). Only 7 yeast isolates showed antagonistic activity against *P. expansum* (Table 3.2). The isolation of yeasts from fruit surfaces with different level of antagonistic biocontrol efficiency against pathogens has been reported in several studies (Lima *et al*., 1997; Viňas *et al*., 1998).

In secondary screening, some yeast isolates provided postharvest control of *B. cinerea* and *P. expansum* (Table 3.3 and Table 3.4, respectively). The effectiveness of some yeast isolates was poor probably because the test was done on mature, late season apples. Fruit maturity influences susceptibility to decay. El-Ghaouth *et al.* (2002) showed that the antagonist yeast, *Candida saitoana* (Nakasa and Suzuki), was more effective in controlling decay on early-season "Golden Delicious" apples than on late-season fruits.

In a preventative trial, the yeast Isolates YP16, YP24, YP25, and YieldPlus® provided the best control of grey mould infection, and reduced grey mould incidence compared to the untreated control (Table 3.5). The biofungicide YieldPlus® and yeast Isolate YP60 provided the best preventative control of blue mould infection (Table 3.6). In general, it was found that the earlier the antagonist application and the later the pathogen application, the better the control of grey mould and blue mould, agreeing with the results of other researchers. De Capdeville *et al.* (2002) reported that yeasts reduced the area under the disease progress curve (AUDPC) of *Penicillium expansum* (Link) Thom. on apple fruit more effectively when applied to the fruit 24 hrs (134%) or 96 hrs (41%) before inoculation with the pathogen than when applied 24 hrs after inoculation.

Chalutz and Wilson (1990) also found that the efficacy of a yeast isolate of *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij against green and blue moulds of grapefruit was reduced if application was delayed by three hours or more after inoculation of the pathogen.

The biofungicide, YieldPlus® and yeast Isolate YP25 showed a greater control efficacy against *B. cinerea* at a concentration of 1×10^8 cells $m\ell^{-1}$ compared to 1×10^6 cells $m\ell^{-1}$ and 1×10^7 cells $m\ell^{-1}$ (Figure 3.3). Furthermore, YieldPlus® and yeast Isolate YP60 were not significantly different in controlling *P. expansum* when applied at a concentration of 1×10^8 cells $m\ell^{-1}$ (Figure 3.4). Usually, the higher the concentration of an antagonist, the better the biocontrol activity (Zhang *et al.,* 2009). Zheng *et al.* (2005) reported the control of green mould of oranges with yeast *Rhodoturula glutinis* (Harrison) when applied at a concentration of 1×10^9 cells $m\ell^{-1}$.

Janisiewicz and Korsten (2002) showed that biocontrol could be improved by manipulation of the antagonist's environment, the antagonist mixture, and manipulation of antagonists to increase their ecological fitness and biocontrol function. In the antagonist mixture trial, a mixture of YP25+YP60 provided complete control of *B. cinerea* and *P. expansum* development when applied at concentrations of 1×10^6 cells $m\ell^{-1}$, 1×10^7 cells $m\ell^{-1}$and 1×10^8 cells $m\ell^{-1}$, compared to independent applications of each antagonist. The use of antagonist mixtures may provide certain advantages: 1) Increase in the effectiveness under different conditions such as cultivars, maturity stages, and location (Sharma *et al.*, 2009); 2) It can result in the control of more than one postharvest disease (Sharma *et al.*, 2009); 3) Reduction in application rates and cost treatments (Sharma *et al.*, 2009); 4) Combination of different biocontrol traits without the transfer of alien genes through genetic transformation (Sharma *et al.*, 2009); and 5) Enhancement in efficiency and reliability of biocontrol as the components of the mixture acts through different mechanisms like antagonism, parasitism, and induction of resistance in the host (Sharma *et al.*, 2009). A mixture of the antagonists *Candida sake* (Saito and Oda) van Uden and H.R. Buckley and *Pantoea agglomerans* (Ewing and Fife) Gavini. controlled fruit rot completely in 'Blanquilla' pear and provided the highest

level of control in blue mould rot on "Golden Delicious" apples (Nunes *et al.,* 2002). Leibinger *et al.* (1997) showed that a mixture of *Aureobasidium pillulans* (de Bary) Arnaud and *Bacillus subtilis* (Ehrenberg) provided control of *P. expansum* and *B. cinerea* on apples. The coefficient of variation in trials was too high and this could be due to (i) variation of laboratory conditions; and (ii) data was not normally distributed.

3.5 CONCLUSION

This study has indicated that yeast antagonists may be used as a non-chemical alternative treatment against postharvest grey mould and blue mould of pome fruits; mixed cultures of yeast Isolates YP25 and YP60 exhibited complete biocontrol activity on *P. expansum* and *B. cinerea* than either yeast applied alone.

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CHAPTER 4

PREVENTATIVE EFFECT OF POTASSIUM SILICATE, POTASSIUM CHLORIDE AND POTASIUM HYDROXIDE POSTHARVEST TREATMENTS FOR THE CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* ON APPLES

ABSTRACT

The effects of potassium silicate, potassium chloride and potassium hydroxide for the control of postharvest grey and blue moulds were tested on “Golden Delicious” apples. The incidence of grey mould and blue mould was significantly (P≤0.001) reduced by the application of potassium silicate, potassium chloride and potassium hydroxide on wounds of apple fruit. Fruit treated with potassium silicate at a concentration of 100,000 mg ℓ^{-1} had the lowest incidence of grey and blue moulds (77.5% and 65%, respectively) when it was applied as a preventative treatment (24 hrs before inoculation with either *Penicillium expansum* or *Botrytis cinerea*). Scanning electron microscopy studies confirmed that potassium silicate applications to apple wounds stopped germination of conidia of both *B. cinerea* and *P. expansum*. Curative treatment (inoculated with either *P. expansum* or *B. cinerea* 4 hrs before treatment) of apples with potassium silicate at a concentration of 100,000 mg ℓ^{-1} resulted in reduced incidence of grey and blue moulds (52.5% and 55%, respectively). Both potassium chloride and potassium hydroxide reduced the incidence of blue mould (77.5% and 70%, respectively) and grey mould (65% and 62.5%, respectively) on “Golden Delicious” apples when applied as a preventative treatment (24 hours before inoculation with either *P. expansum* or *B. cinerea*).

4.1 INTRODUCTION

Postharvest diseases cause economic losses for producers of pome fruit. Losses during storage are mainly caused by *Penicillium expansum* (Link) Thom. and *Botrytis cinerea* Pers.:Fr. Postharvest fungicide treatments are the primary means of controlling these losses. However, the development of resistance to most postharvest fungicides by

pathogens (Conway *et al.*, 2004) and public concern over the potential impact of fungicides on human health and the environment (Droby *et al.*, 2009), have motivated the need to develop an alternative strategy for disease control.

Several additives have shown potential for disease control, e.g., being silicates (Belanger *et al*., 1995), essential oils (Thompson, 1989; Ryu and Holt, 1993), carbonates and bicarbonates (Palmer *et al*., 1997; Smilanick *et al*., 1999, Conway *et al*., 2007; Janisiewicz *et al*., 2008; Smilanick *et al*., 2008), calcium salts (Conway, 1982; Conway *et al*., 1988), chitosan (El-Ghaouth *et al*., 1992) and the sugar analogue, 2-deoxy-D-glucose (El-Ghaouth *et al*., 1995; El-Ghaouth *et al*., 1997).

Research by Palou *et al.* (2001) has shown that carbonic acid salts, sodium bicarbonate (baking powder) and sodium carbonate, have the potential to reduce levels of postharvest diseases of fruit. Several researchers have reported that salt additives, including calcium propionate, calcium chloride, sodium carbonate, and ammonium molybdate were effective when used in combination with microbial antagonists for the control of postharvest diseases of fruits and vegetables (Plaza *et al*., 2001; Teixido *et al*., 2001; Karabulut *et al*., 2005; Qin *et al*., 2006; Janisiewicz *et al*., 2008). Conway *et al.* (2005) have shown that improving biocontrol using antagonist mixtures with heat and/ or sodium bicarbonate has the potential to control postharvest decay of apples. A 10% ethanol solution was effective in controlling *Monilinia fructicola* (G. Winter) Honey. and *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. on peaches and nectarines (Margosan *et al*., 1997) and *Penicillium digitatum* (Pers:Fr. Sacc.) on lemons (Smilanick *et al*., 1995). Sodium silicate and calcium chloride have been shown to control blue and green moulds of Clementine mandarins (Ligorio *et al.,* 2007).

Silicon (Si) plays a major role in the physiology of some plants. It influences plant development, growth and disease resistance (Epstein, 1999). Several researchers have reported that silicon applications reduce plant diseases when applied as a fertilizer, using soil or foliar applications (Cherif *et al*., 1992; Menzies *et al*., 1992; Cherif *et al*., 1994, Belanger *et al*., 1995; Rodrigues *et al*., 2003). The mechanisms involved in Si-induced resistance of plants to fungal diseases are not well understood (Guo *et al.,* 2007). Silicon may be effective by eliciting biochemical defense reactions, involving the accumulation of lignin, phenolic compounds, and pathogenesis-related proteins in infected plants (Cherif *et al.,* 1992; Epstein, 1999).

The objective of this study was to investigate whether potassium silicate, potassium chloride and potassium hydroxide postharvest applications could control *P. expansum* and *B. cinerea* infections on “Golden Delicious” apples.

4.2 MATERIALS AND METHODS

4.2.1 Potassium solutions

A liquid formulation of dissolved potassium silicate (K_2SiO_3), with a concentration of 20.5%, provided by PQ Silicas[1], was used as the silicon source. Potassium chloride (KCℓ) and potassium hydroxide (KOH) salts were also used to evaluate their efficacy for the control of grey and blue moulds. The pH of potassium chloride (Table 4.1), potassium silicate (Table 4.2), and potassium hydroxide (Table 4.3) at different concentrations was determined.

[1]PQ Silicas South Africa (Pty) Ltd, 169 Tedstone Road. Wadeville, 1407, P.O. Box 12062, Gauteng, South Africa.

Table 4.1 pH of potassium chloride (KCℓ) at different concentrations

Concentration (mg $ℓ^{-1}$)	Dilution (g/L)	pH
10	0.038	9.00
100	0.381	9.89
1,000	3.813	10.36
10,000	38.13	10.53
100,000	381.3	10.82

Table 4.2 pH of potassium silicate (K_2SiO_3) at different concentrations

Concentration (mg $ℓ^{-1}$)	Dilution (mℓ/L)	pH
10	0.1	9.23
100	1.0	9.61
1,000	10	10.17
10,000	100	10.29
100,000	Absolute K_2SiO_3	11.21

Table 4.3 pH of potassium hydroxide (KOH) at different concentrations

Concentration (mg $ℓ^{-1}$)	Dilution (g/L)	pH
10	0.00229	10.83
100	0.0229	11.22
1,000	0.229	11.39
10,000	2.29	11.60
100,000	22.9	11.75

4.2.2 Evaluation of the preventative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples

"Golden Delicious" apples were purchased at Pick 'n Pay supermarket from Pietermaritzburg, South Africa. The fruit was disinfected with 70% alcohol for 1 minute, washed with tap water, and air dried prior to wounding. Each apple was wounded (5 mm deep by 5mm wide) with a sterile needle at equatorial positions. Four wounds were made in each fruit. The wounds were inoculated with 30 µℓ of potassium silicate (K_2SiO_3) solution at concentrations of 10, 100, 1,000, 10,000 and 100,000 mg $ℓ^{-1}$. After the wound site had dried for 24 hrs, each wound was inoculated with 20 µℓ of conidia of *P. expansum* suspension at a concentration of 1×10^4 conidia m$ℓ^{-1}$. Untreated wounds inoculated with the same amount of *P. expansum* conidia served as a control. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. Disease incidence (DI) of blue mould was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was used to evaluate the preventative effect of K_2SiO_3 against *B. cinerea*. The experiment was conducted twice. There were 3 fruits per replicate and 10 replicates per treatment.

4.2.3 Evaluation of the curative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea* as a postharvest treatment on apples

Similar methods as in Section 4.2.2 were used. The difference was that: individual wounded apples were first inoculated with 20 µℓ of *B. cinerea* conidia at a concentration of 1×10^4 conidia m$ℓ^{-1}$. After the wound site had dried for 24 hrs, each fruit wound was inoculated with a 30 µℓ solution of K_2SiO_3 at the same concentrations described in Section 4.2.2. Untreated wounds inoculated with the same amount of *B. cinerea* conidia served as a control. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. Disease incidence (DI) of grey mould was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was also used to evaluate the curative

activity of potassium silicate against *P. expansum*. The experiment was conducted twice. There were 3 fruits per replicate and 10 replicates per treatment.

4.2.4 Evaluation of the preventative activity of potassium chloride and potassium hydroxide against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples

"Golden Delicious" apples were disinfected with 70% alcohol for 1 minute, washed with tap water, and air dried prior to wounding. Each apple was wounded as described in Section 4.2.2 and treated with 30 µℓ of either potassium chloride or potassium hydroxide solutions, at concentrations of 10, 100, 1,000, 10,000 and 100,000 mg $ℓ^{-1}$. After the wound site had dried for 24 hrs, each wound was inoculated with 20 µℓ of conidia of a *P. expansum* suspension containing a concentration of 1×10^4 conidia $mℓ^{-1}$. Untreated wounds inoculated with the same amount of *P. expansum* conidia served as a control. Treatments were placed on a bench in a complete randomized block design (CRBD) for 7 days at 25°C and at a RH of 95%. Disease incidence (DI) of blue mould was determined as follow: DI (%) = (number of decayed wounds ÷ number of total wounds) × 100. The same procedure was used to evaluate the preventative effect of potassium silicate and potassium hydroxide against *B. cinerea*. The experiment was conducted twice. There were 3 fruits per replicate and 10 replicates per treatment.

4.2.5 Observations of *in vivo* interaction of potassium silicate with *Penicillium expansum* and *Botrytis cinerea* in apple wounds using scanning electron microscopy

Apples were wounded, then treated with K_2SiO_3 at a concentration of 100,000 mg $ℓ^{-1}$, and then inoculated with either *P. expansum* or *B. cinerea,* as described in Section 4.2.2. Fruit were incubated at 25°C for seven days. After the incubation period, tissue from the treated, and the control wounds, was excised. The tissue samples were fixed overnight in 3% glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.2). The samples were dehydrated in an ethanol series (10 minutes each in 30%, 50%, 70%, 80%, 90%, and 3 × 10 minutes in 100% ethanol) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% ethanol and placed in

a pre-cooled critical point drier. Following critical point drying (CPD) and gold-palladium sputter coating (Polaron Equipment Limited ESEM, coating unit E5100), the samples were viewed in an environmental scanning electron microscopy (ESEM) (Philips, FEI XL 30) at an accelerating voltage of 15 keV. Samples from three fruit from each treatment were viewed.

4.2.6 Statistical analysis

All data were subjected to statistical analysis of variance (ANOVA) using Genstat 14th edition. To determine differences between treatments, Fisher's Least Significant Difference Test was used (P = 0.05).

4.3 RESULTS

4.3.1 Preventative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea*, when applied as a postharvest treatment on apples

Potassium silicate treatments at all concentrations significantly reduced ($P<0.001$) grey mould incidence on "Golden Delicious" apples (Figure 4.1). The control treatment had the highest incidence of grey mould (100%) and it was significantly different ($P<0.001$) from fruit inoculated with 10, 100, 1,000, 10, 000, and 100,000 mg ℓ^{-1} (Figure 4.1). Grey mould incidence was reduced from 75% at a concentration of 10 mg ℓ^{-1} to 72.5% at a concentration of 100 mg ℓ^{-1} (Figure 4.1). Increasing K_2SiO_3 concentrations from 1,000 to 100,000 mg ℓ^{-1}, reduced grey mould incidence from 47.5% to 22.5% (Figure 4.1). Grey mould incidence decreased as a function of dose of potassium silicate (R^2 = 0.930) (Figure 4.2).

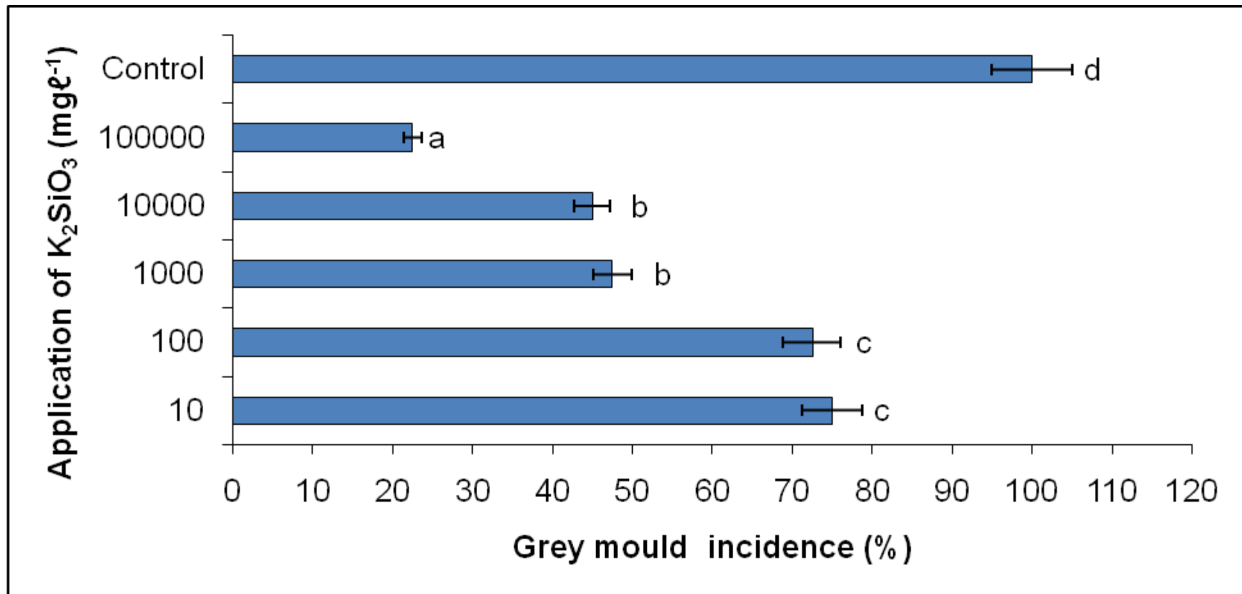

Figure 4.1 Disease incidence of *Botrytis cinerea* on "Golden Delicious" apples treated preventatively with various concentrations of potassium silicate. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

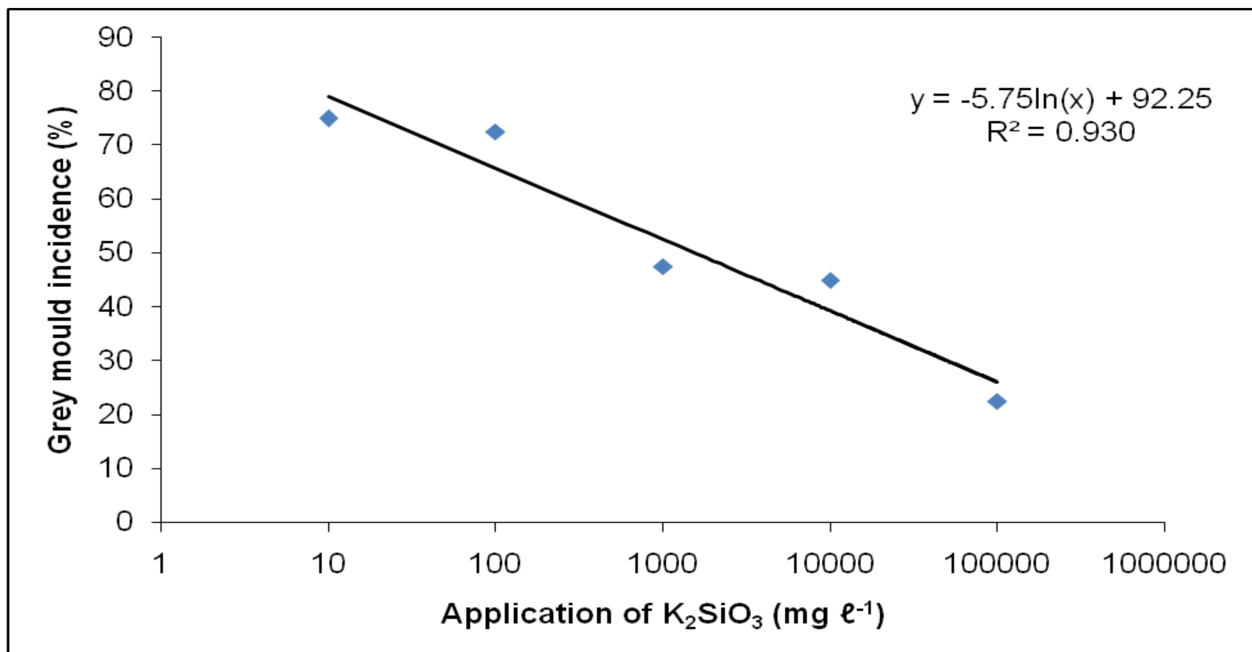

Figure 4.2 Logarithmic regression of dose of potassium silicate on grey mould incidence.

