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Studies on production and ecophysiology of fungal antagonists for improved biological control of *Fusarium oxysporum* f.sp. *lycopersici* and *Monilinia laxa*.

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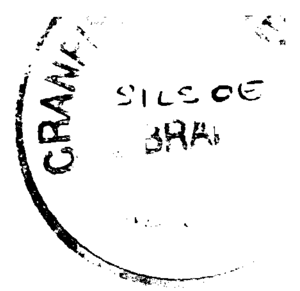

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ABSTRACT

This work investigated the production of the biocontrol agents: *Penicillium oxalicum* to control *Fusarium oxysporum* f.sp. *lycopersici* in the rhizosphere; and *Epicoccum nigrum* and *Penicillium frequentans* to control *Monilinia laxa* in the phyllosphere. Ecophysiological studies were carried out to categorise different inoculum types, and to test their biocontrol efficacy.

A method was developed for the induction of submerged conidiation of *P.oxalicum* for the first time. This was achieved by transferring 24 h cultures to a nitrogen free medium, and further stimulated by a high C:N ratio with 20 mM calcium. Optimum submerged conidial concentrations obtained were $35x10^6$ spores ml^{-1}. The hydrophobicity of submerged and aerial conidia of *P.oxalicum* was similar. However, different results were obtained depending on the method used. Appearance of both spore types under the SEM was similar in size and shape. However, submerged spores were covered by a filamentous matrix, absent in aerial ones. Viability of aerial and submerged *P.oxalicum* spores was higher than 80% after 27 weeks, when stored fresh at either 4 or 25°C, but aerial spores survived slightly better. Freeze-drying severely affected viability, especially of submerged spores. Aerial spores effectively colonised sterile soil in tomato seedbeds with water potentials in the range 1-7 (-MPa), and this was further favoured by the addition of nutrients. Aerial conidia of *P.oxalicum* applied to seedbeds were able to significantly ($P<0.05$) reduce *Fusarium* wilt of tomato at concentrations as low as $6x10^4$ spores ml^{-1} substratum, indicating that the amount of the antagonist needed is not a limiting factor for the practical application of this antagonist. Aerial spores were slightly more effective than submerged ones in the control of the disease. However, mycelium was ineffective. Coating of tomato seeds with formulations of aerial spores of *P.oxalicum* in alginate or methyl cellulose significantly ($P<0.05$) enhanced the growth promotion effect of the antagonist *in vitro*, which may be related to the ability of the fungus to control the disease.

E.nigrum spores were produced by solid fermentation on wheat grains at different water activities (a_w). Maximum levels of sporulation ($7\text{-}11x10^6$ spores g^{-1} grain) were obtained at high a_w (0.996) or reduced a_w (0.98) adjusted with a

mixture glycerol/water. *E.nigrum* and *P.frequentans* were both produced in culture medium at reduced a_w, to improve their ecological competence in the phyllosphere and therefore their biocontrol ability. *E.nigrum* produced at reduced a_w showed improved germ tube extension and in some cases colony growth rate when placed on medium at reduced a_w, showing water stress tolerance of such modified inocula. Furthermore, such inocula showed an enhanced ability to compete with the pathogen, *M.laxa*, at reduced a_w, shown by a higher Niche Overlap Index (the proportion of the carbon compounds utilised by *M.laxa* that were also utilised by *E.nigrum*). *E.nigrum* spores produced at reduced a_w had improved survival when stored fresh at 4 or 25°C. Freeze-drying severely affected the viability of both spore types (produced at high or reduced a_w).

E.nigrum and *P.frequentans* produced at reduced a_w accumulated low molecular weight polyols as compatible solutes. Improvement of biocontrol of peach twig blight was obtained in the case of *E.nigrum*, which is more sensitive to conditions of low water availability than the xerotolerant *P.frequentans*. However, both fungi accumulated glycerol as the main compatible solute, indicating that different accumulation mechanisms may be responsible for the different tolerance to low water availability. Glycerol was also the main compatible solute in *E.nigrum* spores produced by solid fermentation at reduced a_w. *E.nigrum* produced at high or reduced a_w was also able to control brown rot of cherries, under optimum conditions for the development of the disease.

The results presented in this work show that the conditions during the production of biocontrol agents are critical in determining their efficacy. Therefore, when developing mass-production systems it is necessary to aim not only for high propagule numbers but also for inoculum quality, defined by parameters such as ecological competence or survival during storage.

TABLE OF CONTENTS

LIST OF FIGURES AND PLATES I

LIST OF TABLES VIII

LIST OF ABBREVIATIONS XII

CHAPTER 1. LITERATURE REVIEW AND OBJECTIVES 1

1.1. GENERAL INTRODUCTION 2

1.2. FERMENTATION AND FORMULATION OF BIOCONTROL AGENTS (BCAs) 8

1.2.1. Solid fermentation versus liquid fermentation 8
1.2.2. Sporulation of fungi in submerged culture 11
1.2.3. Characteristics of submerged spores: Hydrophobicity 13
1.2.4. Formulation of biological control agents: Seed coating. 15

1.3. THE ECOLOGICAL APPROACH IN BIOCONTROL 17

1.3.1. Fungal competition 19
1.3.2. Ecological constraints in soil and the rhizosphere 21
1.3.3. Ecological constraints in the phyllosphere 23