Potassium silicate treatments at all concentrations significantly ($P<0.001$) reduced blue mould incidence on "Golden Delicious" apples (Figure 4.3). The control treatment had the highest incidence of blue mould (100%) and it was significantly different ($P<0.001$) from fruit inoculated with 10, 100, 1,000, 10, 000, and 100,000 mg ℓ^{-1} (Figure 4.3). Blue mould incidence was reduced from 75% at a concentration of 100 mg ℓ^{-1} to 50% at a concentration of 1,000 mg ℓ^{-1} (Figure 4.3). The incidence of blue mould on apples treated with K_2SiO_3 at 1,000 mg ℓ^{-1} was not significantly different ($P<0.001$) 100,000 mg ℓ^{-1} (Figure 4.3). Increasing K_2SiO_3 concentrations from 10,000 to 100,000 mg ℓ^{-1}, reduced blue mould incidence from 60% to 35% (Figure 4.3). Blue mould incidence decreased as a function of dose of potassium silicate (R^2 = 0.701) (Figure 4.4).

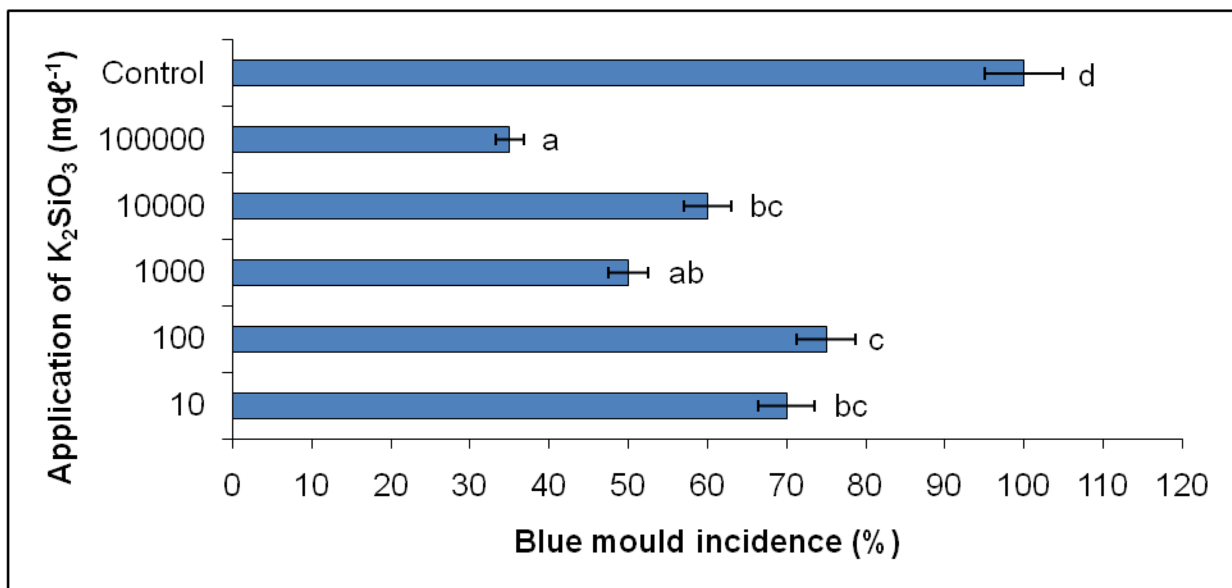

Figure 4.3 Disease incidence of *Penicillium expansum* on "Golden Delicious" apples treated preventatively with various concentrations of potassium silicate. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test ($P = 0.05$).

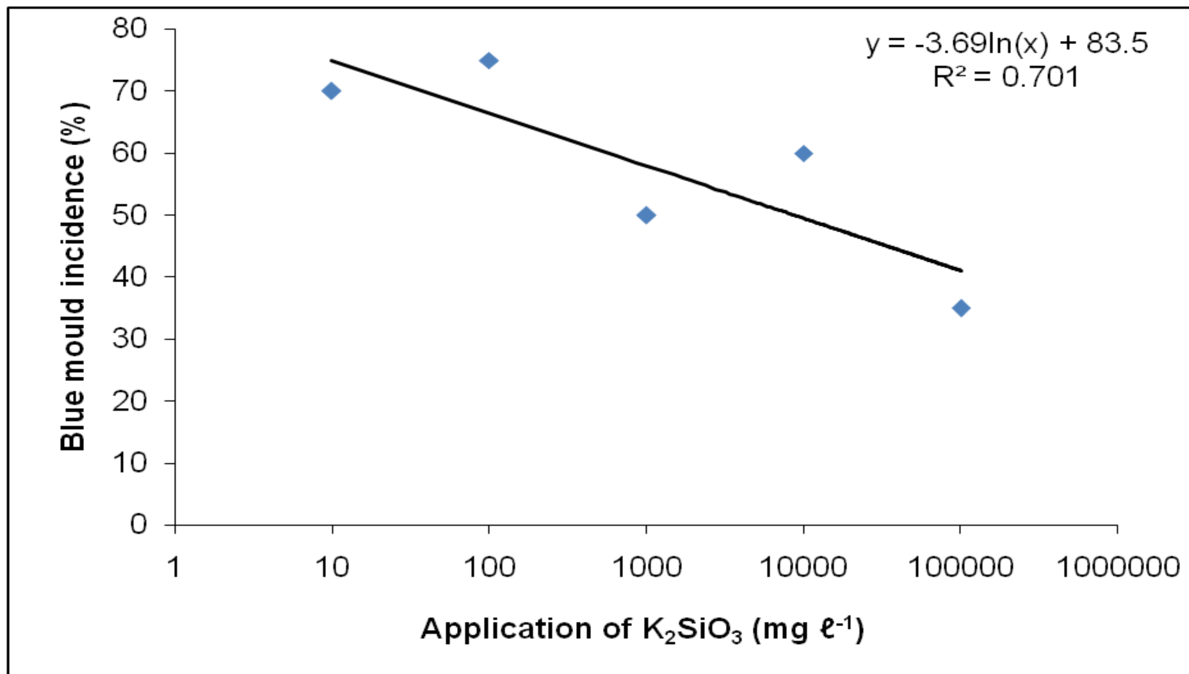

Figure 4.4 Logarithmic regression of dose of potassium silicate on blue mould incidence.

4.3.2 Curative activity of potassium silicate against *Penicillium expansum* and *Botrytis cinerea,* applied as a postharvest treatment on apples

Application of potassium silicate significantly (P<0.001) reduced grey mould on “Golden Delicious” apples, except at a concentration of 10 mg $ℓ^{-1}$ (Figure 4.5). The control treatment had the highest incidence of grey mould (100%) and it was significantly different (P<0.001) from fruit inoculated with 100, 1,000, 10, 000, and 100,000 mg $ℓ^{-1}$ (Figure 4.5). K_2SiO_3 concentrations, from 10 to 100,000 mg $ℓ^{-1}$, reduced grey mould incidence from 82.5% to 47.5%, respectively (Figure 4.5). Grey mould incidence decreased as a function of dose of potassium silicate ($R^2 = 0.883$) (Figure 4.6).

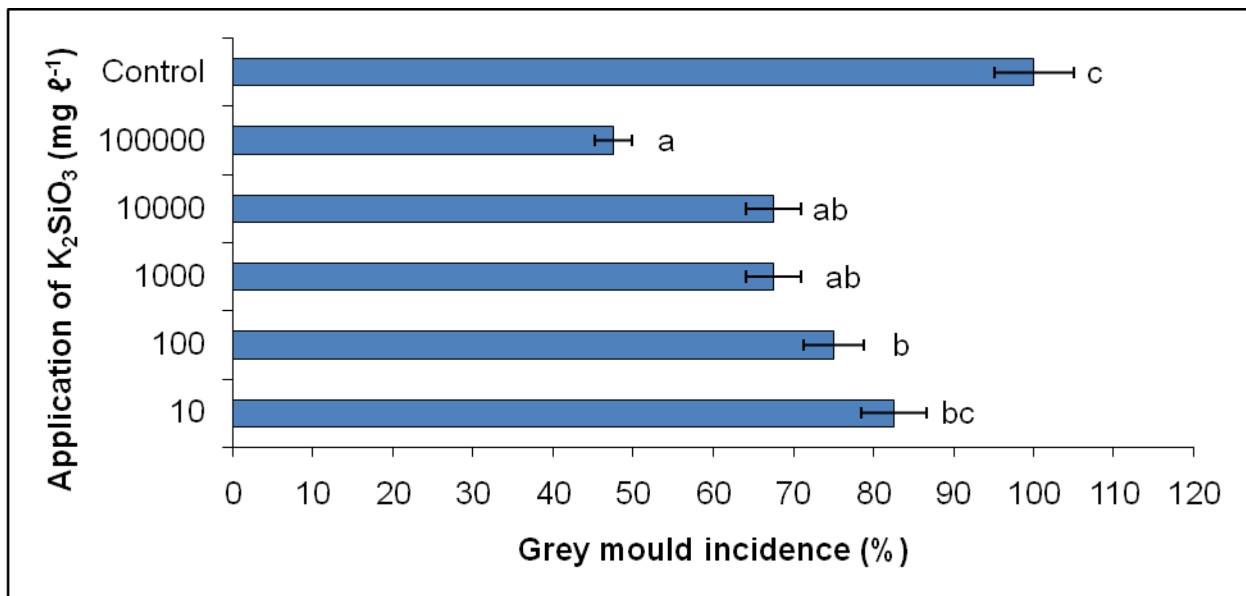

Figure 4.5 Disease incidence of *Botrytis cinerea* on “Golden Delicious” apples treated curatively with various concentrations of potassium silicate. Means followed by the same letter are not significantly different according to the Fisher’s Least Significant Difference Test (P = 0.05).

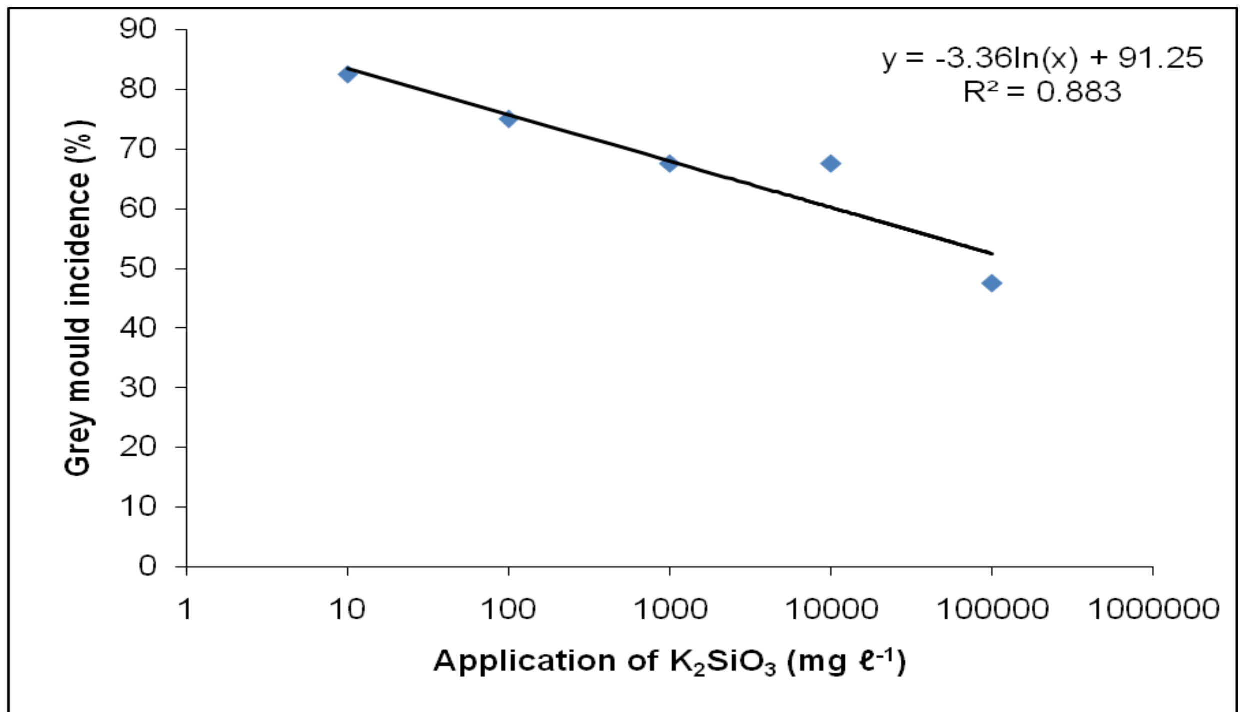

Figure 4.6 Logarithmic regression of dose of potassium silicate on grey mould incidence.

Blue mould incidence was significantly reduced (P<0.001) by the application of K_2SiO_3 at all concentrations, compared to 100% incidence in control treatment (Figure 4.7). Curative effects of K_2SiO_3 on "Golden Delicious" apples at concentrations of 100 mg ℓ^{-1}, 1,000 mg ℓ^{-1}, 10,000 mg ℓ^{-1}, and 100,000 mg ℓ^{-1} were not significantly (P<0.001) different from each other (Figure 4.7). Blue mould incidence decreased as a function of dose of potassium silicate (R^2 = 0.946) (Figure 4.8).

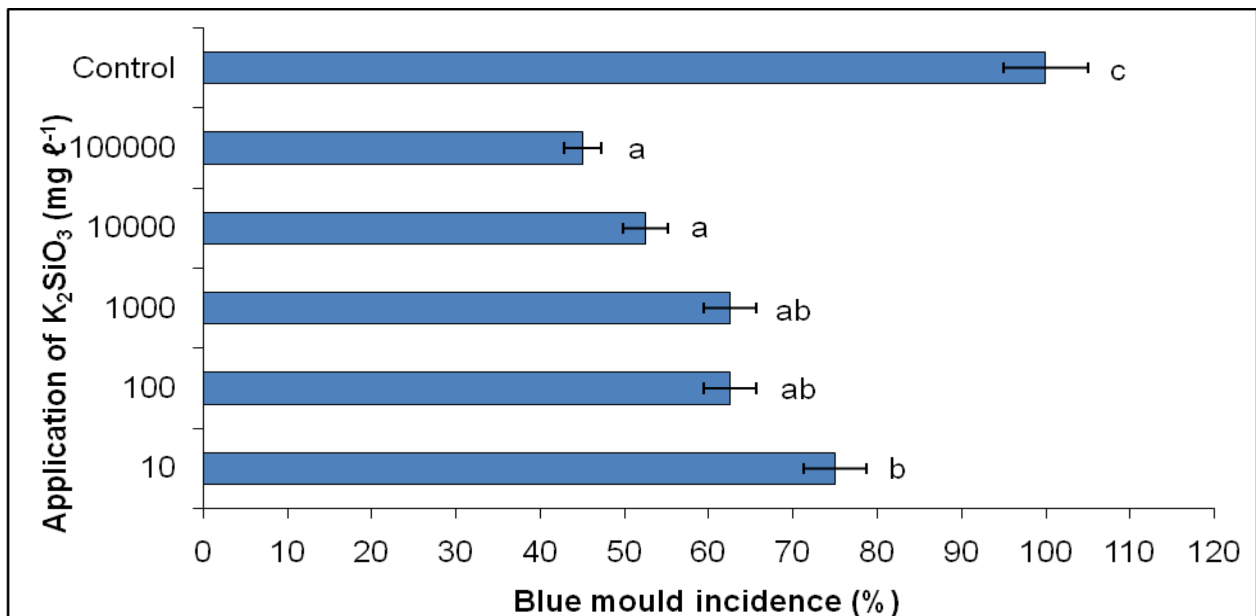

Figure 4.7 Disease incidence of *Penicillium expansum* on "Golden Delicious" apples treated curatively with various concentrations of potassium silicate. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

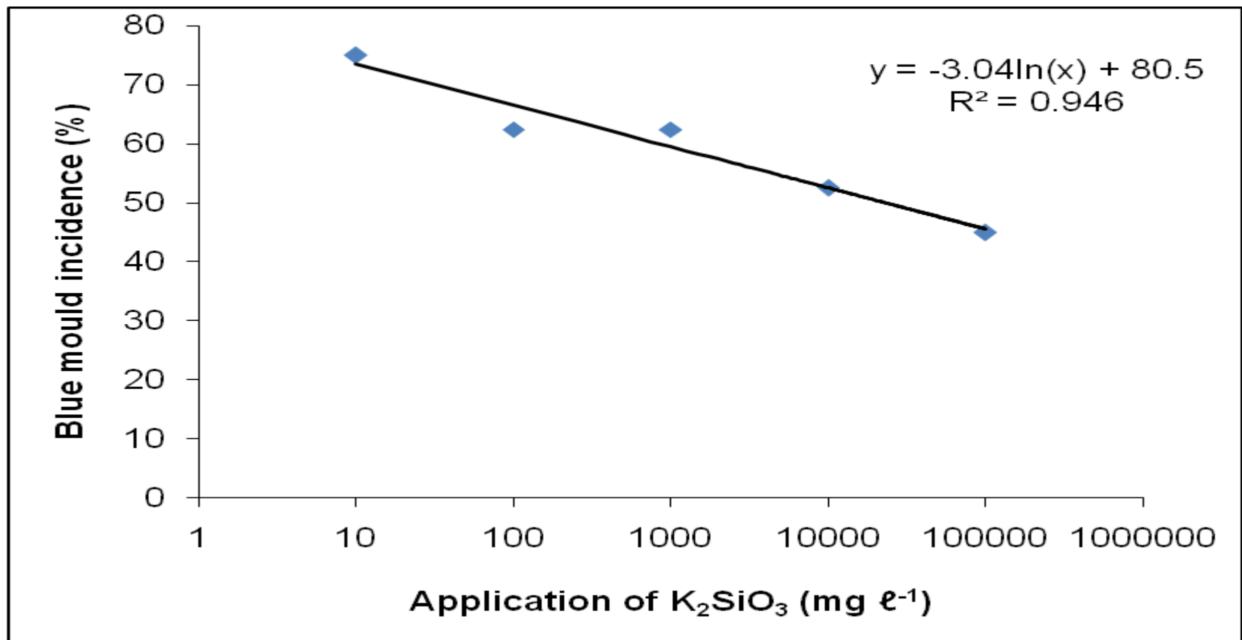

Figure 4.8 Logarithmic regression of dose of potassium silicate on blue mould incidence.

4.3.3 Preventative activity of potassium chloride and potassium hydroxide against *Penicillium expansum* and *Botrytis cinerea* when applied as a postharvest treatment on apples 24 hrs before pathogen inoculation

Potassium chloride treatment at all concentrations significantly (P<0.001) reduced blue mould incidence on “Golden Delicious” apples (Figure 4.9). Increasing KCℓ concentrations from 100 to 100,000 mg $ℓ^{-1}$, reduced blue mould incidence from 45% to 22.5%, respectively, compared to 100% incidence in control fruit (Figure 4.9). Preventative effects of KCℓ on “Golden Delicious” apples at concentrations of 10, 100, 1,000, and 10, 000 mg$ℓ^{-1}$ were not significantly (P<0.001) different from each other (Figure 4.9). Blue mould incidence decreased as a function of dose of potassium chloride (R^2 = 0.757) (Figure 4.10).

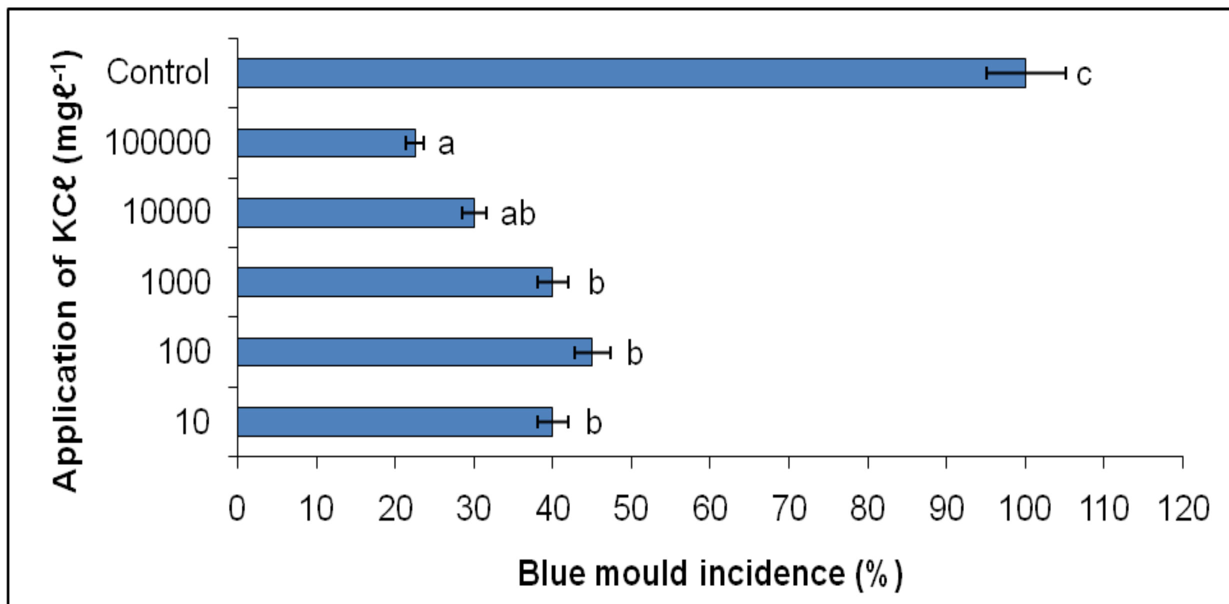

Figure 4.9 Disease incidence of *Penicillium expansum* on “Golden Delicious” apples treated preventatively with various concentrations of potassium chloride. Means followed by the same letter are not significantly different according to the Fisher’s Least Significant Difference Test (P = 0.05).

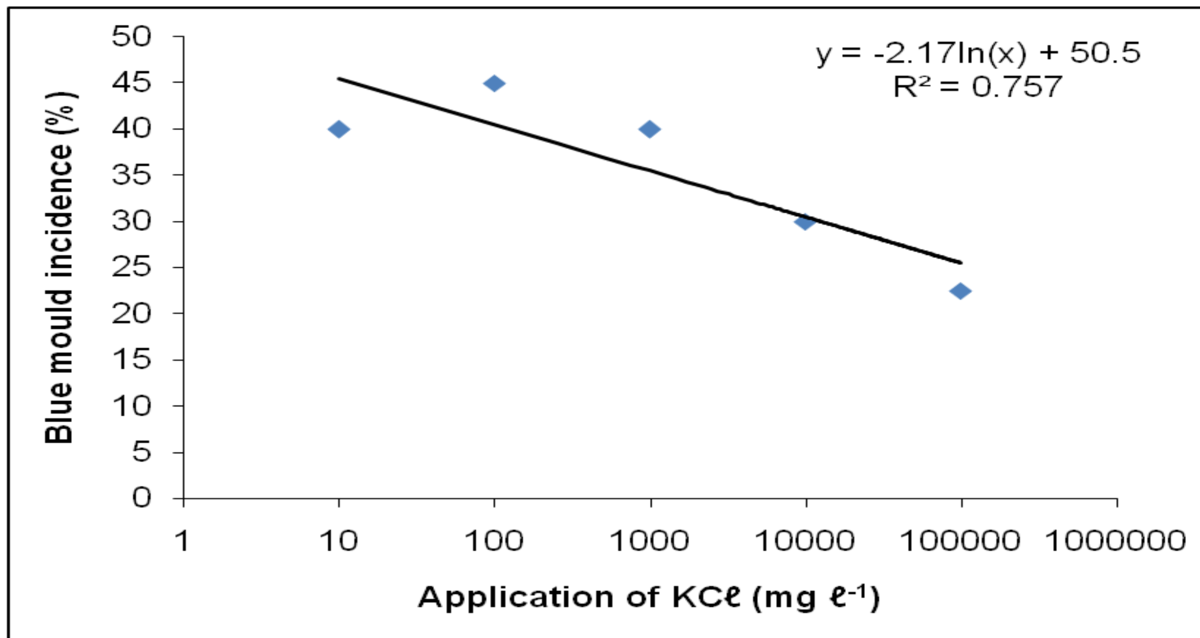

Figure 4.10 Logarithmic regression of dose of potassium chloride on blue mould incidence.

Application of KCℓ at concentrations of 100 to 100,000 mg $ℓ^{-1}$ was not significant (P<0.001) and grey mould incidence was reduced from 40% to 35%, respectively (Figure 4.11). Preventative effects of KCℓ on "Golden Delicious" apples at concentrations of 10, 100 and 10, 000 mg $ℓ^{-1}$ were not significantly different (P<0.001) from each other (Figure 4.11). Grey mould incidence decreased as a function of dose of potassium chloride (R^2 = 0.625) (Figure 4.12).

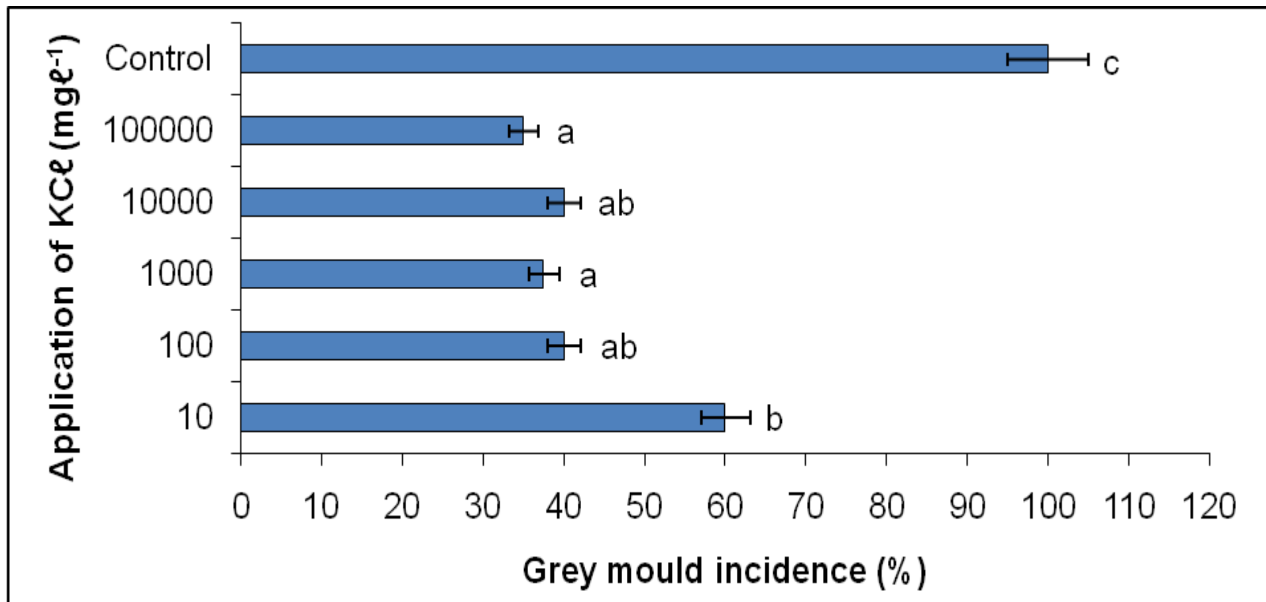

Figure 4.11 Disease incidence of *Botrytis cinerea* on "Golden Delicious" apples treated preventatively with various concentrations of potassium chloride. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

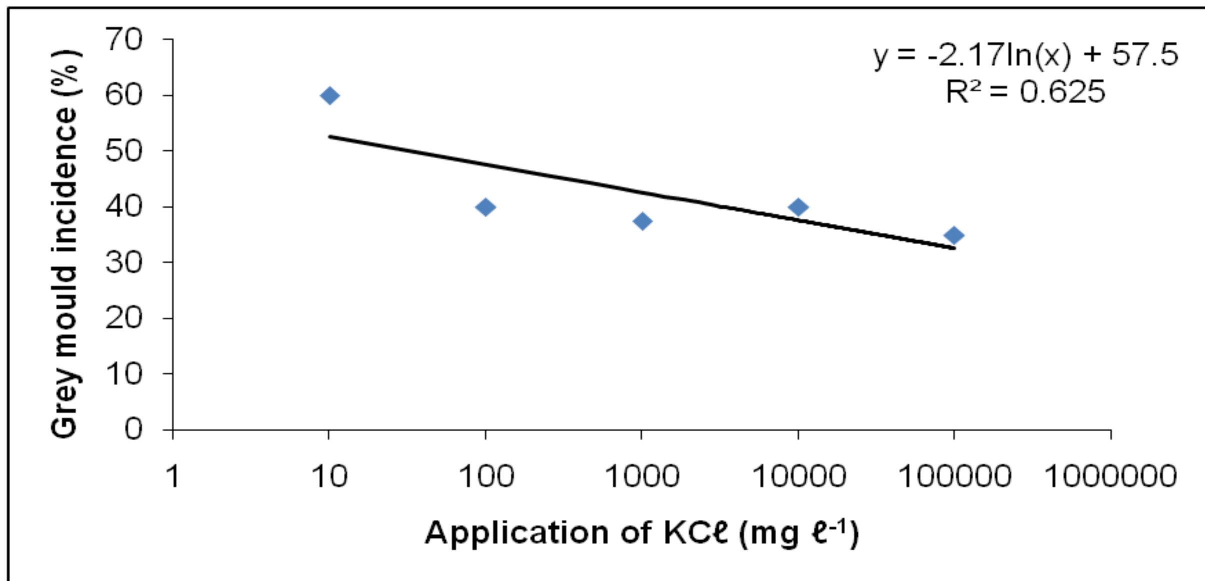

Figure 4.12 Logarithmic regression of dose of potassium chloride on blue mould incidence.

Application of KOH at all concentrations significantly (P<0.001) reduced blue mould incidence on "Golden Delicious" apples, compared to control treatment (Figure 4.13). Increasing KOH concentrations from 10 to 100,000 mg ℓ^{-1}, reduced blue mould incidence from 55% to 30%, respectively (Figure 4.13). Preventative effects of KOH on "Golden Delicious" apples at concentrations of 1,000, 10, 000 and 100, 000 mg ℓ^{-1} were not significantly different (P<0.001) from each other (Figure 4.13). Blue mould incidence decreased as a function of dose of potassium hydroxide ($R^2 = 0.820$) (Figure 4.14).

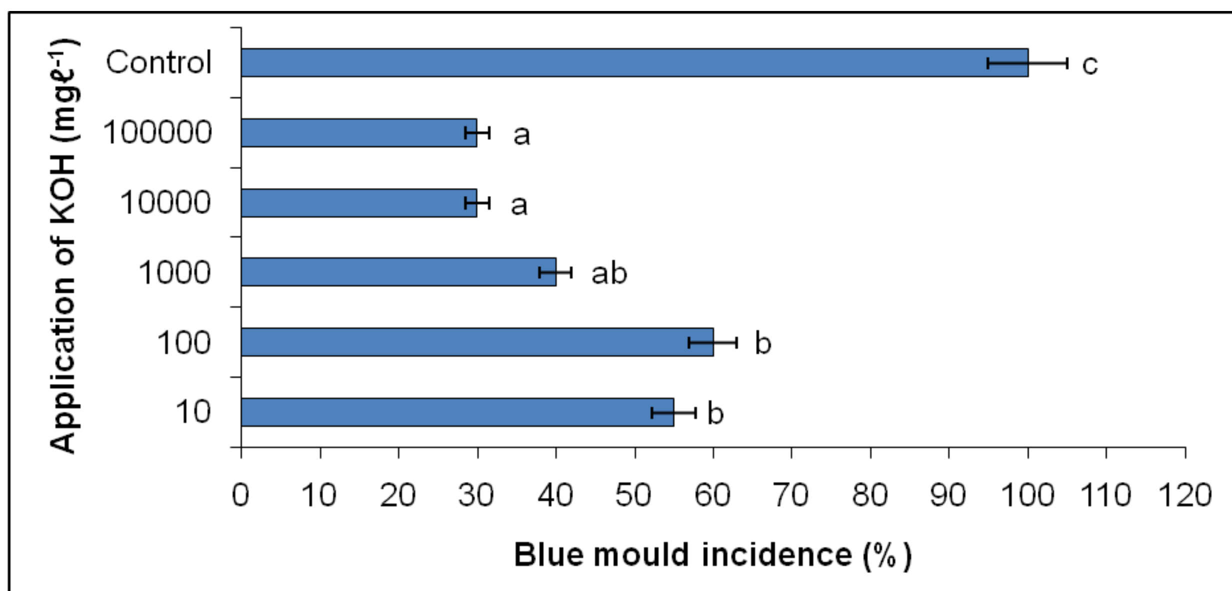

Figure 4.13 Disease incidence of *Penicillium expansum* on "Golden Delicious" apples treated preventatively with various concentrations of potassium hydroxide. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test (P = 0.05).

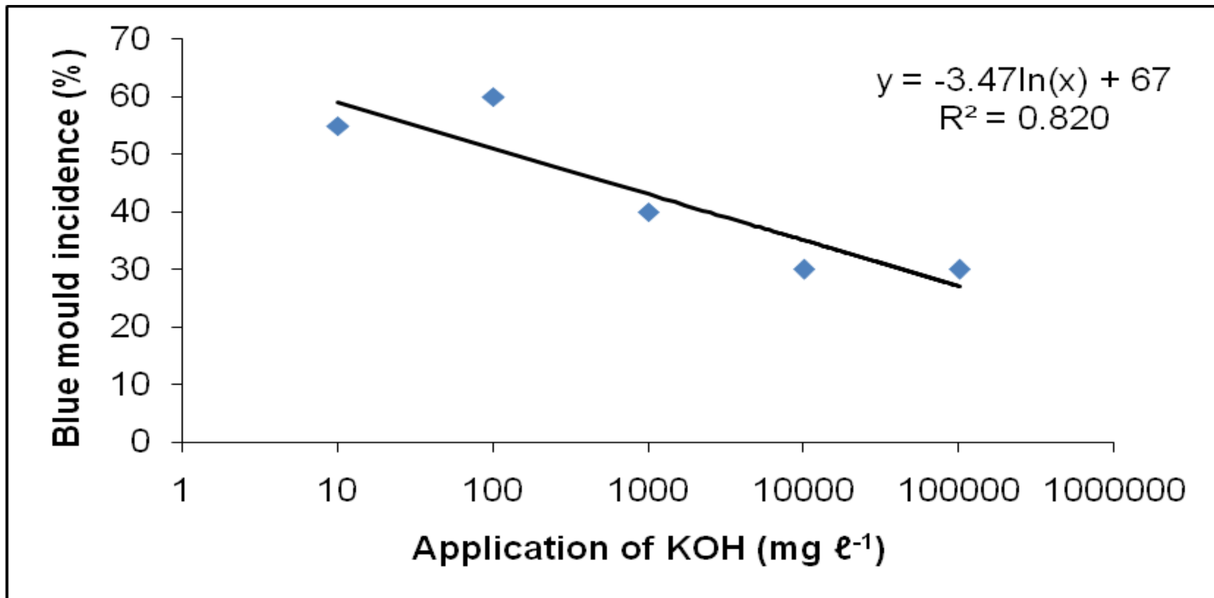

Figure 4.14 Logarithmic regression of dose of potassium hydroxide on blue mould incidence.

Application of KOH at all concentrations significantly ($P<0.001$) reduced grey mould incidence on "Golden Delicious" apples, compared to control treatment (Figure 4.15). Increasing KOH concentration from 10 to 100, 000 mg ℓ^{-1}, reduced grey mould incidence from 57.5% to 37.5%, respectively (Figure 4.15). Preventative effects of KOH on "Golden Delicious" apples at concentrations of 10, 100, 1,000, 10, 000 and 100, 000 mg ℓ^{-1} were not significantly different ($P<0.001$) from each other (Figure 4.15). Grey mould incidence decreased as a function of dose of potassium hydroxide ($R^2 = 0.459$) (Figure 4.16).

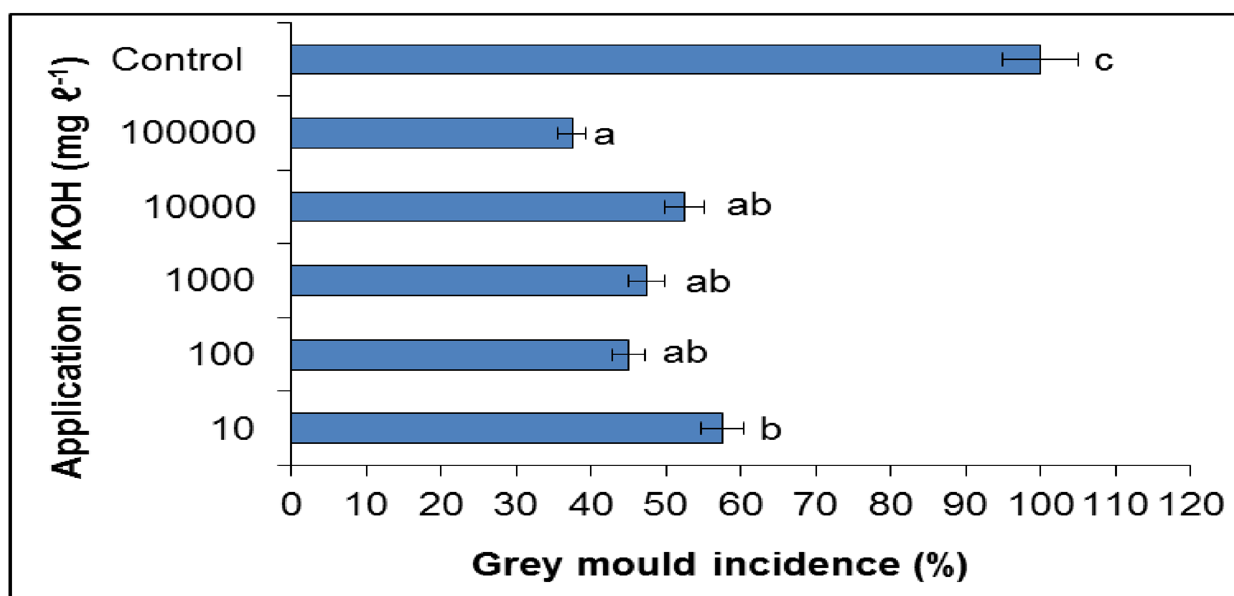

Figure 4.15 Disease incidence of *Botrytis cinerea* on "Golden Delicious" apples treated preventatively with various concentrations of potassium hydroxide. Means followed by the same letter are not significantly different according to the Fisher's Least Significant Difference Test ($P = 0.05$).

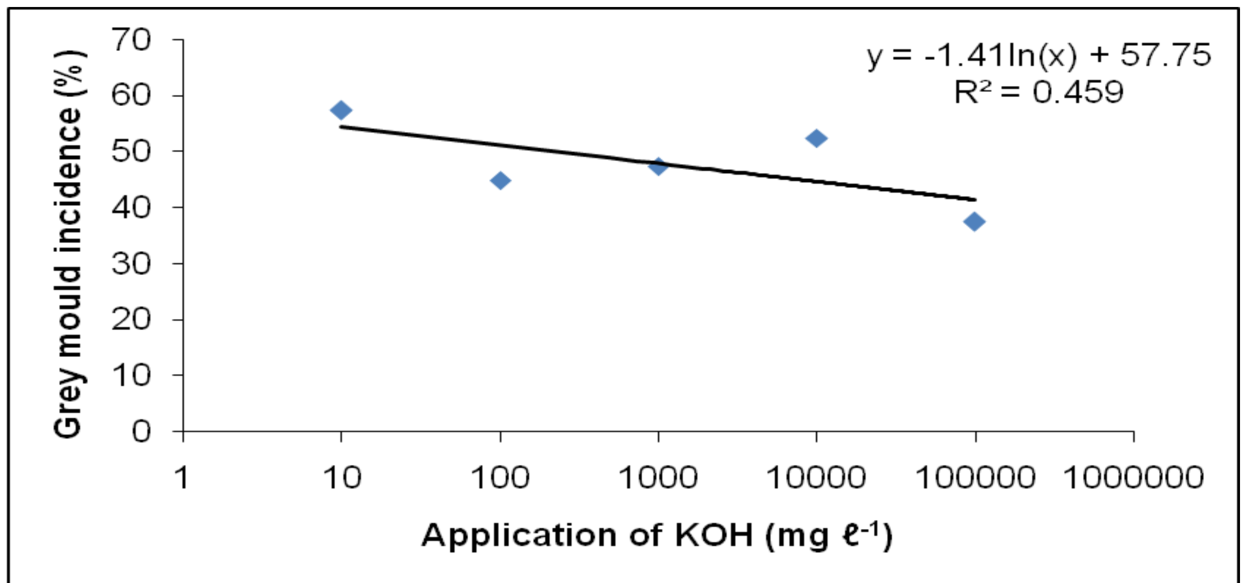

Figure 4.16 Logarithmic regression of dose of potassium hydroxide on grey mould incidence.

4.3.4 Visual observation of wounds of apples treated with potassium silicate and inoculated with either *Penicillium expansum* or *Botrytis cinerea*

Apples inoculated with *Botrytis cinerea* showed visible grey conidia of *B. cinerea* and /or softening of the fruit surface (Figure 4.17A). Apples inoculated with *Penicillium expansum* showed visible green conidia of *P. expansum* and /or softening of the fruit surface (Figure 4.17B).

Figure 4.17 Control apple fruits inoculated with *Botrytis cinerea* only (A) or *Penicillium expansum* only (B).

Apples showed less infection when potassium silicate was applied to wounds 24 hrs before inoculation with either *B. cinerea* (Figure 4.18A) or *P. expansum* (Figure 4.18B).

Figure 4.18 Postharvest control of *Botrytis cinerea* and *Penicillium expansum* on “Golden Delicious” apples treated with potassium silicate at a concentration of 100,000 mg ℓ^{-1} and with *B. cinerea* (A) or *P. expansum* (B).

Application of K_2SiO_3 at a concentration of 100,000 mg ℓ^{-1} into wounds of apples inhibited conidial germination of *Penicillium expansum* (Figure 4.19A) and *Botrytis cinerea* (Figure 4.19B). Deposition of the K_2SiO_3 was evident in wounds of the fruit (Figures 4.19A and 4.19B).