1.4. HOST-PATHOGEN-BIOCONTROL SYSTEMS STUDIED 29

1.4.1. Rhizosphere system: *Lycopersicon esculentum* Mill. - *Fusarium oxysporum* Schlecht f.sp. *lycopersici* (Sacc.) Snyder et Hans. - *Penicillium oxalicum* Currie & Thom. 29
1.4.2. Phyllosphere system: *Prunus persicae* L. - *Monilinia laxa* (Aderh et Ruhl) Honey -*Epicoccum nigrum* Link/*Penicillium frequentans* Westling. 33

1.5. OBJECTIVES OF THIS WORK 39

CHAPTER 2. MATERIALS AND METHODS 42

2.1. CULTURE MEDIA 43

2.2. APPARATUS 46

2.3. BIOLOGICAL MATERIAL 47

2.3.1. Fungal isolates 47
2.3.2. Plant material 48

2.4. PRODUCTION OF *Penicillium oxalicum* INOCULUM 49
2.4.1. Production on solid culture medium 49
2.4.2. Production in submerged culture: induction of submerged conidiation 49

2.5. CHARACTERISATION OF *Penicillium oxalicum* SPORES 54

2.5.1. Study on growth of *Penicillium oxalicum* inoculum in sterile soil 54
2.5.2. Surface hydrophobicity and adhesion of aerial and submerged spores 56
2.5.3. Scanning electron microscopy of aerial and submerged spores 59
2.5.4. Viability of *Penicillium oxalicum* spores after storage 59

2.6. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum* 60

2.6.1. Minimum concentration of aerial conidia for disease control in growth chamber studies 60
2.6.2. Efficacy of aerial and submerged spores in growth chamber studies 63
2.6.3. Efficacy of *Penicillium oxalicum* in greenhouse experiments 64
2.6.4. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion 68

2.7. PRODUCTION OF *Epicoccum nigrum* AND *Penicillium frequentans*..........70

2.7.1. Production in culture media..........70

2.7.2. Production of *Epicoccum nigrum* by solid substrate fermentation..........71

2.8. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans*..........72

2.8.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions..........72

2.8.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w..........73

2.8.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans*..........74

2.8.4. Determination of turgor pressure in *Epicoccum nigrum* mycelium..........77

2.8.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa*..........78

2.8.6. Viability of *Epicoccum nigrum* spores after storage..........78

2.9. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans*..........79

2.9.1. Efficacy of *Epicoccum nigrum* as a post-harvest BCA for brown rot of fruit..........79

2.9.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans*..........80

CHAPTER 3. RESULTS..........84

I: STUDIES ON *Penicillium oxalicum* FOR BIOCONTROL OF *Fusarium* WILT OF TOMATO IN THE RHIZOSPHERE..........84

3.1. PRODUCTION OF *Penicillium oxalicum* INOCULUM: INDUCTION OF SUBMERGED CONIDIATION..........85

3.1.1. Spore production in different media 85
3.1.2. Studies using Morton's method 85
3.1.3. Sporulation in modified Potato Dextrose Broth (PDB) 87

3.2. CHARACTERISATION OF *Penicillium oxalicum* SPORES 90

3.2.1. Study on growth of *Penicillium oxalicum* inoculum in sterile soil 90
3.2.2. Surface hydrophobicity and adhesion of aerial and submerged spores ... 94
3.2.3. Scanning electron microscopy of aerial and submerged spores 98
3.2.4. Viability of *Penicillium oxalicum* spores after storage 98

3.3. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum* 101

3.3.1. Minimum concentration of aerial conidia for disease control in growth chamber studies 101
3.3.2. Efficacy of aerial and submerged spores in growth chamber studies 105
3.3.3. Efficacy of *Penicillium oxalicum* in greenhouse experiments 110
3.3.4. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion 118

II: STUDIES ON *Epicoccum nigrum* AND *Penicillium frequentans* FOR BIOCONTROL OF *Monilinia laxa* IN THE PHYLLOSPHERE 127

3.4. PRODUCTION OF *Epicoccum nigrum* BY SOLID SUBSTRATE FERMENTATION 127

3.5. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans* 129

3.5.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions 129

3.5.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w 132
3.5.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans* 140
3.5.4. Turgor pressure in *Epicoccum nigrum* mycelium 157
3.5.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa* 157
3.5.6. Viability of *Epicoccum nigrum* spores after storage 158

3.6. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans* 160

3.6.1. Efficacy of *Epicoccum nigrum* as a post-harvest BCA for brown rot of fruit 160
3.6.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans* 163

CHAPTER 4. DISCUSSION 169

I: STUDIES ON *Penicillium oxalicum* FOR BIOCONTROL OF *Fusarium* WILT OF TOMATO IN THE RHIZOSPHERE 170

4.1. PRODUCTION OF *Penicillium oxalicum* INOCULUM: INDUCTION OF SUBMERGED CONIDIATION 170

4.2. CHARACTERISATION OF *Penicillium oxalicum* SPORES 174

4.2.1. Study on growth of *Penicillium oxalicum* inoculum in sterile soil 174
4.2.2. Surface properties and adhesion of aerial and submerged spores 177
4.2.3. Viability of *Penicillium oxalicum* spores after storage 182

4.3. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum* 184

4.3.1. Concentration of the *Penicillium oxalicum* treatment and populations of *Penicillium oxalicum* and *Fusarium oxysporum* f.sp. *lycopersici* in the rhizosphere 185
4.3.2. Efficacy of different inoculum forms of *Penicillium oxalicum* 188
4.3.3. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion 191
4.3.4. Possible relationships to induced resistance 194