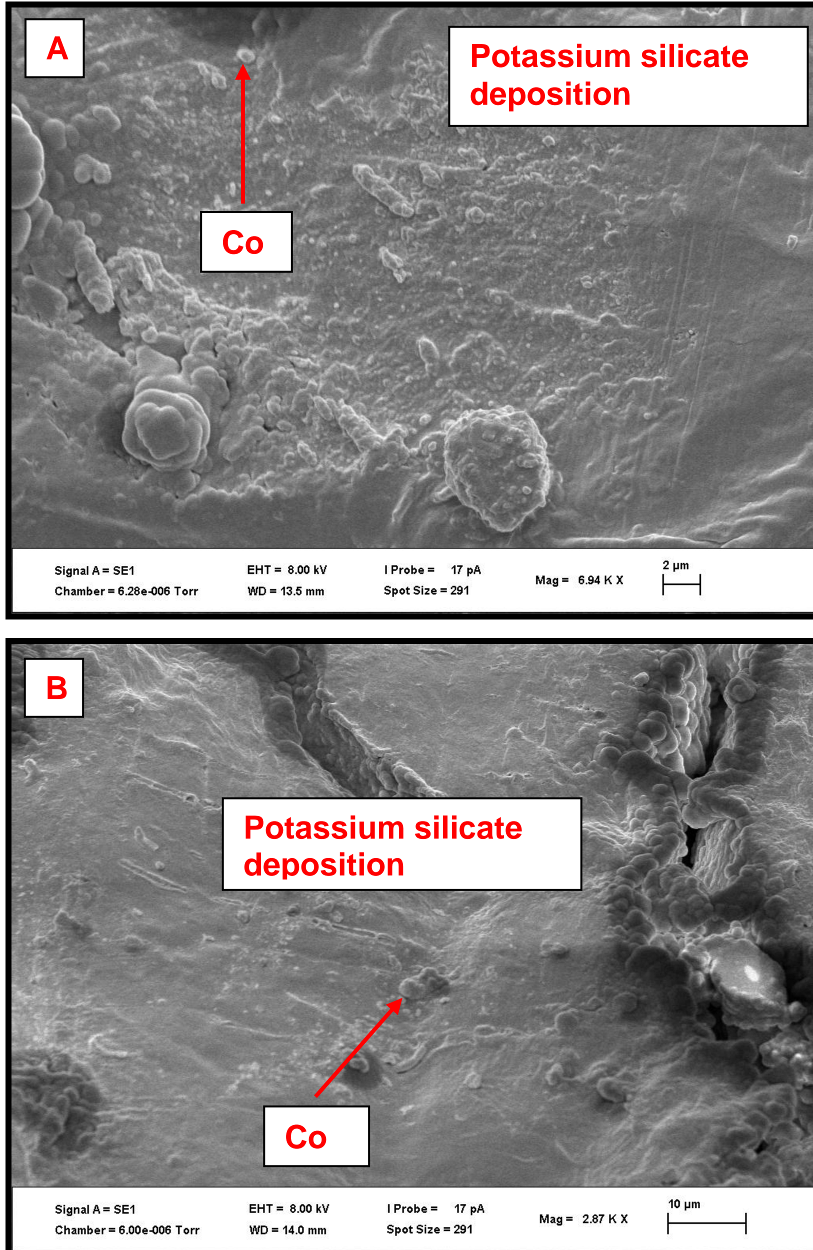

Figure 4.19 Scanning electron micrograph of potassium silicate with either *Penicillium expansum* (A) or *Botrytis cinerea* (B) in wounds of "Golden Delicious" apples. Co= Conidium.

4.4 DISCUSSION

Potassium silicate was effective in suppressing grey and blue mould infections caused by *Botrytis cinerea* and *Penicillium expansum*, respectively, in "Golden Delicious" apples (Figures 4.1, 4.3, 4.5 and 4.7 and 4.19), compared with control fruit (Figures 4.17). The incidence of grey and blue moulds on control apples were significantly higher than on fruit treated with K_2SiO_3. Increasing the concentration of K_2SiO_3 influenced the growth of grey and blue moulds.

These results are similar to those of Menzies *et al.* (1991), who found that powdery mildew caused by *Sphaerotheca fuliginea* (Schltdl.) in cucumber plants was reduced significantly with increasing concentration of Si in nutrient solutions. Similar results were also observed with *P. expansum* (Link) Thom. and *Monilinia fructicola* (G. Winter) Honey. on sweet cherry fruit (Biggs *et al.*, 1997; Qin and Tian, 2005). Postharvest application of silicon oxide and sodium silicate suppressed postharvest pink rot severity caused by *T. roseum* in muskmelons (Guo *et al.*, 2007). Postharvest treatment with Si proved effective in inhibiting pathogen growth as well as inducing disease resistance in melons (Bi *et al.*, 2006). The suppression could have been due to induction of phenolic compounds produced within the 24 hr time period before inoculation with *B. cinerea* or *P. expansum*. Furthermore, this could be due to a direct K_2SiO_3 effect on conidia and subsequently affect conidial germination as a result of fungicidal effect as reported by Bekker *et al*. (2006) on several phytopathogenic fungi from avocado. These authors showed that soluble potassium silicate suppressed fungal growth effectively *in vitro* (plate test) of several phytopathogenic fungi from avocado and the effect was largely a fungicidal effect.

Several studies have shown that deposition of silicon in the cell wall around infection sites provides a physical barrier to pathogen penetration (Heath and Stumpf, 1986). Potassium silicate was found to be more effective in controlling grey and blue moulds of apple at a concentration of 100,000 mg ℓ^{-1}. Application of potassium silicate at highest concentrations (100, 000 mg ℓ^{-1}) resulted in the formation of white residues on the fruit surface. Bowen *et al.* (1992) reported that it is not unusual for a chemical to support

fungal growth at lower concentrations and yet be fungistatic at higher concentrations. Guo *et al.*, (2007) reported that sodium silicate solutions are highly alkaline and it is therefore possible that their fungistatic property is due to the strong alkalinity.

Potassium chloride and potassium hydroxide were observed to be effective in preventing *P. expansum* and *B. cinerea* infection on "Golden Delicious" apples (Figures 4.9, 4.11, 4.13 and 4.15). Grey and blue mould incidence on fruit treated with either KCℓ or KOH, was generally significantly lower than the controls. The use of soluble Si as silicate salts of Ca and K or N has become popular in greenhouse hydroponic systems (Deliopoulos *et al.,* 2010). Cook (1997) indicated that foliar application of KCℓ on wheat suppressed *septoria* blotch and powdery mildew at early stem extension and after flag leaf emergence, respectively. Several authors also concluded that the mode of action of KCℓ is contact and it has both protectant and curative effects (Deliopoulos *et al.,* 2010). Mann (1999) and Kettlewell *et al.* (2000) indicated that potassium chloride can suppress the leaf area of wheat affected by *Septoria tritici* (Berk. and M.A. Curtis), possibly by a contact osmotic mode of action at the phylloplane level. Future research is required to test the potential of K_2SiO_3, KCℓ and KOH as postharvest treatments on other fruit for the control of postharvest fungal diseases and also for identification of optimum dose.

4.5 CONCLUSION

This work has demonstrated the potential of potassium silicate, potassium chloride and potassium hydroxide for the control of postharvest blue mould and grey mould of "Golden Delicious" apples caused by *P. expansum* and *B. cinerea*, respectively.

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CHAPTER 5

EFFECT OF HOT WATER DIP TREATMENT ON POSTHARVEST CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* OF APPLES

ABSTRACT

A hot water dip treatment was used to evaluate its efficacy for the reduction of *Botrytis cinerea* and *Penicillium expansum* development on "Golden Delicious" apples. Fruit were artificially wounded and inoculated with 30 µℓ of 1×10^4 conidia $m\ell^{-1}$ suspension of either *P. expansum* or *B. cinerea*. The inoculated apples were left to air dry at 24±1°C. After 2 hrs, the fruit were immersed in hot water for 50-75°C x 30-900 seconds. Control fruit were dipped in tap water (20°C) or were not treated. Treated fruit were subsequently stored at 25°C for seven days after which wounds were examined and the percentage of disease incidence determined. *In vivo* tests showed that the disease incidence of *P. expansum* was reduced by hot water dip treatments at 60°C for 60 seconds (40%), compared to the control fruit (100%), without causing any skin injuries. Furthermore, *in vivo* studies also indicated that a hot-water dip at 60°C for 60-90 seconds, reduced grey mould development in inoculated wounds to 30% compared with control fruit treated with tap water (100%), without causing any skin injuries.

5.1 INTRODUCTION

The susceptibility of freshly harvested produce to postharvest diseases increases during storage due to physiological changes that enable pathogens to develop (Fallik, 2004). Pre-storage heat treatments have been used to free plant materials from pathogens (Paull and Chen, 2000). These treatments may be applied to the commodity by means of hot water dips and sprays, hot vapour, dry air, infrared or microwave radiation (Couey, 1989). Water treatments are affordable and easy to operate (Tsang *et al.,* 1995). Pre-storage hot water dips of fruit at temperatures above 40°C are effective in controlling storage decay, not only by reducing the pathogen inoculum but also by enhancing the resistance of the fruit tissue, influencing host metabolism and ripening (Barkai-Golan and Philips, 1991). Lurie (1998) reported that postharvest dips are

applied for a few minutes at high temperatures because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit. All fresh and vegetables for domestic or export markets should be free of dirt, dust, pathogens and chemicals before they are packaged.

According to Garcia *et al.* (1995), hot treatment with water dips was effective against *Botrytis cinerea* Pers.:Fr. for Spanish strawberry cultivars. Janisiewicz *et al.* (2003) reported that heat treatment (hot air) at 38°C for 4 days reduced the decay of apple fruit caused by *Colletotrichum acutatum* (J.H. Simmonds) and *Penicillium expansum* (Link) Thom. Fallik *et al.* (1993, 1995) showed that heating, without forced air, can reduce decay caused by *B. cinerea* Pers.:Fr. in tomatoes and *P. expansum* (Link) Thom. in apples. Dimitris *et al.* (2005) indicated that the decay incidence of cactus pear was reduced by 86-91% without damaging fruit quality, when treated at 60°C for 30 seconds or 65°C for 20 seconds. Schirra *et al.* (2000) indicated that hot water treatment on citrus may eliminate incipient infections by removing spores from wounds and acting directly on their viability as well as inducing fruit defence mechanisms in the outer layers of the epicarp which inhibit pathogen growth. Several researchers have shown that immersion of citrus fruit in 50-53°C water for 2-3 minutes controls *Penicillium* and/or *Alternaria* spp., which causes infection in oranges, lemons, and grapefruit (Couey, 1989; Schirra *et al.,* 1997; Nafussi *et al.,* 2001).

The objectives of this study were: 1) To identify a narrower range of temperature x exposure period combinations that would control *P. expansum* and *B. cinerea* without causing damage to skin quality of “Golden Delicious” apples, by conducting an unreplicated observational trial (Rayner, 1967) over a wide range of temperature x exposure period combinations. 2) To conduct a replicated trial in the narrower range of temperatures x exposure period combinations to find the best combination of temperature x exposure period that would control *P. expansum* and *B. cinerea* infections on “Golden Delicious” apples without causing skin damage.

5.2 MATERIALS AND METHODS

5.2.1 Fruit

Apples (*Malus domestica* Borkh.) cultivar “Golden Delicious” were purchased at a Pick ’n Pay supermarket from Pietermaritzburg, South Africa. The fruit was stored for three days at 9±1°C and 90% relative humidity (RH) before use.

5.2.2 Observational Trial 1: hot water treatments to determine a safe temperature x exposure time for “Golden Delicious” apples

Tap water was heated to test temperatures in a 100ℓ water bath with an electronic control unit to maintain water at a constant temperature for the duration of the trial. The proportional integral derivative (PID) controller used has an accuracy of less than 0.2% of the displayed temperature value, plus one digit, with a sampling time of 250mS. The components of the hot water bath include: a temperature controller with full PID functions, with solid state relay (SSR) output to control the heating element, a pressure switch, a circulating pump, the heating element, fuses, a main switch and a contactor. The water bath was insulated, and water was pumped around the bath at 30 L s^{-1} to keep an even distribution of water temperature. “Golden Delicious” apples were placed in stainless steel wire baskets to immerse them in the water bath.

In the first experiment, the tested temperatures were 20 (control), 50, 55, 60, 65, 70, 75 and 80°C (±0.1°C). For each temperature, the “Golden Delicious” apples were exposed for a period of 30, 60, 90, 120, 150, 180, 300, 600 and 900 seconds. Each treatment was applied to three apples as a single replicate. The treated fruit were then air dried, placed in open carton board boxes and stored at 25°C for 7 days. After storage, the fruit were classified into one of two categories: (1) presence of slight or moderate skin blemishes or severe skin injury, marked as “YES”; (2) no visible skin damage, which were marked as “NO”.

5.2.3 Observational Trial 2: hot water treatments followed by cold water dip for the control of skin injury of "Golden Delicious" apples

The experiment was conducted to determine whether the cold water treatment could stop the heat energy damaging the fruit skin. A similar experimental set up was followed as for the first experiment, as described in Section 5.2.2. An unreplicated, observational trial was also conducted. The only difference was that, following the hot water treatment, the "Golden Delicious" apples were immediately immersed for 1 minute into tap water cooled to 1°C in a hydro-cooling tank. Each treatment was applied to three apples as a single replicate.

5.2.4 Replicated Trial 1: efficacy of hot water treatments for the control of *Penicillium expansum* and *Botrytis cinerea* infection of "Golden Delicious" apples

A uniform 5 mm deep by 5 mm wide wound was made at the equator of each fruit using the tip of a sterile needle. Wounds were then inoculated with 30 µℓ of 1×10^4 conidia $mℓ^{-1}$ suspension of *P. expansum*. The inoculated "Golden Delicious" apples were left to air dry at 24±1°C. After 24 hrs, the apples were immersed in hot water for the test temperatures and time periods of 50-75°C x 30-900 seconds, as described in Section 5.2.2. Controls were dipped in tap water (20°C) or were left untreated. Each treatment was applied to 10 apples independently, as ten replicates. Treated fruit was air dried and placed in open carton board boxes and kept at 25°C for 7 days. After 7 days of storage the percentage incidence of *P. expansum* infection on the fruit was counted. A similar procedure was used to determine the efficacy of hot water treatment for the control of *B. cinerea* infection.

5.3 RESULTS

5.3.1 Observational Trial 1: effect of hot water dip treatments at different temperatures and exposure periods on the quality of "Golden Delicious" apples

The best temperature range without skin damage was 50-58°C at all exposure times (Table 5.1). There was no skin damage noted at 60°C when subjected to 120 seconds or less (Table 5.1). There was no skin damage observed on the controls at 20°C (Table 1 and Figure 5.1 C). "Golden Delicious" apples dipped at 60°C for a period of 180 seconds, and at 80°C for a period of 60 seconds, showed skin blemishes and browning (Figures 5.1 A and B), respectively.

Table 5.1 Effect of hot water dip treatments at different temperatures and exposure periods, on the quality of skin of "Golden Delicious" apples stored at 25°C for one week

Exposure temperature (°C)	Exposure time (seconds)								
	30	**60**	**90**	**120**	**150**	**180**	**300**	**600**	**900**
20	No	No	No	No	No	No	No	No	No
50	No	No	No	No	No	No	No	No	No
55	No	No	No	No	No	No	No	No	No
56	No	No	No	No	No	No	No	No	No
58	No	No	No	No	No	No	No	No	No
60	No	No	No	No	Yes	Yes	Yes	Yes	Yes
62	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
64	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
65	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
66	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
68	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
70	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
75	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
80	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Yes = Presence of damage

No= No damage

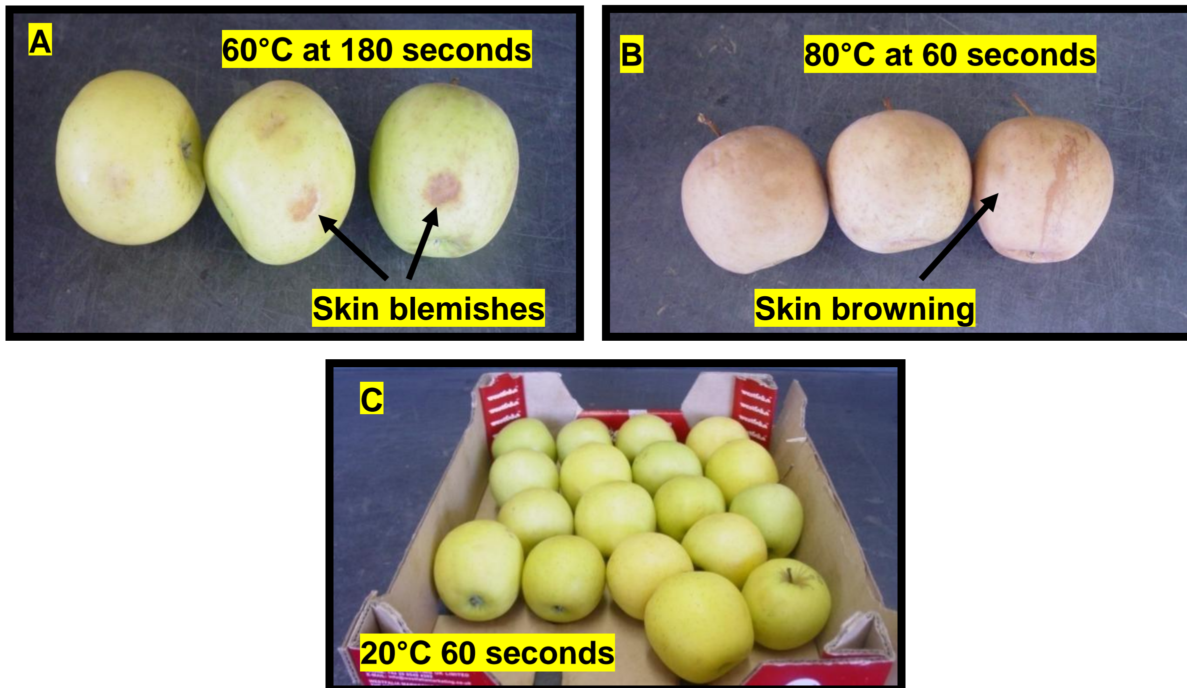

Figure 5.1 “Golden Delicious” apples treated with hot water, after one week of storage. Fruit were dipped into water at 60°C for 180 seconds (A), and into water at 80°C for 60 seconds (B), and into water at 20°C for 60 seconds (C).

5.3.2 Observational Trial 2: effects of hot water dip treatments at different temperatures and exposure periods followed by a cold water dip for one minute, on skin quality of “Golden Delicious” apples

The results were similar to Table 5.1.The physiological damage caused by hot water treatment on “Golden Delicious” apples was not reduced by cold water dip treatment.

5.3.3 Replicated Trial 1: effectiveness of hot water dip treatments of “Golden Delicious” apples in the control of artificially inoculated *Penicillium expansum* and *Botrytis cinerea*

When apples were immersed at 20°C for 60 seconds, blue mould incidence was 100% (Figure 5.2 A and Table 5.2). Hot water immersion treatment at 50-75°C resulted in differing responses in terms of *P. expansum* control depending on the immersion period (Table 5.2 and Figures 5.2 B, C and D). The skin was severely injured when treated at 70-75°C for all immersion periods (Figures 5.2 C and D). When fruit were exposed to

75°C for 180-900 seconds, incidence of *P. expansum* increased to 100% (Table 5.2). The disease incidence of *P. expansum* was reduced by hot water dip treatments at 60°C for 60 seconds (40%), compared to the control fruit (100%), without causing any skin injuries.

Table 5.2 Efficacy of hot water dip treatments for the control of artificially inoculated *Penicillium expansum* on "Golden Delicious" apples stored at 25°C for one week

Exposure temperature (°C)	**Blue mould incidence (%) Exposure time (seconds)**								
	30	**60**	**90**	**120**	**150**	**180**	**300**	**600**	**900**
20 (not dipped)		100							
20 (dipped)		100							
50	100	90	100	100	90	100	70	70	60
55	100	70	80	60	90	100	70	90	90
56	60	100	70	100	100	100	70	60	50
58	60	50	50	60	70	100	100	80	60
60	70	40	50	50	60	50	40	60	50
62	60	30	40	60	70	60	20	50	30
64	80	90	80	80	100	70	30	60	30
65	90	80	90	80	50	70	70	40	50
66	60	80	90	70	60	50	60	50	20
68	50	90	70	60	50	100	60	70	50
70	70	70	60	90	70	60	50	100	80
75	70	50	60	60	50	100	100	100	100

The disease incidence highlighted with a yellow colour indicates the temperature × exposure time combinations in which blue mould incidence was reduced to ≤ 50% with no skin damage (Table 5.2).