II: STUDIES ON *Epicoccum nigrum* AND *Penicillium frequentans* FOR BIOCONTROL OF *Monilinia laxa* IN THE PHYLLOSPHERE 196

4.4. PRODUCTION OF *Epicoccum nigrum* BY SOLID SUBSTRATE FERMENTATION 196

4.5. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans* 198

4.5.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions 198
4.5.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w 200
4.5.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans* 204
4.5.4. Turgor pressure in *Epicoccum nigrum* mycelium 211
4.5.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa* 213
4.5.6. Viability of *Epicoccum nigrum* spores after storage 214

4.6. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans* 216

4.6.1. Efficacy of *Epicoccum nigrum* as post-harvest BCA for brown rot of fruit 216

4.6.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans* 219

CHAPTER 5. CONCLUSIONS AND FUTURE WORK 224

REFERENCES.................... 229

APPENDIX. PUBLISHED PAPERS.................... 266

LIST OF FIGURES AND PLATES

Figure 1.1. Interactions between factors involved in a biological control system

Figure 1.2. Flow chart showing the different Sections and Phases of work carried out in this thesis

Figure 3.1. A: Time course development of sporulation of *P.oxalicum* in submerged culture. B: Effect of age of mycelium at transference on sporulation of *P.oxalicum* in submerged culture. Morton's method was used. Medium pH was 5. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration

Figure 3.2. Effect of pH (A) and calcium or PEG addition (B) on sporulation of *P.oxalicum* in submerged culture. Morton's method was used. Medium pH was 5 in B. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration

Figure 3.3. Effect of reduction in KNO_3 concentration in Morton's medium amended with Cl_2Ca (2.94 g l^{-1}) on sporulation of *P.oxalicum* in submerged culture. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration

Figure 3.4. Soil adsorption curves at 25°C

Figure 3.5. Effect of soil water potential (-MPa) and nutrient addition on evolution of *P.oxalicum* population (sqrt (10^6 cfu g^{-1} dry soil)) for 60 days. Data are means of three replicates

Figure 3.6. Viability of fresh and freeze-dried *P.oxalicum* spores stored at 4 or 25°C. Data are means of three replicates and indicate percentage germination on Potato Dextrose Agar (PDA). Incubation times were 16 and 20 h for fresh and freeze-dried spores, respectively.

Figure 3.7. Evolution of disease index in tomato plants cv. Lorena inoculated with *F.oxysporum* f.sp. *lycopersici* and treated with aerial spores of *P.oxalicum* at different concentrations. Data are means of five replicates in all cases

Figure 3.8. Populations of *P.oxalicum* and *F.oxysporum* f.sp. *lycopersici* in the rhizosphere of tomato plants reated with *P.oxalicum* aerial spores at different concentrations. Data are means of three replicates

Figure 3.9. Effect of *P.oxalicum* treatments on disease severity and consumption of nutrient solution in tomato plants cv. Lorena inoculated or uninoculated with *F.oxysporum* f.sp. *lycopersici.* Data are means of five replicates. A and B are repetitions. Key to treatments: 1: aerial spores in water, 2: aerial spores in Morton's medium, 3: submerged spores, 4: mycelium, 6: untreated control

Figure 3.10. Effect of *P.oxalicum* treatments on disease severity and consumption of nutrient solution in tomato plants cv. Lorena (A) and cv. Precodor (B and C) inoculated or uninoculated with *F.oxysporum* f.sp. *lycopersici.* Data are means of five replicates. Key to treatments: 1: aerial spores in water, 2: aerial spores in Morton's medium, 3: submerged spores, 5: liquid from submerged cultivation, 6: untreated control

Figure 3.11. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced on solid substrate at a_w = 0.98 (Treatment 1) or a_w = 0.95 (Treatment 2). Treatment 8: untreated control Treatment 9: uninoculated control

Figure 3.12. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced in liquid medium at a_w = 0.998 (Treatment 3), a_w = 0.98 (Treatment 4) or a_w = 0.95 (Treatment 5). Treatment 8: untreated control. Treatment 9 uninoculated control

Figure 3.13. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced on PDA plates and applied as a spore suspension at 10^6 spores ml^{-1} (Treatment 6) or 10^8 spores ml^{-1} (Treatment 7). Treatments 8: untreated control. Treatement 9: uninoculated control.

Figure 3.14. Percentage germination of tomato seeds cv. Precodor formulated with *P.oxalicum*, on Hoagland agar. Data are means of five replicates. Bars indicate Least Significant Differences (LSD) for each date. Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated

Figure 3.15. Percentage of tomato seeds cv. Precodor (A) and cv. Lorena (B) bearing cotyledons from seeds formulated with *P.oxalicum* and sown on Hoagland agar. Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated

Figure 3.16. Percentage of tomato seeds cv. Precodor bearing leaves from seeds formulated with *P.oxalicum* and sown on Hoagland agar (A) or water agar (B). Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated

Figure 3.17. Percentage of tomato seeds cv. Lorena bearing leaves from seeds formulated with *P.oxalicum* and sown on Hoagland agar (A) or water agar (B). Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated

Figure 3.18. Weight of tomato seedlings cv. Precodor (A) and cv. Lorena (B) 15 days after sowing on Hoagland agar or water agar. Seeds were formulated with *P.oxalicum*. Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated

Figure 3.19. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **PDA** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates

Figure 3.20. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **1/10PDA** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates

Figure 3.21. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **MM** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates