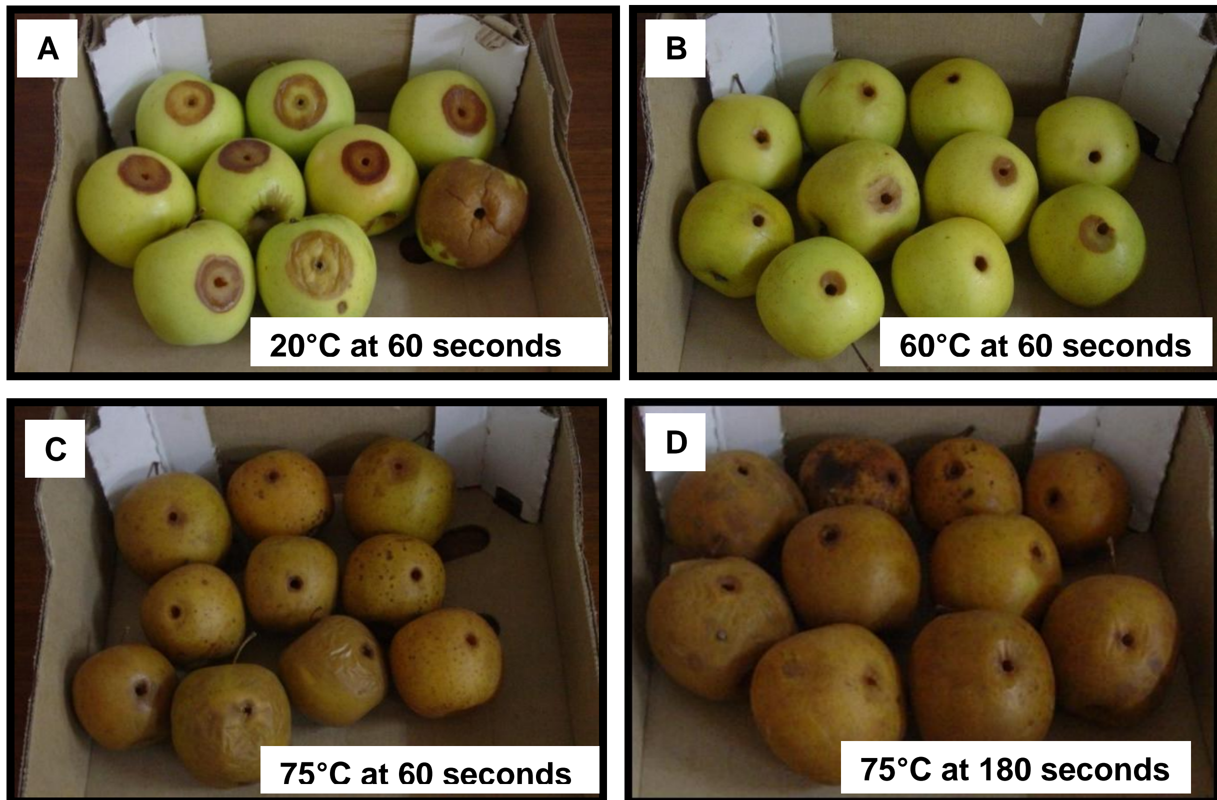

Figure 5.2 Effects of hot water dip treatments using various temperatures x exposure periods for the control of *Penicillium expansum* on "Golden Delicious" apples.

When apples were dipped at 20°C for 60 seconds, incidence of *B. cinerea* was 100% (Figure 5.3A and Table 5.3). Hot water immersion treatment at 50-75°C resulted in differing responses in terms of *B. cinerea* control depending on the immersion period (Table 5.3 and Figures 5.3 B, C and D). The skin of "Golden Delicious" apples was injured when treated at 70-75°C for all immersion periods (Figures 5.3 D). When apples were exposed at 75°C for 2-15 minutes, incidence of *B. cinerea* increased to 100% (Table 5.3). *In vivo* studies also indicated that a hot-water dip at 60°C for 60-90 seconds, reduced grey mould development in inoculated wounds to 30% compared with control fruit treated with tap water (100%), without causing any skin injuries.

Table 5.3 Efficacy of hot water dip treatments for the control of artificially inoculated *Botrytis cinerea* on “Golden Delicious” apples stored at 25°C for one week

Exposure temperature (°C)	**Grey mould incidence (%) Exposure period (seconds)**								
	30	**60**	**90**	**120**	**150**	**180**	**300**	**600**	**900**
20 (not dipped)		100							
20 (dipped)		100							
50	90	70	100	100	90	80	90	70	50
55	100	80	90	70	80	80	60	90	70
56	50	80	80	90	70	90	60	70	80
58	80	60	50	60	90	80	100	60	60
60	80	30	30	50	70	50	50	20	40
62	70	30	30	60	80	90	50	60	20
64	90	60	60	70	50	80	60	50	30
65	70	80	50	100	50	40	60	30	40
66	100	50	70	80	60	90	70	50	80
68	70	70	100	90	70	60	60	40	40
70	60	100	80	60	70	30	40	80	100
75	50	70	30	100	100	100	100	100	100

The disease incidence highlighted with a yellow colour indicates the temperature × exposure time combinations in which grey mould incidence was reduced to ≤ 50% with no skin damage (Table 5.3).

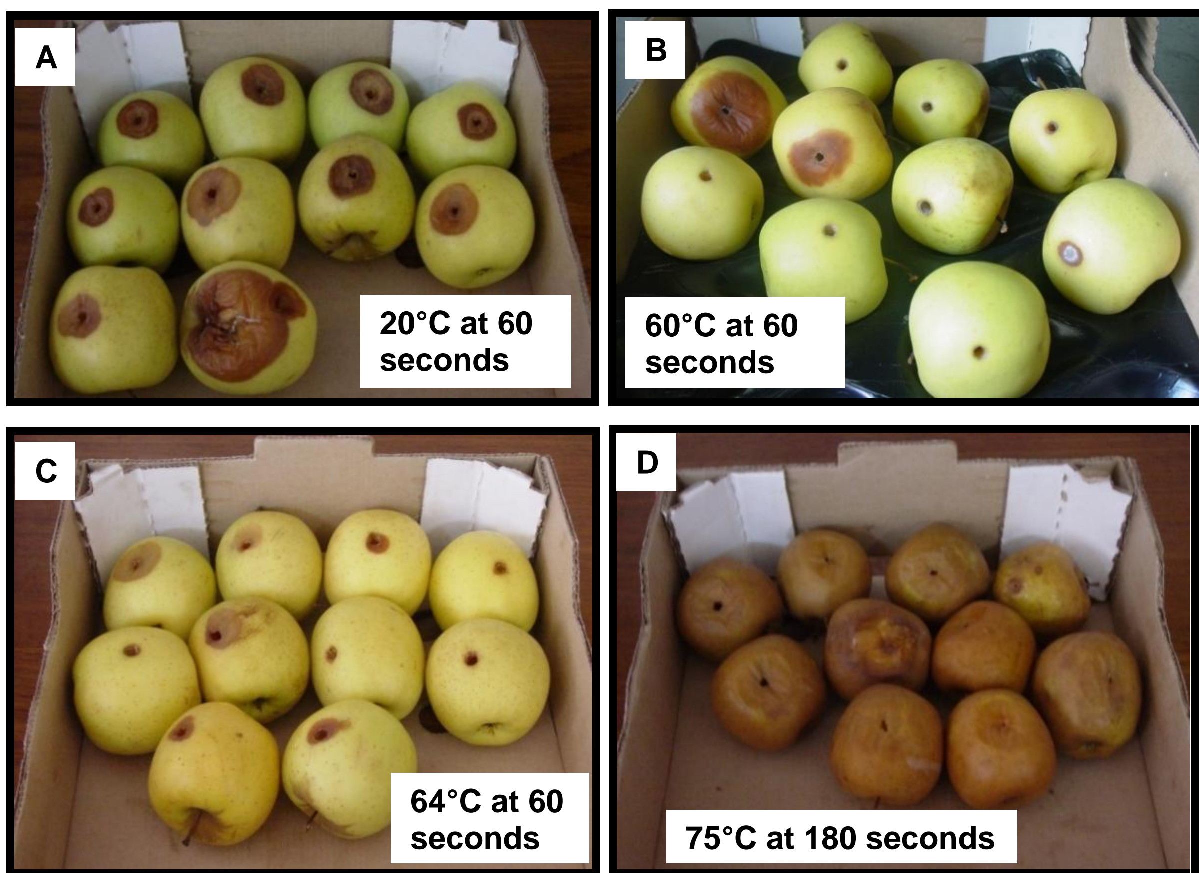

Figure 5.3 Effects of hot water dip treatments using various temperatures x exposure periods for the control of *Botrytis cinerea* on "Golden Delicious" apples.

5.4 DISCUSSION

Postharvest diseases are currently controlled by the use of synthetic fungicides, but non-chemical treatments are needed because of the development of resistant strains of pathogens and increasing public concerns over human health and environmental risks (Spotts and Cervantes, 1986). Postharvest heat treatment has been shown to be a promising method for reducing postharvest diseases (Lurie, 1998).

From this study it was observed that the best temperature range without skin damage was 50-58°C for 30-900 seconds (Table 5.1). Blue and grey mould incidence was not completely controlled by the application of hot water dip treatment but it was reduced to ≤ 50% and skin damage was zero (Table 5.1 and Table 5.2). The best temperature ×

time combination for the control of blue mould was 58°C for 60-90 seconds (50% control) and 60°C for 60-120 seconds (60% to 40% control) (Table 5.2). Furthermore, grey mould incidence was reduced to 50% at 58°C for 90 seconds (50% control) and 60°C for 60-120 second (70% to 50% control) (Table 5.3). According to Teitel *et al.* (1989), a reduced temperature of 52°C and a longer dip time of 2 minutes controlled decay of caused by *Alternaria* spp., *Fusarium* spp., *Rhizopus* spp. and *Murcor* spp. without causing external heat injury of "Galia" melons.

Hot water treatment of "Golden Delicious" apples at higher temperatures caused damage of the fruit skin (Table 5.1). This resulted in an increased susceptibility of the fruit tissue to the pathogen. The higher disease incidence when fruit were subjected to hot water treatments at high temperatures indicates that these treatments were ineffective in killing conidia of *B. cinerea* or *P. expansum,* and the mycelium of either *P. expansum* or *B. cinerea* survived treatment of these high temperatures (Table 5.2 and Table 5.3). Hot water treatment at the higher temperatures also caused damage to the fruit skin, which may have increased the susceptibility of the tissue to the pathogen. Karabulut *et al*. (2002) also showed that hot water treatments were not able to kill conidia of *P. expansum* (Link) Thom. of apples.

The results obtained in this study suggested that heat treatment can reduce postharvest blue and grey moulds infection of "Golden Delicious" apples. Limiting spore germination of pathogens may be one of the mechanisms of action of heat treatment (Zhang *et al*., 2008). Hot water treatment may reduce fungal decay by affecting the survival of the pathogen spores and modifying the physiology of the host (Conway *et al.,* 2005). The potential effects of hot water include induction of antifungal-like substances that inhibit fungal development in fruit tissue (Lurie, 1998; Schirra *et al*., 2000), induction of proteins such as chitinase and β-1,3-glucanase, stabilization of membranes (Lurie, 1998; Schirra *et al*., 2000), inhibition of synthesis of cell wall hydrolytic enzymes (Lurie, 1998; Schirra *et al*., 2000), and delay of degradation of preformed antifungal compounds that are present in unripe fruit (Lurie, 1998; Schirra *et al*., 2000).

Fallik (2004b) reported the advantages achieved by using hot water treatments on fruit. These include: a) slowing the ripening of fruit to obtain a longer shelf life; b) reducing the sensitivity of subtropical fruit to low temperatures, thereby allowing a longer storage period at a temperature which would normally cause chilling injury; c) reducing postharvest rots by either inactivation of pathogenic or enhancement of host resistance; d) controlling insect pests as a quarantine treatment; and e) making possible the use of postharvest fungicides at lower concentrations.

Nafussie *et al.* (2001) showed that after hot water treatment of lemons, lignin-like materials were produced at the pathogen inoculation site, followed by an accumulation of phytoalexins. Therefore, the mode of action of heat treatment on fruits is via induction of resistance, which results in increased lignin formation, release of heat shock related proteins and phytoalexins production against *P. expansum* and *B. cinerea* (Schirra *et al.,* 2000).

5.5 CONCLUSION

The disease incidence of *P. expansum* and *B. cinerea* on "Golden Delicious" apples was reduced by hot water treatments at 58-60°C for 60 to 120 seconds, compared to the control fruit.

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CHAPTER 6

SCANNING ELECTRON MICROSCOPY STUDIES OF THE INTEARCTIONS OF YEAST ISOLATES YP60 AND YP25 WITH *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* IN APPLE WOUNDS

ABSTRACT

Biological control agents may compete with pathogens for nutrients and space to delay or prevent decay of fruit after harvest. The ability of yeast Isolates YP25 and YP60 to colonize fruit wounds and their mode of action were studied by using environmental scanning electron microscopy (ESEM). Based on ESEM observations, when applied preventatively to control *Botrytis cinerea* and *Penicillium expansum*, yeast isolates YP25 and YP60 inhibited conidial germination of *B. cinerea* and *P. expansum*, respectively. The yeast Isolates YP25 and YP60 demonstrated good colonization of apple wounds and provided protection against *P. expansum* and *B. cinerea,* respectively.

6.1 INTRODUCTION

A variety of microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Mari and Guizzardi, 1998; Fravel, 2005). Yeast antagonists have been efficacious as biological control agents (Qing and Shiping, 2000; Irtwange, 2006).

Several researchers found many yeasts and a few bacteria that controlled blue mould on pome fruits (Janisiewicz and Marchi, 1992; Janisiewicz *et al.,* 1994; Janisiewicz and Bors, 1995; Filonow *et al.,* 1996; Janisiewicz, 1996; and Janisiewicz *et al.,* 1998). *Cryptococcus laurentii* (Strain 317) *and Candida ciferrii (*Strain 283*)* isolated from the surface of healthy apples, controlled blue mould of apple caused by *Penicillium expansum* (Vero *et al.,* 2002). *Pichia guilliermondii* Strain R13 showed efficacy in reducing disease incidence on *Colletotrichum capsici* infected chilli fruits to as low as 6.5% (Chanchaichaovivat *et al.,* 2007).

Most postharvest fruit pathogens are necrotrophs which require nutrients for germination and initiation of the pathogenic process (Janisiewicz *et al.,* 2000). Competition for nutrients and space between the pathogen and the antagonist is considered as the major mode of action by which microbial agents control pathogens causing postharvest decay (Droby *et al.,* 1992; Wilson *et al.,* 1993; Filonow, 1998; Ippolito *et al.,* 2000 and Jijakli *et al.,* 2001). Past studies to identify the modes of action of biocontrol yeasts have shown to be safe to consumers (Arras and Arru, 1997; Arras *et al.,* 1999). The objective of this study to determine the mode of action of yeast Isolates YP25 and YP60; using environmental scanning electron microscopy.

6.2 MATERIALS AND METHODS

6.2.1 *In vitro* reaction of yeast Isolates YP60 and YP25 against *Penicillium expansum* and *Botrytis cinerea*

Yeast Isolates YP60 and YP25, were grown on nutrient yeast dextrose agar (NYDA) plates and incubated at 25°C for 3 days. Yeast isolates were then streaked on potato dextrose agar (PDA) plates on both sides at a distance of about 10 mm from the edge of the Petri dish. Agar with a 5 mm plug of either *P. expansum* or *B.* cinerea was grown on PDA, occupying the center of the plate and incubated at 25°C for seven days. The treatments were evaluated for evidence of inhibition of either *P. expansum* or *B. cinerea* by Isolates YP25 and YP60, by measuring the width of the inhibition zone between the two fungi. There were 5 plates for each treatment. This experiment was conducted twice.

6.2.2 Preparation of the yeast Isolates YP25 and YP60 on agar using scanning electron microscopy

Cultures of the yeast Isolates YP25 and YP60 were streaked onto nutrient agar (NA) plates. The plates were incubated (Incubator: Labotec, South Africa) at 25°C for four days. Blocks of 10 mm × 10 mm agar with a colony of the yeast on the surface were prepared for electron microscopy studies.

6.2.3 Preparation of the yeast Isolates YP25 and YP60 to examine their interactions in apple wounds, using scanning electron microscopy

Apples were disinfected with 70% alcohol for one minute, dried, and then wounded (20 mm in length × 3mm in depth) at one site at the equator of the fruit using a sterile dissecting needle. One wound was made in each apple. Each wound site was inoculated with 30 μℓ of cell suspension of either YP25 or YP60 at a concentration of 1×10^8 cells $m\ell^{-1}$. Fruit were incubated at 25°C. After 4 days, colonized wounds on the fruit were excised and prepared for observation by scanning electron microscopy. Control apples were inoculated with the same amount of distilled water. After 48 hours, all wounds were inoculated with 20 μℓ of either *Penicillium expansum* or *Botrytis cinerea* at a concentration of 1×10^4 conidia $m\ell^{-1}$. Fruit were incubated at 25°C. After seven days, wounded tissue from the treated and control fruit were excised and used for electron microscopy studies.

6.2.4 Scanning electron microscopy studies of the growth of the yeast Isolates YP25 and YP60 on nutrient agar and their colonization ability in apple wounds

Samples were taken of yeast Isolates YP25 and YP60 grown on NA, together with excised wounds of apples treated with combinations of Isolates YP25 and *Botrytis cinerea*, YP60 and *Penicillium expansum,* plus a control treatment. The samples were fixed overnight in 2% buffered osmium tetroxide (10 ml 4% OsO_4, 5ml 0.2M sodium cacodylate buffer and 5 ml distilled water). The samples were dehydrated in an ethanol series (10 minutes each in 30%, 50%, 70%, 80%, 90%, and 3 × 10 minutes in 100% ethanol) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% ethanol and placed in a pre-cooled Hitachi HCP-2 critical point drier. Following critical point drying (CPD) and gold-palladium sputter coating (Polaron Equipment Limited ESEM, coating unit E5100), the samples were viewed in an environmental scanning electron microscopy (ESEM) (Philips, FEI XL 30, Holland) at an accelerating voltage of 15 keV. Samples from five fruit from each treatment, and a sample of either yeast Isolates YP25 or YP60 on the surface of nutrient agar were viewed.

6.2.5 Scanning electron microscopy studies of the interactions of yeast Isolate YP25 and *Botrytis cinerea*, and Isolate YP60 and *Penicillium expansum* in wounds of apple fruit

Sample tissue from wounded apples treated with *P.* or *B. cinerea*, and from untreated wounded apples, was excised for ESEM observations. The tissue samples were held overnight in a fixative of 3% (v v-1) glutaraldehyde in 0.05 M sodium cocodylate buffer (pH 7.2) and dehydrated in an ethanol series (refer to Section 6.2.4), transferred into critical point drier baskets and gold-palladium sputter coated. The samples were viewed in an ESEM (Philips, FEI XL 30, Holland) at an accelerating voltage of 15 keV. Samples from five fruit from each treatment were viewed.

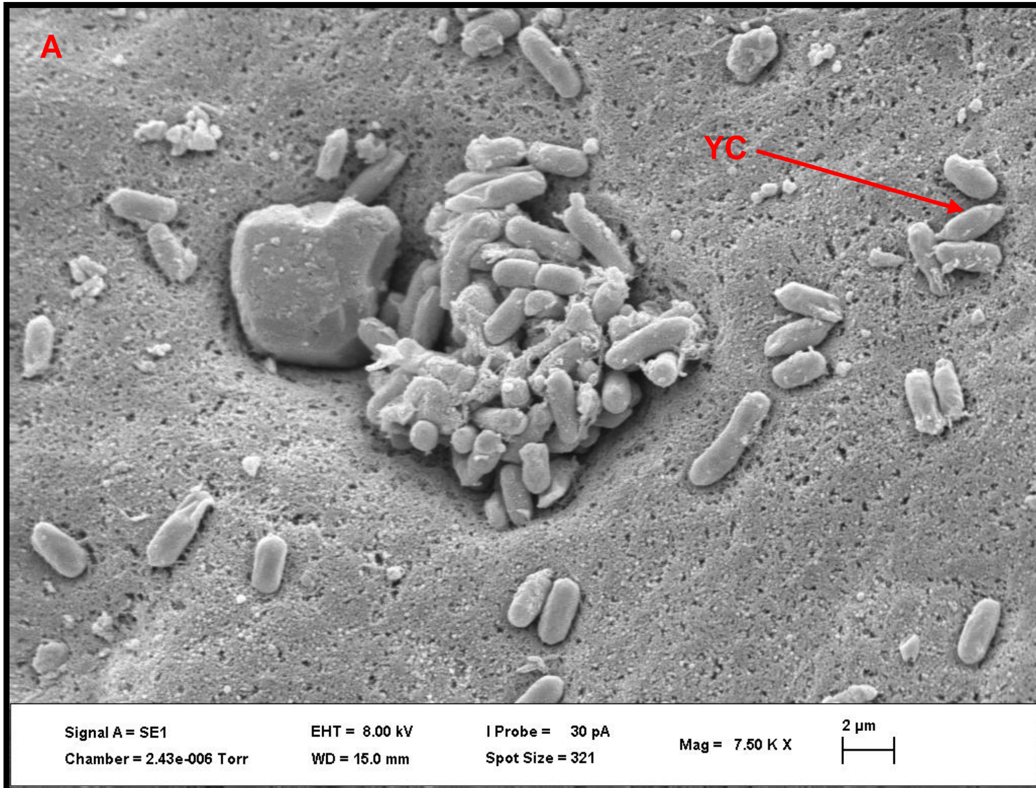
6.3 RESULTS

6.3.1 *In vitro* reaction of yeast Isolates YP60 and YP25 against *Penicillium expansum* and *Botrytis cinerea*

Yeast Isolates YP25 and YP60 did not inhibit the growth of *B. cinerea* and *P. expansum* on NA plates. There was no gap between colonies of YP25 and YP60 and the pathogens (*B. cinerea* and *P. expansum*). ESEM studies showed no hyperparasitism between yeast isolates (YP25 and YP60) and pathogens (*B. cinerea* and *P. expansum*).