Figure 3.22. Calibration curves for the solutes analysed

Figure 3.23. Accumulation of trehalose and glucose in mycelium of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

Figure 3.24. Accumulation of glycerol, arabitol and mannitol in mycelium of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

Figure 3.25. Accumulation of in trehalose and glucose in spores of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

Figure 3.26. Accumulation of glycerol, arabitol and mannitol in spores of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

Figure 3.27. Accumulation of trehalose and glucose in biomass of *P.frequentans*996 and *P.frequentans*95. Data are means of three replicates

Figure 3.28. Accumulation of glycerol, erythritol and mannitol in biomass of *P.frequentans*996 and *P.frequentans*95. Data are means of three replicates

Figure 3.29 . Viability of fresh and freeze-dried *E.nigrum* spores stored at 4 or 25°C. Data are means of three replicates and indicate percentage germination on Potato Dextrose Agar (PDA). Incubation times were 8 and 20 h for fresh and freeze-dried spores, respectively.

Figure 3.30. Control of brown rot of cherries by *E.nigrum*996 and *E.nigrum*98, at different relative humidities (%RH), assessed 6, 7 and 10 days after inoculation with *M.laxa*. Data are means of five replicates. Bars indicate LSD for each evaluation date

Figure 3.31. Evolution of lesions induced by *M.laxa* on peach twigs treated with different preparations of the antagonists *E.nigrum* and *P.frequentans*.

Treatment 1: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.996

Treatment 2: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.98

Treatment 3: *E.nigrum*; conidia + mycelium; a_w = 0.996

Treatment 4: *E.nigrum*; conidia + mycelium; a_w = 0.98

Treatment 5: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.996

Treatment 6: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.95

Treatment 7: captan

Treatment 8: untreated control

Figure 3.32. Evolution of disease index of peach twigs inoculated with *M.laxa* and treated with different preparations of the antagonists *E.nigrum* and *P.frequentans*.
Treatment 1: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.996
Treatment 2: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.98
Treatment 3: *E.nigrum*; conidia + mycelium; a_w = 0.996
Treatment 4: *E.nigrum*; conidia + mycelium; a_w = 0.98
Treatment 5: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.996
Treatment 6: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.95
Treatment 7: captan
Treatment 8: untreated control

Figure 4.1. Mean water activity of polyol solutions over a range of concentrations, at 25°C. Data taken from Chirife *et al* (1984) and Hallsworth (1995)

Plate 3.1. Aspect of phialides and conidia of *P.oxalicum* observed by scanning electron microscopy. 1: from aerial culture, 2: from submerged culture

Plate 3.2. Germination of spores of *E.nigrum* on water agar at 0.935 a_w after 23 h incubation A: *E.nigrum*996. B: *E.nigrum*98

Plate 3.3. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on PDA. a) at 0.990 a_w and 10°C, b) at 0.990 a_w and 25°C

Plate 3.4. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on 1/10PDA a) at 0.996 a_w and 17°C, b) at 0.990 a_w and 25°C

Plate 3.5. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on MM a) at 0.984 a_w and 10°C, b) at 0.990 a_w and 17°C, c) at 0.990 a_w and 25°C

Plate 3.6. Disease control of rot of cherries (*M.laxa*) by *E.nigrum* at 75% (a) and 33% (b) relative humidity, ten days after inoculation. 1: fruits treated with *E.nigrum*98, 2: fruits treated with *E.nigrum*996, 3: untreated fruits and inoculated with the pathogen.

Plate 3.7. Symptoms of disease induced by *Monilinia laxa* on peach twigs. Rating as described in section 2.9.2.

LIST OF TABLES

Table 1.1. Commercially available products for biocontrol of plant pathogens

Table 1.2. Water activity, equilibrium relative humidity and water potentials at 25°C

Table 3.1. Sporulation of *P.oxalicum* in submerged culture in different culture media

Table 3.2. Effect of soil water potential (Ψ_w) and nutrient addition on percentage germination of *Penicillium oxalicum* 5 days after inoculation

Table 3.3. Percentage adhesion of aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces

Table 3.4. Percentage adhesion of aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces

Table 3.5. Percentage adhesion of germ tubes from aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces

Table 3.6. Percentage adhesion of aerial and submerged *P.oxalicum* spores to tomato roots

Table 3.7. Hydrophobicity indices for aerial and submerged *P.oxalicum* spores, calculated by the phase distribution method

Table 3.8. Effect of aerial spores of *P.oxalicum* on consumption of nutrient solution and number of leaves on tomato plants cv. Lorena inoculated with *Fusarium oxysporum* f.sp. *lycopersici*. Spore suspensions were applied at different concentrations

Table 3.9. Population of *Fusarium oxysporum* f.sp. *lycopersici* (cfu g^{-1} fresh root)) in the rhizosphere of tomato plants cv. Lorena and cv. Precodor, treated with different spore preparations of *Penicillium oxalicum*

Table 3.10. Populations of *Penicillium oxalicum* (cfu g^{-1} fresh root) in the rhizosphere of tomato plants cv. Lorena and cv. Precodor inoculated (I) or uninoculated (U) with *Fusarium oxysporum* f.sp. *lycopersici*, and treated with different spore preparations of the antagonist

Table 3.11. Disease indices in plants treated with different spore preparations of *P.oxalicum*, 36, 50 and 64 days after inoculation with *F.oxysporum* f.sp. *lycopersici*

Table 3.12. Number of leaves of tomato plants treated with different spore preparations of *P.oxalicum* 64 days after inoculation with *Fusarium oxysporum* f.sp. *lycopersici*

Table 3.13. Populations of *Penicillium oxalicum* (cfu g^{-1} fresh root) in the rhizosphere of tomato plants treated with different spore preparations of the antagonist, 1 and 64 days after inoculation with *F. oxysporum* f.sp. *lycopersici*

Table 3.14. Populations of *P.oxalicum* (cfu x 10^4 $seed^{-1}$) on tomato seeds cv. Lorena and Precodor formulated with *P.oxalicum* before (B) and after (A) drying

Table 3.15. Spore production by *E.nigrum* on wheat grain cv. Rendeveau at different levels of water activity (a_w), expressed as millions ($x10^6$) spores g^{-1} grain

Table 3.16. Spore production by *E.nigrum* on wheat grain cv. Brigadier at different levels of water activity (a_w), expressed as millions ($x10^6$) spores g^{-1} grain

Table 3.17. Percentage germination of *E.nigrum* spores on water agar at 0.996 a_w (A) and 0.935 a_w (B)

Table 3.18. Germ tube extension (μm) from *E.nigrum* spores on water agar at 0.996 a_w (A) and 0.935 a_w (B)

Table 3.19. Contents of sugars and polyols in 10-d-old mycelium and spores of *Epicoccum nigrum* subjected to homogenization treatment

Table 3.20. Contents of sugars and polyols in mycelium of *Epicoccum nigrum* without homogenization treatment

Table 3.21. Contents of sugars and polyols in spores of *Epicoccum nigrum* without homogenization treatment

Table 3.22. Accumulation of solutes in spores of *E.nigrum* produced on wheat grain cv. Rendeveau at two levels of water activity (a_w). Data indicate mg g^{-1} freeze dried spores

Table 3.23. Accumulation of solutes in spores of *E.nigrum* produced on wheat grain cv. Brigadier at two levels of water activity (a_w). Data indicate mg g^{-1} freeze dried spores

Table 3.24. Concentration (ppm) of solutes in the washings used to extract *E.nigrum* spores from wheat grain cv. Rendeveau at two levels of water activity (a_w)

Table 3.25. Concentration (ppm) of solutes in the washings used to extract *E.nigrum* spores from wheat grain cv. Brigadier at two levels of water activity (a_w)

Table 3.26. Contents of sugars and polyols in mycelium and spores of *Penicillium frequentans* subjected to homogenization treatment

Table 3.27. Contents of sugars and polyols in mycelium and spores of *Penicillium frequentans* without homogenization treatment

Table 3.28. Water potential (Ψ), osmotic potential (Ψ_{Π}) and turgor pressure (Ψ_P) of *E.nigrum* mycelium

Table 3.29. NOIs for *E.nigrum* paired with *M.laxa*, derived from carbon source utilization data, at two a_w levels

Table 3.30. Contrast analysis of different treatments applied to control *Monilinia laxa*. Comparison of lesion lengths

Table 3.31. Contrast analysis of different treatments applied to control *Monilinia laxa*. Comparison of disease index rating the appearance of the twig

LIST OF ABBREVIATIONS

a_w: Water activity

BCA: Biological Control Agent

NOI: Niche Overlap Index

Ψ_w: Water potential

SSF: Solid Substrate Fermentation

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Biological control is the practice in which, or process whereby, the undesirable effects of an organism are reduced through the agency of another organism that is not the host plant, pathogen or man (Deacon, 1983). The first attempt to use microorganisms to control plant diseases was by Hartley (1921), who used antagonistic fungi to control damping-off of pine seedlings. Several publications have extensively reviewed and analysed the early studies on biological control (Baker and Cook, 1974; Cook and Baker 1983; Chet 1987).

Although much research on biological control has been carried out in the last two decades, very few products based on antagonistic microorganisms to control plant diseases have been commercialised. They are listed in Table 1.1.

This lack of practical and consistent success has been of major concern, and many plant pathologists have started to debate which basic principles should be followed in order to find and develop efficient biocontrol agents (BCAs). Numerous publications have in recent years concentrated on overcoming the hurdles of environmental fluctuations in the field (Waage and Greathead, 1988; Boland, 1990; Campbell, 1990; Deacon, 1991; Lewis and Papavizas, 1991; Andrews, 1992; Cook, 1993; Deacon and Berry, 1993; Fokkema, 1993).

Table 1.1. Commercially available products for biocontrol of plant pathogens[a].