6.3.2 Scanning electron microscopy observations of the yeast Isolates YP25 and YP60 on nutrient agar

Figures 6.1A and B showed the growth of yeast Isolates YP25 and YP60 on NA, under ESEM.



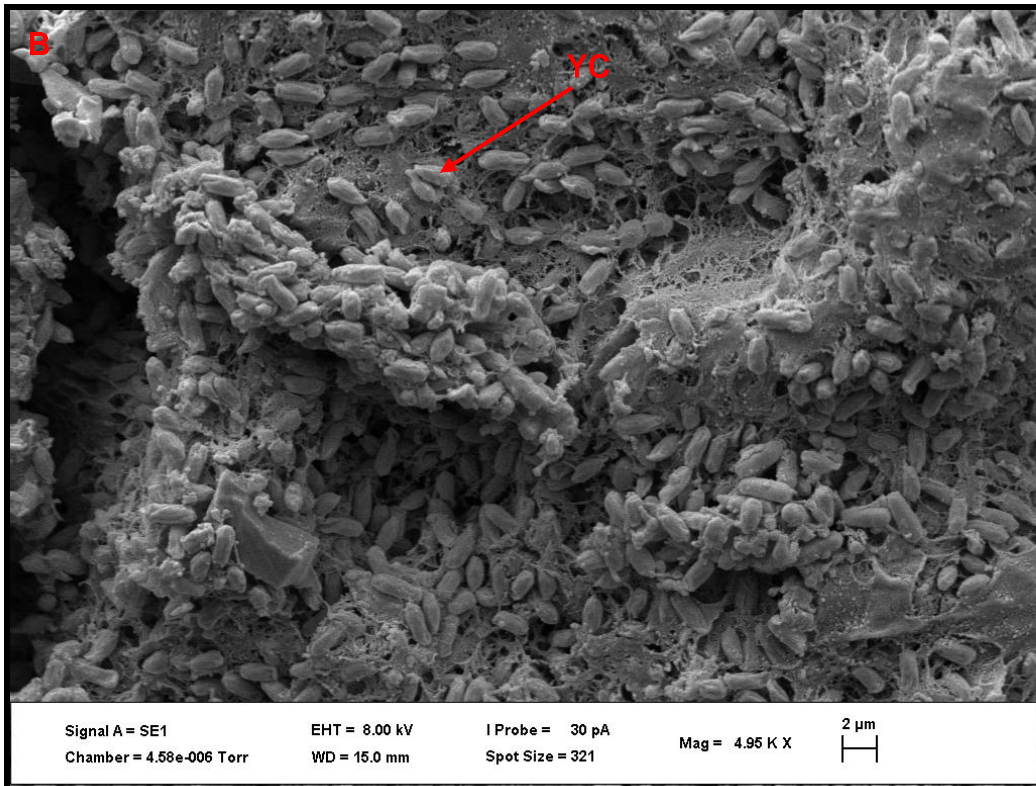


Figure 6.1 Scanning electron micrographs of yeast Isolates YP25 (A) and YP60 (B) on nutrient agar. YC= yeast cells.

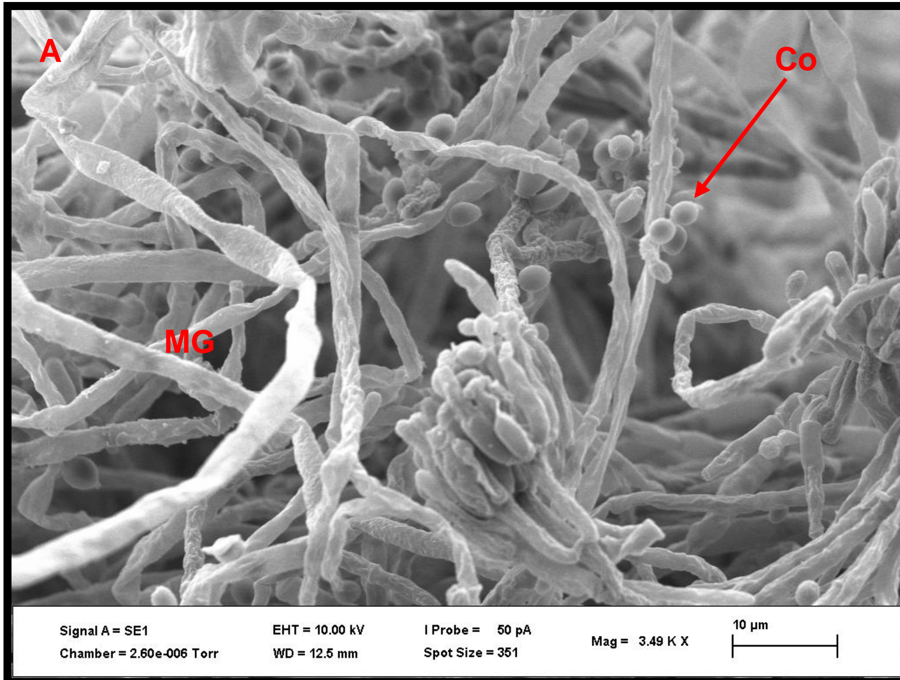
6.3.3 Visual observation of wounds of apples inoculated with either *Penicillium expansum* and *Botrytis cinerea*

Apples treated with either *Penicillium expansum* or *Botrytis cinerea* developed either green conidia (Figure 6.2A) or grey conidia (Figure 6.2B), respectively. The decayed tissue was soft and watery and lesions had very sharp margins between diseased and healthy tissues (Figure 6.2A and B).

Figure 6.2 "Golden Delicious" apples infected with *Penicillium expansum* (A) and *Botrytis cinerea* (B) 7 days after inoculation.

6.3.4 Scanning electron microscopy studies of wounds of apple inoculated with either *Penicillium expansum* or *Botrytis cinerea* only

Mycelial growth and conidia of *P. expansum* and *B. cinerea* was observed using the ESEM (Figures 6.3A and B, and Figures 6.4A and B, respectively).



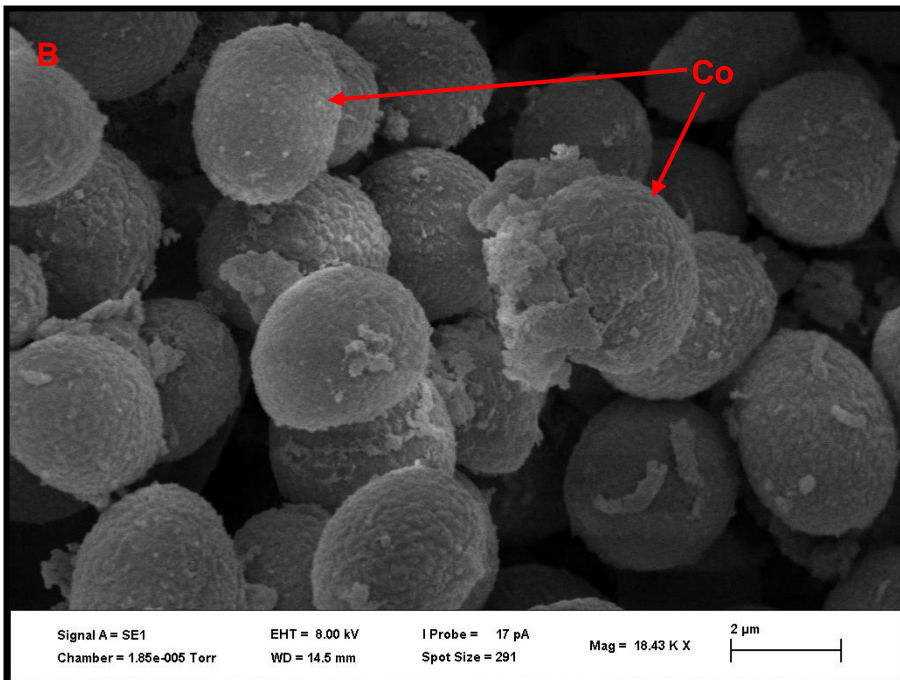
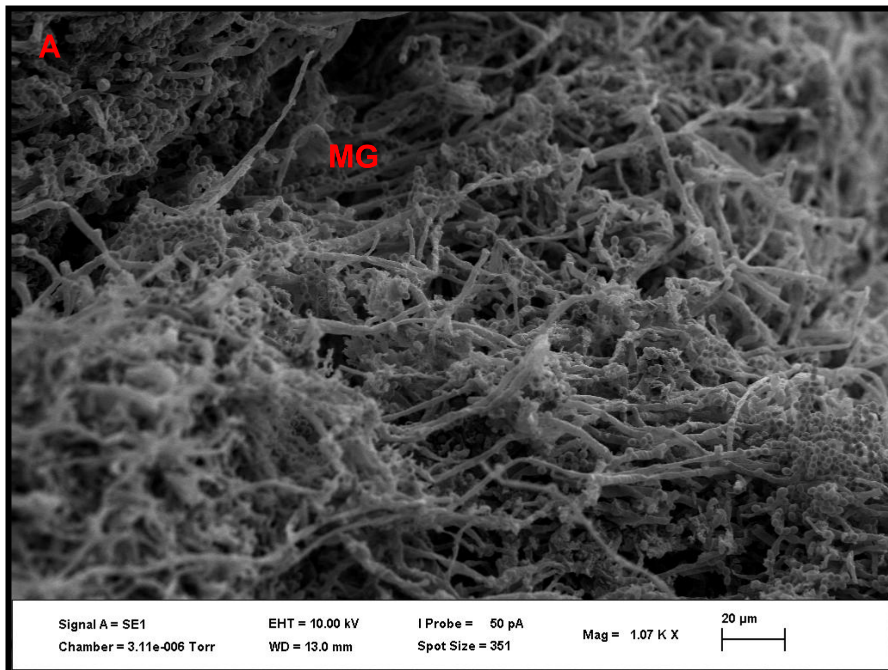


Figure 6.3 Scanning electron micrograph of untreated control fruit: (A) mycelia of *Penicillium expansum* in the wounds of apple; (B) conidia of *P. expansum* in apple wounds. MG= mycelial growth; Co= conidia.



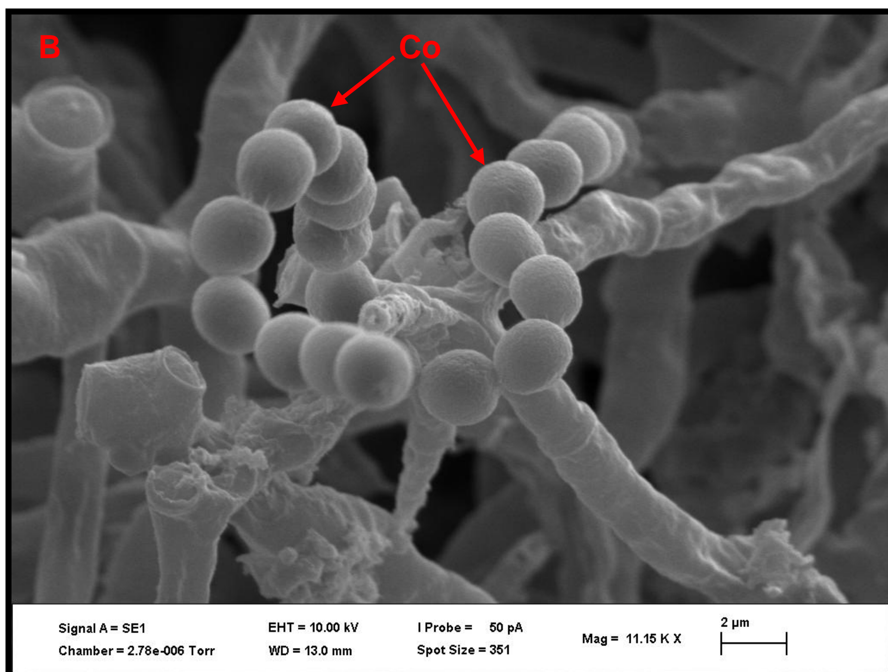


Figure 6.4 Scanning electron micrograph of untreated control fruit: (A) mycelia of *Botrytis cinerea* in the wounds of apple; (B) conidia of *B. cinerea* in apple wounds. MG= mycelial growth; Co= conidia.

6.3.5 Visual observation of wounds of apples treated with the yeast Isolate YP25 and *Botrytis cinerea*, and YP60 and *Penicillium expansum*

Treatment of "Golden Delicious" apples with YP25 and *B. cinerea*, and YP60 and *P. expansum* showed less infection 7 days after incubation (Figures 6.5A and B).

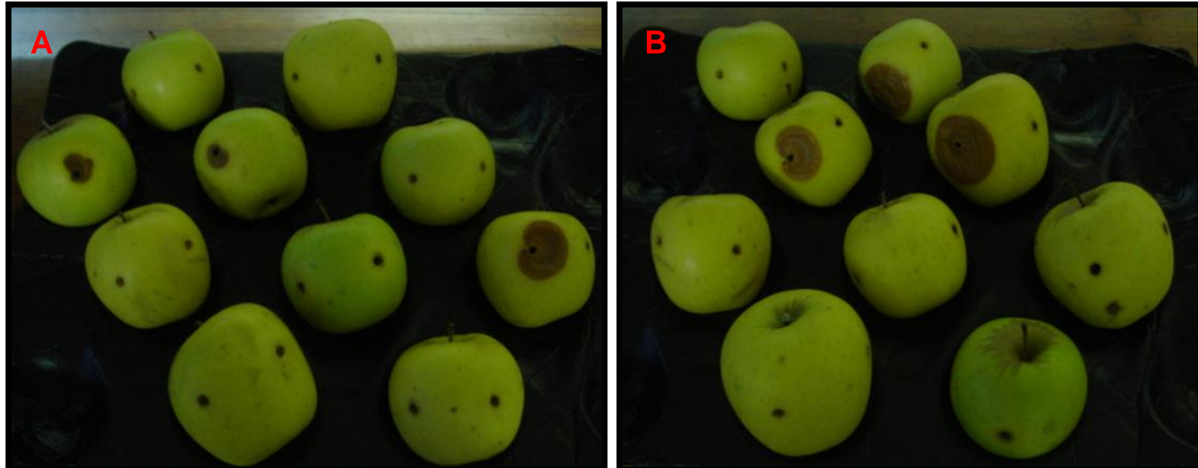
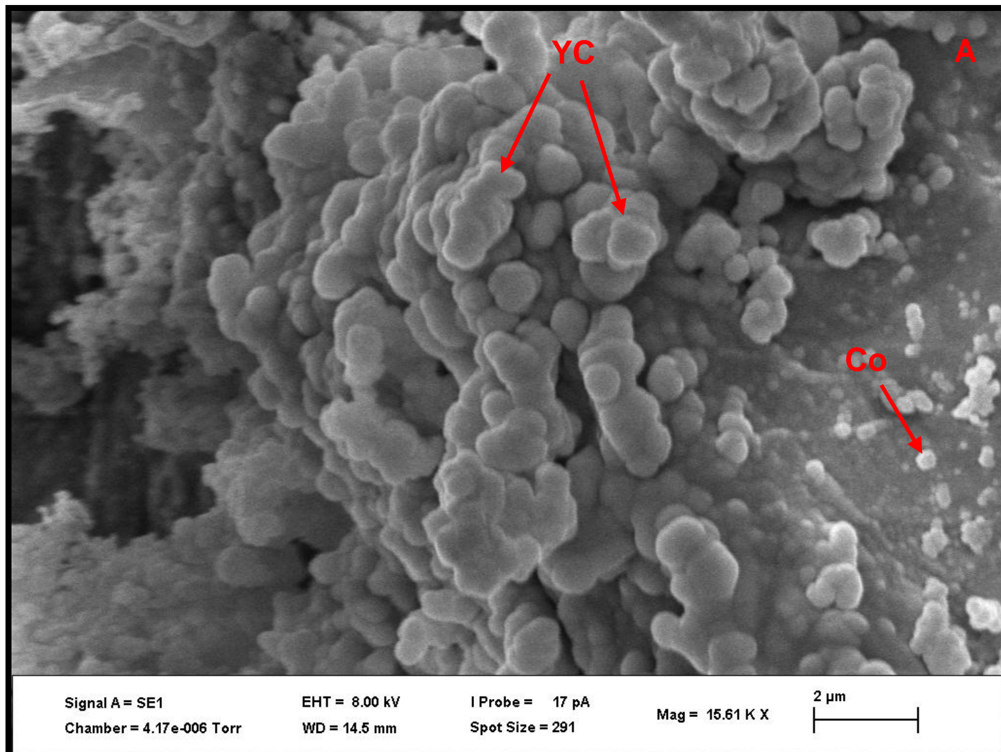


Figure 6.5 "Golden Delicious"apples preventatively treated with yeast Isolates and either *Botrytis cinerea* or *Penicillium expansum:* (A) fruit inoculated with YP25 and *B. cinerea,* (B) fruit inoculated with YP60 and *P. expansum*.

6.3.6 Scanning electron microscopy observations of interaction of the yeast Isolates YP25 and YP60 on *Penicillium expansum* and *Botrytis cinerea*, respectively in apple wounds

Conidial germination of *B. cinerea* was inhibited by yeast Isolate YP25 (Figure 6.6A), compared to the control treatment (Figures 6.4A and B). Similarly, conidial germination of *P. expansum* was inhibited by yeast Isolate YP60 (Figure 6.6B), compared to the control treatment (Figures 6.3A and B).



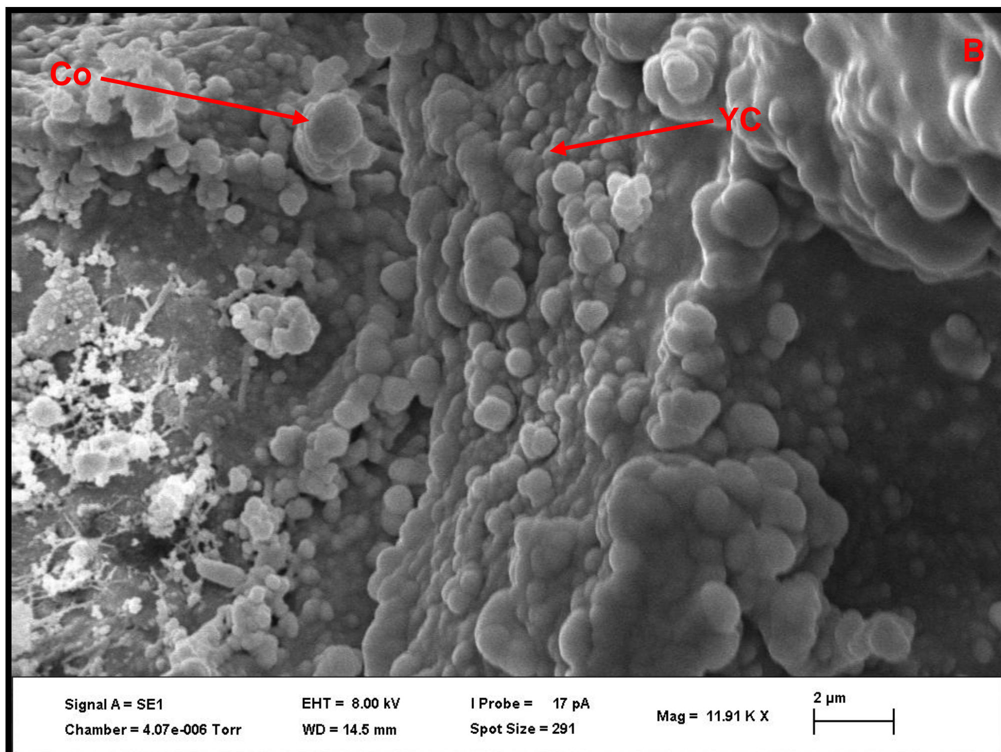


Figure 6.6 Scanning electron micrographs showing interaction of the yeast Isolate YP25 and *Botrytis* cinerea (A), yeast Isolate YP60 and *Penicillium expansum* (B) in apple wounds. Co= conidia; YC= yeast cells.

6.4 DISCUSSION

An environmental scanning electron microscopy was used to study the germination of *B. cinerea* and *P. expansum* in apple wounds, preventatively treated with yeast Isolates YP25 and YP60. From this study, it was observed that conidial germination of *B. cinerea* and *P. expansum* was inhibited by Isolates YP25 and YP60, respectively (Figures 6.6A and B), compared to the control treatments which showed extensive mycelial growth and production of conidia of *P. expansum* and *B. cinerea* (Figures 6.3A and B, and Figures 6.4A and B).

Yeasts possess many properties that make them useful for control purposes. Yeasts generally do not produce mycotoxins as do many mycelial fungi, nor antibiotic metabolites that are likely to be produced by bacterial antagonists (Droby and Chalutz, 1994). Yeast Isolates YP25 and YP60 competitively colonized wounds of apple in the presence of *B. cinerea* and *P. expansum,* respectively (Figures 6.6A and B).

Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time, as well as withstand many pesticides used in the postharvest environment (Wilson and Wisniewski, 1989; El-Tarabily and Sivasithamparam, 2006). In addition, yeasts can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Druvefors, 2004).