Product name	Biocontrol agent	Pathogen/Disease	Crop
AQ10	*Ampelomyces quisqualis* isolate M-10	Powdery mildews	apples, grapes, vegetables, ornamentals, strawberries
Bio-Fungus (formerly Anti-Fungus)	*Trichoderma* spp.	*Sclerotinia, Pythium* spp., *Phytophthora, Rhizoctonia solani, Fusarium, Verticillium*	flowers, strawberries, trees, vegetables
Aspire	*Candida oleophila* I-182	*Penicillium* spp., *Botrytis* spp.	citrus, pome fruit
Binab T	*Trichoderma harzianum/polysporum*	wilt, take-all, root rot and decay of wood	flowers, fruit, ornamentals, turf, and vegetables
Biofox C	*F. oxysporum* (nonpathogenic)	*F. oxysporum, F. moniliforme*	basil, carnation, cyclamen, tomato
Bio-save 10	*Pseudomonas syringae* ESC-10	*B. cinerea, Penicillium* spp., *Mucor pyroformis, Geotrichum candidum*	citrus, pome fruit
Bio-save 11	*P. syringae* ESC-11	*B. cinerea, Penicillium* spp., *M. pyroformis, G. candidum*	citrus, pome fruit
BlightBan A506	*P. fluorescens* A506	*Erwinia amylovora*	almond, apple, cherry, peach, pear, potato, strawberry, tomato
Blue Circle	*Burkholderia cepacia, (Pseudomonas) cepacia*	*Fusarium, Pythium,* spiral, lance, sting nematodes	vegetables
Conquer	*P. fluorescens*	*Pseudomonas tolassii*	mushrooms
Contans	*Coniothyrium minitans*	*Sclerotinia sclerotiorum, Sclerotinia minor*	canola, sunflower, peanut, soybean, vegetables (lettuce, bean, tomato)
Deny (formerly Precept)	*Burkholderia cepacia, (Pseudomonas) cepacia* type Wisconsin	*Rhizoctonia, Fusarium, Pythium,* spiral, lance, sting nematodes	alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops, wheat
Epic	*Bacillus subtilis*	*R. solani, Fusarium* spp., *Alternaria* spp., *Aspergillus* spp.	cotton, legumes
Fusaclean	*F. oxysporum* (nonpathogenic)	*F. oxysporum*	basil, carnation, cyclamen, gerbera, tomato
Galltrol-A	*Agrobacterium radiobacter* strain 84	*Agrobacterium tumefaciens*	fruit, nut, ornamental nursery stock
Intercept	*P. cepacia*	*R. solani, Fusarium* spp., *Pythium* sp.	maize, vegetables, cotton
Kodiak, Kodiak HB, Kodiak AT	*B. subtilis*	*R. solani, Fusarium* spp., *Alternaria* spp., *Aspergillus* spp.	cotton, legumes
Mycostop	*Streptomyces griseoviridis* strain K61	*Fusarium* spp., *Alternaria brassicola, Phomopsis* spp., *Botrytis* spp., *Pythium* spp., *Phytophthora* spp.	field, ornamental and vegetable crops
Phagus	Bacteriophage	*P. tolaasii*	*Agaricus* spp., *Pleurotus* spp.

Table 1.1 (cont.)

Product name	Biocontrol agent	Pathogen/Disease	Crop
Polygandron	*Pythium oligandrum*	*Pythium ultimum*	sugarbeet
PSSOL	*Pseudomonas solanacearum* (nonpathogenic)	*P. solanacearum*	vegetables
RootShield (Bio-Trek T-22G)	*T. harzianum* Rifai strain KRL-AG2 (T-22)	*Pythium* spp., *R. solani, Fusarium* spp.	trees, shrubs, transplants, all ornamentals, cabbage, tomato, cucumber
Rotstop, P.g. Suspension	*Phlebia gigantea*	*Heterobasidion annosum*	trees
SoilGard (GlioGard)	*Gliocladium virens* GL-21	*R. solani, Pythium* spp.	ornamental and food crop plants
Supresivit	*T. harzianum*	various fungi	—
System 3	*B. subtilis* GB03 and chemical pesticides	seedling pathogens	barley, beans, cotton, peanut, pea, rice, soybean
T-22G, T-22 Planter Box, (Bio-Trek)	*T. harzianum* Rifai strain KRL-AG2	*Pythium* spp., *R. solani, Fusarium* spp., *Sclerotinia homeocarpa*	bean, cabbage, corn, cotton, cucumber, peanut, potato, sorghum, soybean, sugarbeet, tomato, turf, greenhouse ornamentals
Trichodex	*T. harzianum*	*Collectotrichum* spp., *Fulvia fulva, Monilia laxa, Plasmopara viticola, Pseudoperonospora cubensis, Rhizopus stolonifer, S. sclerotiorum, B. cinerea,*	cucumber, grape, nectarine, soybean, strawberry, sunflower, tomato
Trichopel, Trichoject, Trichodowels, Trichoseal	*T. harzianum* and *T. viride*	*Armillaria, Botryosphaeria, Chondrosterium, Fusarium, Nectria, Phytophthora, Pythium, Rhizoctonia*	—
Trichoderma 2000 (formerly "TY")	*Trichoderma* sp.	*R. solani, Sclerotium rolfsii, Pythium* spp., *Fusarium* spp.	nursery and field crops
Victus	*P. fluorescens* strain NCIB 12089	*P. tolaasii*	mushrooms

[a]This list is produced by the U.S. Department of Agriculture and can be found at: http://www.barc.usda.gov/psi/bpdl/bioprod.htm

There has, for example, been much criticism of *in vitro* methods for the selection of antagonistic microorganisms. *In vitro* screening has often involved growth inhibition and the production of antibiotic substances which are often not produced in natural conditions, and thus antagonism *in vitro* may not always be reflected in disease suppression *in vivo.* For example, different *Pseudomonas* spp. were shown to inhibit radial growth of *M.fructicola* on culture media, but they had little or no effect on the pathogen *in vivo* (Pusey and Wilson, 1984). Lindow (1987) described the lack of correlation between *in vitro* antibiosis with antagonism of ice nucleation active bacteria. Kraus and Loper (1991) reported a lack of evidence for the role of pyoluteorin produced by *Pseudomonas fluorescens* in the biocontrol of *Pythium ultimum* damping-off and Boland and Inglis (1988) found no correlation between *in vitro* inhibition of *Sclerotinia sclerotiorum* by *E.purpurascens* and disease suppression. On the other hand, evidence for the role of antibiotic production in biocontrol has been shown in the case of phenazine-1-carboxylic acid from *P.fluorescens* (Thomashow *et al.*, 1990) and pyoluteorin and 2,4-diacetyl-phloroglucinol from strain CHAO of *P.fluorescens* (Maurhofer *et al.*, 1992). A relationship between flavipin production by *E.nigrum* and antagonism against *M.laxa* on peach twigs has been also suggested by Madrigal *et al.* (1991).

More recently, emphasis has been placed on using more realistic natural systems, which take account of all the factors that affect the environment in which the biocontrol agent is expected to work. Thus many studies have in recent years attempted to use more environmental screens to obtain useful candidate biological control agents. Indeed Deacon (1991) argued that ecological factors were crucial in developing successful biocontrol agents. Knowledge of the ecology of

microorganisms (both biocontrol agents and pathogens) in the phyllosphere and rhizosphere is now considered an essential prerequisite for the practical and successful use in agriculture of beneficial microbial colonisers of plant surfaces (Fokkema and Schippers, 1986). It has now been recognised that the limited knowledge of the ecology of introduced BCAs has constrained the practical success of biocontrol fungi (Fokkema, 1993).

It is surprising, therefore, that the ecological competence of microbial antagonists has received very little study, although this could be critical for developing effective biocontrol agents. For a microorganism to be effective it has to be able to grow and develop in the environment where it is expected to control the disease. For example, it is generally assumed that root colonisation by introduced bacteria is essential for biocontrol of root pathogens (Weller, 1988) and that the inability of introduced bacteria to became established effectively in the rhizosphere is the major cause of inconsistent results in the field (O'Sullivan *et al.*, 1991). In the fluctuating environment of the phyllosphere, the establishment and colonisation by an antagonist is crucial for effective biological control (Andrews, 1992) and suppression of sporulation (Köhl *et al.*, 1995 a, b). Effective colonisation can be, *per se*, the mode of action of the antagonist, as in the case of competitive exclusion of ice nucleation-active strains of *Pseudomonas syringae* by prior inoculation of leaf surfaces with near-isogenic ice nucleation-deficient strains (Lindow, 1987). In cases where the mode of action involves mechanisms of antibiosis or lysis there is still a need for good ecological competence.

Another problem in the development of effective BCAs is the mass-production of ecologically competent inoculants. In many cases microorganisms grown in nutrient rich media in fermenters result in high spore concentration, but poor viability and quality. In other cases a low yield in fermentation systems, a low survival in formulation, or a high uneconomic dose is required, making the production cost too high to justify the industrial production of the antagonist (Powell, 1992). Industrial production of ecologically competent strains of biocontrol agents has been overlooked for a long time, but is now considered to be a fundamental step in improving the efficacy of biocontrol agents (Cook, 1993). Inoculum quality has to a large extent been neglected, with work having been mainly focused on obtaining high yields.

After fermentation, an adequate formulation can enable the antagonist to become established in the environment. Formulations may include a food base, wetting agents, inert diluting agents, water binding or desiccating agents, as well as the actual microorganism (Campbell, 1989).

It is important to realise that BCAs cannot be expected to behave as chemical methods of control. As pointed out by Cook (1993) microorganisms intended for use as introduced BCAs must be considered in a biological and not the current chemical paradigm where, if a strain works, it is called a 'biopesticide' and possibly expected to replace a chemical pesticide. BCAs could in many cases be part of an integrated crop protection strategy in combination with chemicals. A change in the attitude of both researchers and industry is occurring according to Whipps (1997), and this could produce an increase in the available biological control products in the market.

In the following pages a more detailed analysis of the problems associated with a) the fermentation and formulation of biological control agents and b) ecophysiological approaches to improve the quality of biocontrol agents will be considered. This will be followed by a review of the host-pathogen-biocontrol systems studied in this thesis, in the phyllosphere and rhizosphere: *Epicoccum nigrum* and *Penicillium frequentans* for the control of *Monilinia laxa* on peach twigs and cherry fruits, and *Penicillium oxalicum* for the control of *Fusarium oxysporum* f. sp. *lycopersici* on tomato, respectively.

1.2. FERMENTATION AND FORMULATION OF BIOCONTROL AGENTS (BCAs)

Both solid and liquid fermentation systems have been used for the mass production of BCAs (Lewis and Papavizas, 1991). The nutritional and cultural conditions have been studied in detail for some agents but most studies have emphasised optimisation of fungal propagule numbers only, seldom considering inoculum quality. Comparisons of solid-state with submerged fermentation, and applications as dry versus wet inocula with or without a carrier or adjuvant have been studied (Cook, 1993). Some workers have started to consider the amenability of systems for industrial production as a criterion for the selection of antagonists (Schisler *et al.*, 1992). Aspects of solid and liquid fermentation relevant to this study will be discussed in the following sections.

1.2.1. Solid fermentation versus liquid fermentation

Solid-state fermentation (SSF) is probably the oldest method used to make microorganisms work for man (Hesseltine, 1977). It involves the growth of microorganisms on moist solid substrate in the absence of free-flowing water. The

solid substrate acts as a heterogeneous source of carbon, nitrogen, and minerals as well as growth factors, and they have a capacity to absorb water (Murthy *et al.*, 1993). SSF offers the greatest possibilities when fungi are used, because unlike other microorganisms, their growth habit in nature is to colonise solid substrates such as pieces of wood, seed, stems, roots and leaves of plants; and drier parts of animals (Hesseltine, 1977). SSF has been used for the production of some BCAs, such as *Talaromyces flavus* (Fravel *et al.*, 1985) or entomopathogenic fungi. For example, Jenkins (1996) developed a method for the production of *Metarhizium flavoviride* on rice, obtaining yields of about $1x10^9$ conidia g^{-1} rice.