Fungal growth of *Botrytis cinerea* and *Penicillium expansum* was not inhibited by yeast Isolates YP25 and YP60 on nutrient agar plates. Furthermore, there was no visual or microscopic evidence of hyperparasitism by Isolates YP25 and YP60 on *B. cinerea* and *P. expansum*. Similar findings were reported by Zheng *et al*. (2005) that tests on agar plates with yeast isolate *Rhodotorula glutinis* (Harrison) did not inhibit growth of *P. digitatum* (Pers.Fr. Sacc.). The control of *B. cinerea* and *P. expansum* on wounds in "Golden Delicious" apples could have been due to yeast Isolates YP25 and YP60 consuming all nutrients required for germination of *B. cinerea* and *P. expansum*. We hypothesize that the prolific colonies of yeast Isolates YP25 and YP60 used up all available nutrients, and that this resulted in the observed control of both *P. expansum*

and *B. cinerea* by inhibiting conidial germination, a theory proposed by Wilson and Wisniewski (1989). Most yeast antagonists do not produce any inhibitory substances during *in vitro* testing and are very efficient in utilizing carbon and nitrogen sources in apple and pear wounds (Janisiewicz *et al.,* 2000).

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CHAPTER 7
INTEGRATED CONTROL OF *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA* OF APPLES USING POTASSIUM SILICATE, YEAST ANTAGONISTS AND YIELDPLUS®

ABSTRACT

The use of potassium silicate (K_2SiO_3), YieldPlus® and yeast antagonists treatments, alone or in combination, was investigated for the control of grey and blue moulds of "Golden Delicious" apples. *In vitro* studies indicated that yeast Isolates YP25 and YP26 were compatible with K_2SiO_3 postharvest treatments. K_2SiO_3 alone or in combination with yeast antagonists and YieldPlus® reduced postharvest grey and blue moulds on apples. YieldPlus® and Imazalil® treatments alone were more effective in reducing grey and blue moulds than yeast Isolates YP25 and YP60. The combination of K_2SiO_3 with an antagonist mixture of YP25+YP60 provided the best control (95%) of grey mould, while the combination of K_2SiO_3 and YieldPlus® provided the best control (85%) of blue mould. The use of potassium silicate and yeasts antagonists in combination resulted in 95% and 77.5% control of *B. cinerea* and *P. expansum*, respectively, in "Golden Delicious" apples compared to 62.5% and 65% on Imazalil® fungicide treated fruits.

7.1 INTRODUCTION

Postharvest diseases can be a limiting factor for long-term storage of pome fruits (He *et al.,* 2003). Grey mould (*Botrytis cinerea* Pers.: Fr.) and blue mould (*Penicillium expansum* (Link) Thom.) are the most destructive postharvest pathogens on pome fruit (Spotts and Chen, 1987). The use of fungicides is the main disease control option that is currently practiced. Biocontrol agents have been widely investigated and promising results have been achieved (Janisiewicz and Korsten, 2002). Microbial antagonists such as bacteria, yeast, and filamentous fungi play an important role in the natural control of numerous postharvest pathogens of fruits (Janisiewicz, 1987; Wilson and Chalutz, 1989; Roberts, 1990; Lima *et al.,* 1997).

Alternatives to chemical control, such as biological control, are often less effective compared with commercial fungicides (Janisiewicz *et al*., 1992; El-Ghaouth *et al*., 2002; Leverentz *et al*., 2003). To achieve a similar level of efficacy provided by conventional chemicals, the use of microbial antagonists combined with commercial chemicals (Droby *et al*., 1998), hot water (Pusey, 1994; Nunes *et al*., 2002; Palou *et al*., 2002; Obagwu and Korsten, 2003), chloride salts (McLaughlin *et al*., 1990), carbonate salts (Smilanick *et al*., 1999; El-Ghaouth *et al*., 2000a; El-Ghaouth *et al*., 2000b; Palou *et al*., 2002; Obagwu and Korsten, 2003), and other physical treatment such as curing and heat treatments (Plaza *et al*., 2003) have been used as part of integrated control strategies.

Integrated approaches have been successfully applied to apples to control *Penicillium expansum* (Link) Thom. Optimum control of postharvest fungal infection has been achieved by combining heat treatment, calcium chloride and the antagonist *Pseudomonas syringae* (Van Hall) (Conway *et al.,* 1999) or a biocontrol mixture, applied with a heat treatment and/or sodium bicarbonate (Conway *et al.,* 2005). The addition of glycochitin enhanced the biocontrol activity of *Candida saitoana* against decay of apple, lemon, and orange, caused by *Botrytis cinerea*, *Penicillium expansum* and *Penicillium digitatum*, respectively (El-Ghaouth *et al*., 2000a; 2000b). The objective of this study was to investigate the individual or combined effect of potassium silicate, yeast antagonists, and the biofungicide YieldPlus® treatments for the control of *B. cinerea* and *P. expansum* on apples.

7.2 MATERIALS AND METHODS

7.2.1 Fruit

Apple (*Malus domestica* Borkh.) cultivar “Golden Delicious” was collected from a Pick ‘n Pay Supermarket in Pietermaritzburg, South Africa (SA). Only undamaged, healthy, mature fruits were used in the experiments.

7.2.2 Yeast antagonists

Yeasts, potentially antagonistic to *B. cinerea* and *P. expansum*, were isolated from the surfaces of apple and pear fruits. The fruit peel was cut into small pieces weighing 80 g and placed in separate 250 mℓ Erlenmeyer flasks containing 100 mℓ sterile distilled water and shaken in a water bath at 130 rotations per minute (rpm) for 1 hrs at 28°C. Fruit peel pieces were removed and the liquid suspension was used to make serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. An aliquot of 0.2 mℓ of each dilution was plated onto nutrient yeast dextrose agar (NYDA) plates and incubated at 25°C for 3 days. Pure cultures of yeast were made by sub-culturing colonies onto fresh (NYDA) plates. Yeast cultures were cultured in 250 mℓ Erlenmeyer flasks containing 50 mℓ of nutrient yeast dextrose broth (NYDB) (8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1 liter of distilled water) on a rotary shaker at 200 rpm for 48 hrs at 25°C. Yeast cells were centrifuged at 4,000 × g for 10 min, re-suspended in sterile distilled water, and adjusted to a concentration of 1×10^7 cells $mℓ^{-1}$ with a haemacytometer.

7.2.3 Fungal pathogens

B. cinerea and *P. expansum* were isolated from infected strawberries and pears, respectively. The cultures were maintained on potato dextrose agar (PDA) at 25°C. The spores were directly plated onto PDA. Plates were incubated at 25°C for 7 days. Pure cultures were obtained by subculturing on fresh PDA plates. The cultures were maintained on McCartney bottles three-quarter filled with double sterilized distilled water, and incubated at room temperature for long term storage. The pathogen inoculum was prepared from 7 day old culture dishes incubated at 25°C, by flooding with sterile distilled water, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. Spore concentrations *B. cinerea* and *P. expansum* were determined with a haemacytometer and adjusted to1 $\times 10^4$ conidia $mℓ^{-1}$ with sterile distilled water.

7.2.4 *In vitro* compatibility of yeast Isolates P60 and YP25 with potassium silicate (K_2SiO_3)

Yeast Isolates (YP60 and YP25) were streaked onto NYDA plates and incubated at 25°C for 3 days. A loop of each yeast isolate was added into 10 mℓ of potassium silicate (K_2SiO_3) diluted with sterile distilled water, to concentrations of 10, 100, 1,000, 10,000 and 100,000 mg $ℓ^{-1}$. Sterile distilled water served as control treatments. Ten minutes later 0.1 mℓ of the suspensions was plated onto nutrient agar (NA) plates. There were three plates per treatment. The plates were incubated at 25°C for three days and the growth of the yeast Isolates YP25 and YP60, with and without K_2SiO_3, was observed.

7.2.5 Assessment of potassium silicate, YieldPlus® and yeast Isolates YP25 and YP60 treatments, applied alone or in combination, for the control of *Penicillium expansum* and *Botrytis cinerea* of "Golden Delicious" apples

Fruit was uniformly wounded, approximately 5 mm deep and 3 mm wide, with a sterile needle at ach equatorial side. Four wounds were made in each apple fruit. The wounds were treated as described in Table 7.1. The wounds were allowed to dry for 24 hours, and then inoculated with a 30 µℓ conidial suspension of *P. expansum* or *B. cinerea* at 1×10^4 conidia $mℓ^{-1}$. There were three controls: an untreated control (with no yeast Isolates, no biofungicide, and no fungicide); a biofungicide control with YieldPlus® (1×10^8 cells $mℓ^{-1}$); and a fungicide control with Imazalil® (0.67g of Imazalil® in 1000 mℓ of water). Ten fruits were used for each treatment. Fruit were kept at room temperature (24±1°C) for 7 days, and the incidence of grey and blue moulds was determined.

Table 7.1 Treatments for screening of yeast Isolates YP25 and YP60, YieldPlus® and potassium silicate, alone or in combination, for control of *Penicillium expansum* and *Botrytis cinerea* on "Golden Delicious" apples.

Treatment	K_2SiO_3	YieldPlus®	Imazalil®	YP60	YP25
1 (control)	No	No	No	No	No
2	No	No	Yes	No	No
3	Yes	No	No	No	No
4	No	Yes	No	No	No
5	No	No	No	Yes	No
6	No	No	No	No	Yes
7	Yes	Yes	No	No	No
8	Yes	No	No	Yes	No
9	Yes	No	No	No	Yes
10	Yes	No	No	Yes	Yes

7.2.6 Statistical analysis

Data for screening of yeast Isolates (YP25 and YP60), YieldPlus® and potassium silicate, alone or in combination, for control of *Penicillium expansum* and *Botrytis cinerea* on "Golden Delicious" apples were subjected to an analysis of variance (ANOVA) using Genstat® Executable 14th edition. To determine differences between treatments, Fisher's Least Significant Difference Test was used (P = 0.05).

7.3 RESULTS

7.3.1 *In vitro* compatibility of yeast isolates (YP60 and YP25) with potassium silicate (K_2SiO_3)

Yeast Isolate YP60 mixed with 100, 000 mg ℓ^{-1} K_2SiO_3 (Figure 7.1A), or without K_2SiO_3 (Figure 7.1B), was able to grow on NA plates. Yeast Isolate YP25 mixed with 100,000 mg ℓ^{-1} K_2SiO_3 (Figure 7.2 A) or without K_2SiO_3 (Figure 7.2B), was able to grow on NA plates.

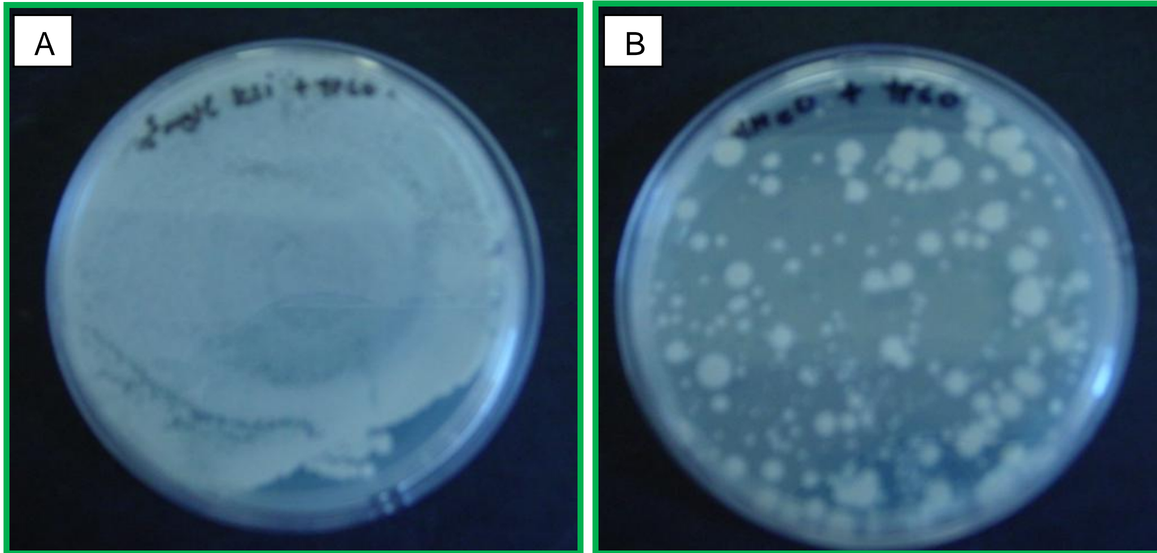

Figure 7.1 Growth of YP60 with potassium silicate at 100,000 mg ℓ^{-1} (A) and without potassium silicate (B) on nutrient agar plates.

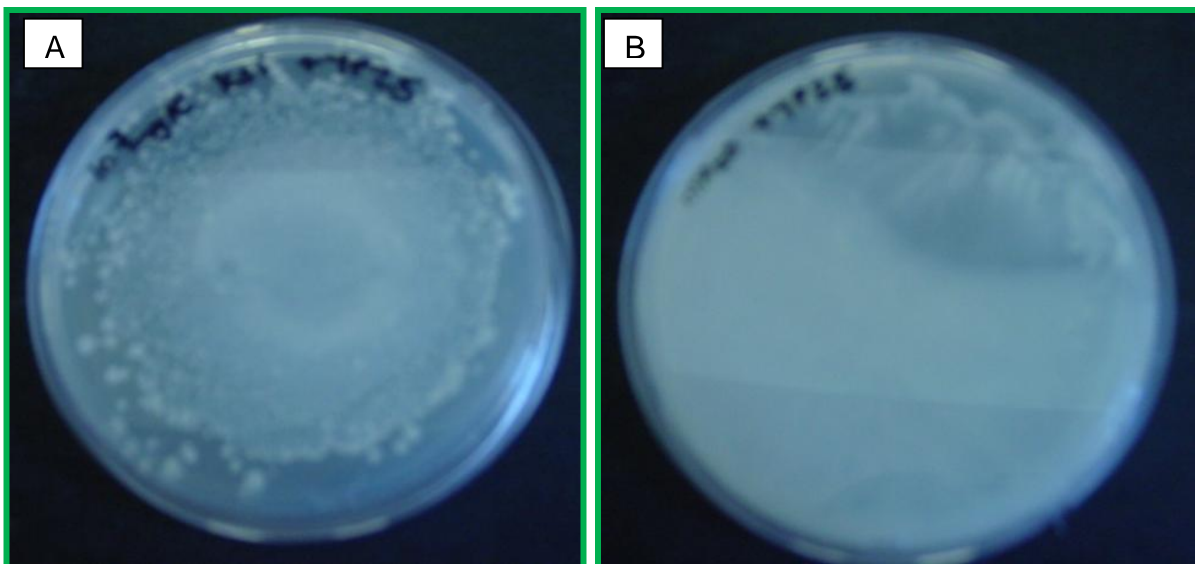

Figure 7.2 Growth of YP25 with potassium silicate at 100, 000 mg ℓ^{-1} (A) and without potassium silicate (B) on nutrient agar plates.

7.3.2 Effects of potassium silicate, yeast Isolate YP25, yeast Isolate YP60 and YieldPlus® treatments, alone or in combination, on the control of *Botrytis cinerea* and *Penicillium expansum* of "Golden Delicious" apples

The incidence of grey mould in all treated fruit was significantly (P<0.001) lower than those of the control fruit (Figure 7.3). The combination of potassium silicate (KSi) and the yeast antagonist mixture was the most effective treatment, and the level of grey mould incidence was 5%, compared to 95.7% incidence in control fruit (Figure 7.3). Imazalil® treated fruit developed an incidence of 37.5% *B. cinerea*, compared to 27.5% incidence in YieldPlus® treated fruit (Figure 7.3). The combination of potassium silicate and either yeast Isolates YP25 and YP60 significantly (P<0.001) reduced grey mould, with a 12.5% incidence and 20% incidence, respectively, compared with 37.5% and 97.5% for the Imazalil® and the control treatments, respectively (Figure 7.3).

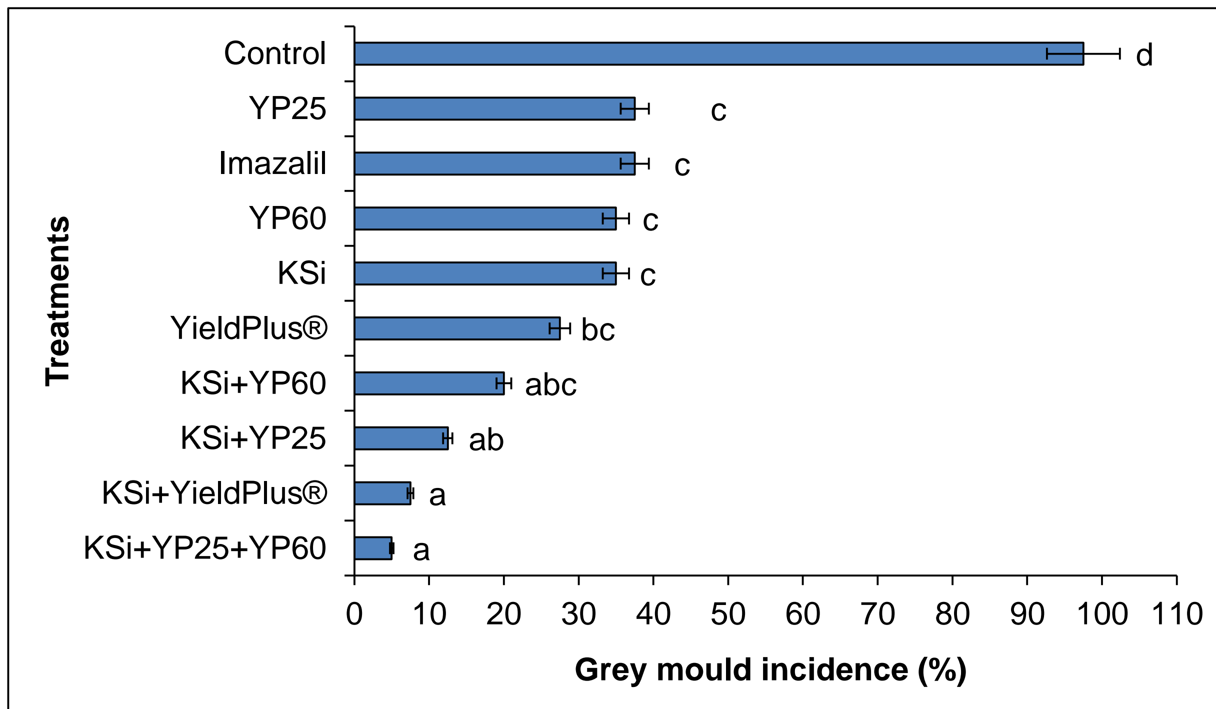

Figure 7.3 Effect of yeast Isolates YP25 and YP26, silicon (KSi) and YieldPlus® on grey mould incidence of "Golden Delicious" apples. Means with the same letters are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

The incidence of blue mould in all treated fruit was significantly (P<0.001) lower than those of the control fruit (Figure 7.4). The combination of potassium silicate and YieldPlus® was the most effective treatment for the control of blue mould (Figure 7.4). The level of blue mould incidence was 15%, compared to 100% incidence in control fruit (Figure 7.4). Imazalil® treated fruit developed an incidence of 35% *P. expansum*, compared to YieldPlus® treated fruit with 37.5% *P. expansum* (Figure 7.4). Furthermore, the incidence of blue mould was significantly (P<0.001) reduced by the combination of potassium silicate and either yeast Isolate YP25 (20%) and YP60 (17.5%), compared to Imazalil® (35%) and control (100%) treatments (Figure 7.4).

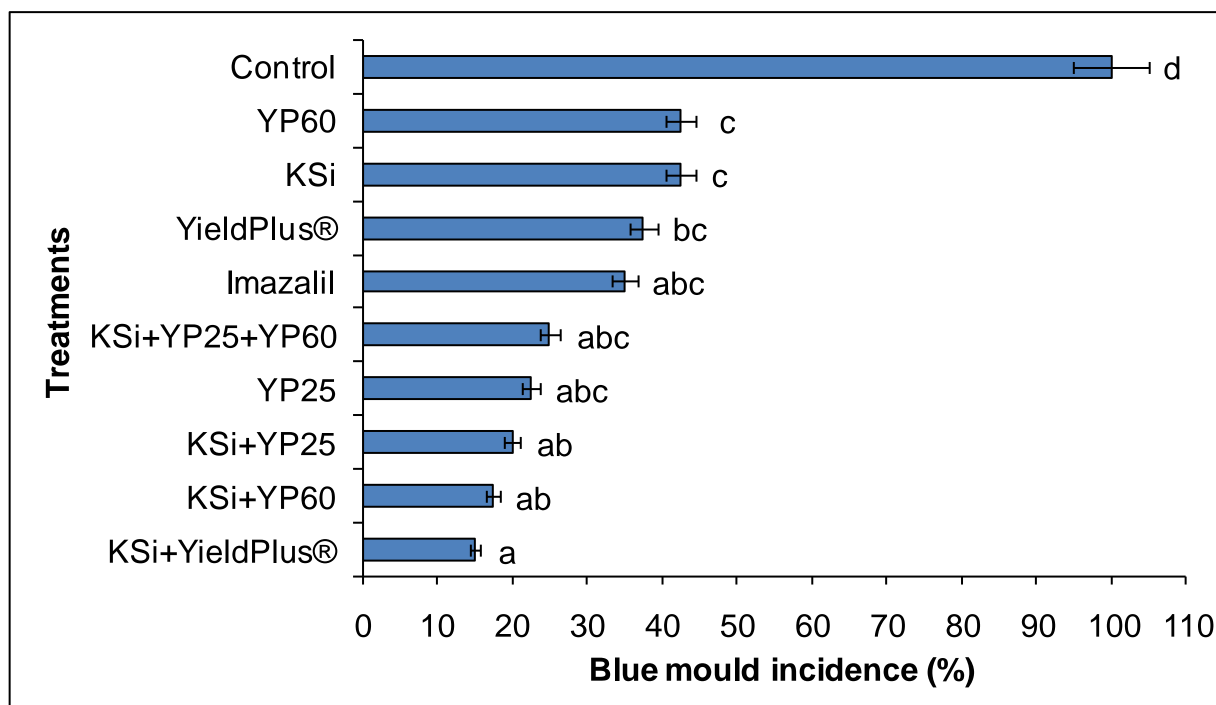

Figure 7.4 Effect of yeast Isolates YP25 and YP26, silicon (KSi) and YieldPlus® on blue mould incidence of "Golden Delicious" apples. Means with the same letters are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

7.4 DISCUSSION

Postharvest application of microbial antagonists is a better, practical and useful method for the control of postharvest diseases of fruit and vegetables (Sharma *et al.,* 2009).

However, microbial antagonists usually do not provide 100% control of postharvest diseases. Several approaches have been useful to increase microbial antagonists effectiveness and to enhance their bioefficacy (Sharma *et al.,* 2009).

Potassium silicate, antagonistic yeasts, YieldPlus® treatments and their combinations were compared. The effectiveness of potassium silicate combined with the yeast antagonist mixture (YP25+YP60) in controlling *Botrytis cinerea* infection was higher than either potassium silicate or the yeast antagonists treatments alone (Figure 7.3). Several researchers have found that a number of pathogens on apple fruit were controlled better by microbial antagonists applied in mixtures than by the same microbial strains applied alone (Leibinger *et al.,* 1997; Calvo *et al.,* 2003; and Conway *et al.,* 2005).

Sharma *et al.* (2009) listed several advantages through the use of antagonist mixtures: 1) Increase in the effectiveness under different conditions such as cultivars, maturity stages, and location; 2) It can result in the control of more than one postharvest disease; 3) Reduction in application rates and cost of treatments; 4) Combination of different biocontrol traits without the transfer of alien genes through genetic transformation; and 5) Enhancement in efficiency and reliability of biocontrol because the components of the mixture acts through different mechanisms such as antagonism, parasitism, and induction of resistance in the host. A mixture of the antagonists *Candida sake* (Berkh.) and *Pantoea agglomerans* (Ewing and Fife) Gavini. controlled fruit rot diameter completely in 'Blanquilla' pear and provided the highest level of control in blue mould rot on "Golden Delicious" apples (Nunes *et al.,* 2001). Leibinger *et al.* (1997) showed that a mixture of *Aureobasidium pullulans* (de Bary) and *Bacillus subtilis* (Ehrenberg) reduced postharvest decay of *P. expansum* and *B. cinerea* on "Golden Delicious" apples. Application of mixture M1, a combination of strains of *A. pullulans* and *R. glutinis*, increased the population sizes of *A. pullulans* and red yeasts more than 10-fold on "Golden Delicious" apple, and they composed 95% of the total population of microorganisms on the fruit surface (Leibinger *et al.* 1997).

Several researchers have shown that salt additives improve the bioefficacy of some microbial antagonists in controlling postharvest decay on fruit and vegetables (Plaza *et al.,* 2001; Teixido *et al.,* 2001; Tian *et al.,* 2002; El-Ghaouth *et al.,* 2004; Zhang *et al.,* 2005; Qin *et al.,* 2006; Torres *et al.,* 2007; Cao *et al.,* 2008; and Janisiewicz *et al.,* 2008). Sharma *et al.* (2009) reported that the bioefficacy of *Candida sake* against *Penicillium expansum* on apples was significantly enhanced by the addition of L-serine and L-aspartic nitrogenous compounds.

7.5 CONCLUSION

The use of potassium silicate, yeast Isolates YP25 and YP60, YieldPlus® and the antagonists mixture (YP25+YP60) in combination, resulted in the control of *B. cinerea* and *P. expansum* of "Golden Delicious" apples compared to Imazalil® treated fruit.

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THESIS OVERVIEW

The purpose of this overview is to look back at the objectives presented in the introduction, to review progress made towards these objectives, to identify future research needs and then recommend future research directions. Finally, it is to make recommendations to the pome industry based on the research outcomes of this thesis.

The research objective and results are summarized. The first objective was to determine the influence of conidial concentration of *B. cinerea* and *P. expansum* on their pathogenicity on pears. There was no significant dose effect on the incidence of grey mould and blue mould at different conidial concentrations (1×10^3, 1×10^4, 1×10^5 conidia $m\ell^{-1}$). The second objective was to determine the sensitivity of *B. cinerea* and *P. expansum* to the biofungicide YieldPlus (*Cryptococcus albidus*). Isolates of both *B. cinerea* and *P. expansum* were reduced by the biofungicide YieldPlus® treatment compared to the untreated control. YieldPlus® is an effective biocontrol agent against grey mould and blue mould of pears, caused by *B. cinerea* and *P. expansum*, respectively.

The third objective was to isolate antagonistic yeasts originating from the surface of apple and pear fruits and evaluate their efficiency for the *in vivo* control of *B. cinerea* and *P. expansum* of pome fruit. A total of 100 epiphytic yeast isolates were obtained from the fruit surface of "Golden Delicious" apples and "Packham's Triumph" pears, and screened against *B. cinerea* and *P. expansum*, the causal agents of grey and blue moulds respectively. Fifteen yeast isolates reduced grey mould incidence by > 50%, when applied four hours before inoculation with *B. cinerea*. Similarly, seven yeast isolates reduced blue mould incidence by > 50%, when applied four hours before inoculation with *P. expansum*. YieldPlus® and yeast Isolate YP25 provided the best control of *B. cinerea*, while Isolate YP60 and YieldPlus® provided the best control of *P. expansum* on "Golden Delicious" apples. A mixture of YP25 and YP60 provided complete control of both *B. cinerea* and *P. expansum,* when applied to "Golden Delicious" apples before inoculation with either *B. cinerea* or *P. expansum*.

The fourth objective was to evaluate the effect of applying potassium silicate, potassium chloride and potassium hydroxide as postharvest treatments for the control *B. cinerea* and *P. expansum* of “Golden Delicious” apples. Potassium silicate as a postharvest treatment resulted in preventative and curative control of *B. cinerea* (77.5% and 52.5%, respectively) and *P. expansum* (65% and 55%, respectively). Both potassium chloride and potassium hydroxide reduced the incidence of blue mould (77.5% and 70%, respectively) and grey mould (65% and 62.5%, respectively) on “Golden Delicious” apples when applied as a preventative treatment (24 hours before inoculation with either *P. expansum* or *B. cinerea*).

The fifth objective was to evaluate the use of hot water treatment for the control of *B. cinerea* and *P. expansum*. *In vivo* tests showed that the disease incidence of *P. expansum* and *B. cinerea* on “Golden Delicious” apples was reduced by hot water dip treatments at 58-60°C for 60 to 120 seconds, without causing skin damage.

The sixth objective was to determine possible modes of action of effective yeast antagonists YP25 and YP60 for the control of *B. cinerea* and *P. expansum* by observing interactions in apple fruit wounds using ESEM. The yeast Isolates YP25 and YP60 demonstrated good colonization of apple wounds and provided protection against *P. expansum* and *B. cinerea*, respectively. Scanning electron microscopy observations demonstrated that yeast Isolates YP25 and YP60 inhibited or restricted conidial germination of *B. cinerea* and *P. expansum*, when it was applied preventatively.

Lastly, the seventh objective was to determine independent and combined effects of potassium silicate, YieldPlus® and yeast Isolates YP25 and YP60 treatments for the control of *B. cinerea* and *P. expansum* of “Golden Delicious” apples. The use of potassium silicate, yeasts (Isolates YP25 and YP60), YieldPlus® and antagonists mixture (YP25+YP60) in combination, resulted in the control of *B. cinerea* and *P. expansum* of “Golden Delicious” apples compared with Imazalil® treated fruit. The combination of K_2SiO_3 with an antagonist mixture of YP25+YP60 provided the best control (95%) of grey mould, while the combination of K_2SiO_3 and YieldPlus® provided

the best control (85%) of blue mould. The use of potassium silicate and yeasts antagonists in combination resulted in 95% and 77.5% control of *B. cinerea* and *P. expansum* respectively in "Golden Delicious" apples compared to 62.5% and 65% on Imazalil® fungicide treated fruits.

Future research will be based on the following steps: 1) Assess the ability of yeast Isolates YP25 and YP60 to colonize pome fruit surfaces and to investigate their efficacy in controlling postharvest infection by *B. cinerea* and *P. expansum*, when applied before harvest in the orchard; 2) Investigate the effects of preharvest applications of potassium silicate for the control of *B. cinerea* and *P. expansum* infections of apple and pear fruits and to determine the silicon content of leaves and fruit of apple and pear after harvest; 3) Investigate the individual or combined effect of hot water dip, yeast Isolates YP25 and YP25, and potassium silicate treatments in commercial packhouses for the control of *B. cinerea* and *P. expansum* of apples and pears; 4) Molecular work to determine the colonization and performance of yeast Isolates YP25 and YP60 in the field trials.

Grey mould (*Botrytis cinerea)* and blue mould (*Penicillium expansum*) are the most destructive postharvest pathogens on pome fruits. These pathogens attack fruits through wounds. Postharvest fungicidal treatment on apples is currently the main control measure applied to decreases losses caused by postharvest pathogens. The residual effect during storage and the appearance of pathogen strains of *P. expansum* and *B. cinerea* resistant to fungicides has increased the need to develop alternative methods. As such, the deciduous fruit industry needs non-chemical control options in order to combat *Penicillium* and *Botrytis*. From this research we have discovered control measures that are effective against *Botrytis cinerea* and *Penicillium expansum* of pome fruit. These control options include using antagonistic yeast isolates, postharvest application of potassium silicate and a hot water dip treatment.

APPENDICES

Appendix 2A: Pathogenicity of *B. cinerea* on "Packham's Triumph" pears after 7 days of storage at 25°C

Concentration of ***B. cinerea***	Grey mould incidence (%)
1×10^3	79.2 a
1×10^4	79.2 a
1×10^5	100.0 a
Control	8.3 b
P-value	<0.001
F-ratio	28.1
SED	10.7
LSD	22.3
%CV	27.8

Appendix 2B: Pathogenicity of *P. expansum* on "Packham's Triumph" pears after 7 days of storage at 25°C

Concentration of ***P. expansum***	Blue mould incidence (%)
1×10^3	87.5 b
1×10^4	87.5 b
1×10^5	95.8 b
Control	8.3 a
P-value	<0.001
F-ratio	29.13
SED	10.8
LSD	22.5
%CV	26.8

Appendix 2C: Sensitivity of *B. cinerea* on "Packham's Triumph" pears after 7 days of storage at 25°C

Concentration of *B. cinerea*	**Grey mould incidence (%)**
1×10^6	41.7 b
1×10^7	29.2 b
1×10^8	4.2 c
Control	95.8 a
P-value	<0.001
F-ratio	24.30
SED	11.1
LSD	23.2
%CV	45.0

Appendix 2D: Sensitivity of *P. expansum* to YieldPlus® on "Packham's Triumph" pears after 7 days of storage at 25°C

Concentration of *P. expansum*	**Blue mould incidence (%)**
1×10^6	37.5 b
1×10^7	20.8 ab
1×10^8	8.3 a
Control	95.8 c
P-value	<0.001
F-ratio	33.8
SED	9.41
LSD	19.6
%CV	40.1

Appendix 3A: Dose effect of selected yeast antagonists on grey mould incidence of "Golden Delicious" apples, when applied 48 hrs before inoculation with *B. cinerea*

Treatment	Grey mould incidence (%)
YieldPlus® (1 x 10^8 cells mℓ^{-1})	16.7 a
YP25 (1 x 10^8 cells mℓ^{-1})	16.7 a
YieldPlus® (1 x 10^7 cells mℓ^{-1})	25.0 a
YieldPlus® (1 x 10^6 cells mℓ^{-1})	33.3 a
YP16 (1 x 10^8 cells mℓ^{-1})	33.3 a
YP24 (1 x 10^8 cells mℓ^{-1})	33.3 a
YP16 (1 x 10^6 cells mℓ^{-1})	41.7 a
YP25 (1 x 10^7 cells mℓ^{-1})	41.7 a
YP24 (1 x 10^6 cells mℓ^{-1})	50.0 ab
YP25 (1 x 10^6 cells mℓ^{-1})	50.0 ab
YP16 (1 x 10^7 cells mℓ^{-1})	58.3 ab
YP24 (1 x 10^7 cells mℓ^{-1})	58.3 ab
Control (1 x 10^4 conidia mℓ^{-1})	100.0 b
P-value	0.96
F-ratio	0.19
LSD	54.6
SED	26.6
%CV	75.7

Appendix 3B: Dose effect of selected yeast antagonists on blue mould incidence of "Golden Delicious" apples, when applied 48 hrs before inoculation with *P. expansum*

Treatment	Blue mould incidence (%)
YieldPlus® (1 x 10^8 cells $m\ell^{-1}$)	16.7 a
YP60 (1 x 10^8 cells $m\ell^{-1}$)	16.7 a
YP60 (1 x 10^7 cells $m\ell^{-1}$)	25.0 a
YieldPlus® (1 x 10^7 cells $m\ell^{-1}$)	33.3 a
YP60 (1 x 10^6 cells $m\ell^{-1}$)	33.3 a
YieldPlus® (1 x 10^6 cells $m\ell^{-1}$)	41.7 a
Control (1 x 10^4 conidia $m\ell^{-1}$)	100.0 b
P-value	0.78
F-ratio	0.08
LSD	51.5
SED	24.0
%CV	77.1

Appendix 3C: Effect of a yeast antagonist mixture (YP25+YP60) on grey mould incidence of "Golden Delicious" apples, when applied 48 hrs before inoculation with *B. cinerea*

Treatment	Grey mould incidence (%)
YP25 (1 x 10^6 cells $mℓ^{-1}$)	0.0 a
YP25+YP60 (1 x 10^6 cells $mℓ^{-1}$)	0.0 a
YP25+YP60 (1 x 10^7 cells $mℓ^{-1}$)	0.0 a
YP25+YP60 (1 x 10^8 cells $mℓ^{-1}$)	0.00 a
YP25 (1 x 10^8 cells $mℓ^{-1}$)	0.0 a
YieldPlus® (1 x 10^8 cells $mℓ^{-1}$)	10.0 ab
YP25 (1 x 10^7 cells $mℓ^{-1}$)	10.0 ab
YieldPlus® (1 x 10^7 cells $mℓ^{-1}$)	20.0 bc
YieldPlus® (1 x 10^6 cells $mℓ^{-1}$)	30.0 cd
YP60 (1 x 10^8 cells $mℓ^{-1}$)	40.0 d
YP60 (1 x 10^6 cells $mℓ^{-1}$)	60.0 e
YP60 (1 x 10^7 cells $mℓ^{-1}$)	60.0 e
Control (1 x 10^5 conidia $mℓ^{-1}$)	100.0 f
P-value	0.31
F-ratio	1.20
LSD	18.7
SED	9.4
%CV	83.0

Appendix 3D: Effect of a yeast antagonist mixture (YP25+YP60) on blue mould incidence of "Golden Delicious" apples, when applied 48 hrs before inoculation with *P. expansum*

Treatment	**Grey mould incidence (%)**
YP25+YP60 (1 x 10^7 cells mℓ^{-1})	0.0 a
YieldPlus® (1 x 10^7 cells mℓ^{-1})	0.0 a
YP25+YP60 (1 x 10^6 cells mℓ^{-1})	0.0 a
YP25+YP60 (1 x 10^8 cells mℓ^{-1})	0.0 a
YP60 (1 x 10^8 cells mℓ^{-1})	0.0 a
YieldPlus® (1 x 10^8 cells mℓ^{-1})	0.0 a
YieldPlus® (1 x 10^6 cells mℓ^{-1})	10.0 ab
YP60 (1 x 10^7 cells mℓ^{-1})	10.0 ab
YP60 (1 x 10^6 cells mℓ^{-1})	20.0 b
YP25 (1 x 10^7 cells mℓ^{-1})	50.0 c
YP25 (1 x 10^8 cells mℓ^{-1})	60.0 c
YP25 (1 x 10^6 cells mℓ^{-1})	60.0 c
Control (1 x 10^5 conidia mℓ^{-1})	100.0 d
P-value	0.41
F-ratio	1.02
LSD	16.8
SED	8.5
%CV	79.5

Appendix 4A: Preventative activity of potassium silicate against *B. cinerea*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of K_2SiO_3 (mg ℓ^{-1})	Grey mould incidence (%)
10	75.0 c
100	72.5 c
1000	47.5 b
10000	45.0 b
100000	22.5 a
0 (Control)	100.0 d
P-value	<0.001
F-ratio	19.0
LSD	17.9
%CV	33.0

Appendix 4B: Preventative activity of potassium silicate against *P. expansum*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of K_2SiO_3 (mg ℓ^{-1})	Blue mould incidence (%)
10	70.0 bc
100	75.0 c
1000	50.0 ab
10000	60.0 bc
100000	35.0 a
0 (Control)	100.0 d
P-value	<0.001
F-ratio	9.31
LSD	20.8
%CV	35.7

4C: Curative activity of potassium silicate against *B. cinerea*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of K_2SiO_3 (mg $ℓ^{-1}$)	Grey mould incidence (%)
10	82.5 bc
100	75.0 b
1000	67.5 ab
10000	67.5 ab
100000	47.5 a
0 (Control)	100.0 c
P-value	0.001
F-ratio	4.80
LSD	22.7
%CV	34.5

Appendix 4D: Curative activity of potassium silicate against *P. expansum*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of K_2SiO_3 (mg $ℓ^{-1}$)	Blue mould incidence (%)
10	75.0 b
100	62.5 ab
1000	62.5 ab
10000	52.5 a
100000	45.0 a
0 (Control)	100.0 c
P-value	<0.001
F-ratio	9.17
LSD	18.2
%CV	30.6

Appendix 4E: Preventative activity of potassium chloride against *P. expansum*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of KCℓ$_2$ (mg ℓ$^{-1}$)	Blue mould incidence (%)
10	40.0 b
100	45.0 b
1000	40.0 b
10000	30.0 ab
100000	22.5 a
0 (Control)	100.0 c
P-value	<0.001
F-ratio	24.4
LSD	15.8
%CV	38.2

Appendix 4F: Preventative activity of potassium chloride against *B. cinerea*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of KCℓ$_2$ (mg ℓ$^{-1}$)	Grey mould incidence (%)
10	60.0 b
100	40.0 ab
1000	37.5 a
10000	40.0 ab
100000	35.0 a
0 (Control)	100.0 c
P-value	<0.001
F-ratio	11.34
LSD	21.2
%CV	45.3

Appendix 4G: Preventative activity of potassium hydroxide against *P. expansum*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of KOH (mg ℓ^{-1})	Blue mould incidence (%)
10	55.0 b
100	60.0 b
1000	40.0 ab
10000	30.0 a
100000	30.0 a
0 (Control)	100.0 c
P-value	<0.001
F-ratio	13.2
LSD	20.60
%CV	43.8

Appendix 4H: Preventative activity of potassium hydroxide against *B. cinerea*, when applied as a postharvest treatment on "Golden Delicious" apples

Application of KOH (mg ℓ^{-1})	Grey mould incidence (%)
10	57.5 b
100	45.0 ab
1000	47.5 ab
10000	52.5 ab
100000	37.5 a
0 (Control)	100.0 c
P-value	<0.001
F-ratio	11.3
LSD	18.8
%CV	37.0

Appendix 7A Effects of potassium silicate, yeast Isolates YP25, yeast Isolate YP60 and YieldPlus® treatments, alone or in combination, on the control of *B. cinerea* of "Golden Delicious" apples

Treatments	Grey mould incidence (%)
Silicon+YP25+YP60	5.0 a
YieldPlus®+Silicon	7.5 a
Silicon+YP25	12.5 ab
Silicon+YP60	20.0 abc
YieldPlus®	27.5 bc
Silicon	35.0 c
YP60	35.0 c
Imazalil®	37.5 c
YP25	37.5 c
Control	97.5 d
P-value	<0.001
F-ratio	14.4
LSD	19.5
%CV	69.7

Appendix 7B Effects of potassium silicate, yeast Isolates YP25, yeast Isolate YP60 and YieldPlus® treatments, alone or in combination, on the control of *P. expansum* of "Golden Delicious" apples

Treatments	**Blue mould incidence (%)**
YieldPlus®+Silicon	15.0 a
Silicon+YP60	17.5 ab
Silicon+YP25	20.0 ab
YP25	22.5 abc
Silicon+YP25+YP60	22.5 abc
Imazalil®	35.0 abc
YieldPlus®	37.5 bc
Silicon	42.5 c
YP60	42.5 c
Control	100 d
P-value	<0.001
F-ratio	11.04
LSD	21.0
%CV	66