SSF, provided the necessary moisture exists in an absorbed or complexed form within the solid matrix, is more advantageous compared with liquid fermentation, because of the possibility for an efficient oxygen transfer process. The higher oxygen transfer associated with the larger interfacial surface-to-liquid volume ratios, resulting from the distribution of liquid film on the surface of the solid substrate, is responsible for a more advantageous enzyme productivity, product recovery, and fermenter volume. The disadvantages of SSF come from technical limitations. For example, problems in the engineering design of SSF exist due to the difficulty in experimentally measuring the key process variables. The amount of solids involved is very high and the medium is heterogeneous, making accurate maintenance and measurement of parameters such as cell biomass level, nutrient concentration, pH, and temperature extremely difficult. More research effort needs to be focused on a) accurate control of water availability b) development of mathematical models accounting for the interactions of transport of heat and mass with bioreaction kinetics in different types of solid state fermentation systems; c) theoretical predictions and experimental

determination of transport parameters for solid state fermentation; d) development of reliable estimation methods, in particular for biomass in the presence of solids, and e) better design of bioreactors enabling accurate measurement and control of variables like temperature, gaseous concentration, and water activity (Murthy *et al.*, 1993).

Some of the substrates used in SSF for the production of biocontrol agents include cereal straws, wheat bran, bagasse, grain seeds and meals, perlite and peanut hull meal. Escande and Echandi (1991) used whole or chopped oat grains and vermiculite + potato broth for the production of binucleate *Rhizoctonia*, with the highest viability of the fungus obtained on chopped oat grains. Some substrates produce biomass that is not effective: Lewis and Papavizas (1993) obtained control of damping-off caused by *Rhizoctonia solani* with *Stilbella aciculosa* grown on various substrates, except from perlite + corn cobs (fragments). Little attention has however been paid to accurately controlling water availability in such SSF systems. Jenkins (1996) studied how increasing water content of rice grains produced an increase in conidial yield of *Metarhizium flavoviride*. However, water content does not necessarily indicate water availability for fungal growth, and studies are needed on the effect of water availability on inoculum yield and quality.

In liquid fermentation the microorganism grows in submerged culture in a medium which supplies nutrients for growth. Liquid fermentation offers the advantages of a longstanding technology and a sophisticated physical plant developed originally for production of microbial products (drugs, organic acids, enzymes) rather than the microorganisms. It is used for the production of bacteria, yeasts and for certain fungi such as *Trichoderma* and *Gliocladium* (Lewis and Papavizas, 1991). The strain Fo47

of *Fusarium oxysporum* is produced by liquid fermentation for the control of fusarium wilts (Alabouvette *et al.*, 1993). It is generally agreed that liquid fermentation is preferred to solid fermentation because it is much easier to establish as an homogeneous system, mixing is accomplished by the design of the system, addition of nutrients can be done simply, heat can be easily removed and environmental conditions changed at will (Powell, 1992). Lappa (1979) compared solid culture with surface liquid culture and submerged fermentation of *Beauveria* sp. and *Metarhizium* sp., and found that submerged culture is by far the most economical in time, productivity and cost of consumables.

Media used in liquid fermentation varies from synthetic defined media to complex media based on industrial byproducts. Harman *et al.* (1991) used minimal media for the production of *Trichoderma harzianum* in liquid culture, but this medium had to be modified by the addition of V8 and an osmoticum to get high overall yields of viable conidia. In some cases the medium needs to have a specific nutrient composition for the production of quality inoculants and to get an adequate yield. However, from an industrial point of view it is important to produce microbial biomass using inexpensive means, whether solid or liquid fermentation systems are used. A suitable medium should consist of inexpensive, readily available agricultural by-products, such as molasses, brewer's yeast, corn steep liquor, sulphite waste liquor, and cottonseed and soya flours (Lewis and Papavizas, 1991).

1.2.2. Sporulation of fungi in submerged culture

One problem encountered when trying to produce a biological control fungus by liquid fermentation is the difficulty of inducing sporulation. Although in some cases the

effectiveness of conidia for inducing biocontrol has been shown to be lower than that of mycelium (Papavizas and Lewis, 1989; Sharma and Singh, 1990), spores are generally preferred to mycelium as inoculants, because they are better able to withstand adverse environmental conditions and have a longer shelf-life (Smith, 1978).

Many filamentous fungi will remain entirely vegetative in submerged culture. This fact is consistent with the observation that most differentiated structures are characteristic of the aerial mycelium in surface cultivation. The cause of the inhibition of sporulation in submerged culture is complex and most probably involves a combination of many factors including not only oxygen tension, but also changes in the physical nature of the hyphal wall associated with submergence, and the direct contact of the sporogenous cells with nutrients and inhibitory factors in the medium (Smith, 1978).

In this study induction of sporulation of the BCA *Penicillium oxalicum* in submerged culture was attempted for the first time. Sporulation of *Penicillium* spp. has been induced in submerged culture by using different methods. Morton *et al.* (1958) induced sporulation of *Penicillium griseofulvum* by adding glucose to the medium after 24 h culture or by transferring 24-h cultures to a medium without a nitrogen source. Sekiguchi *et al.* (1975) studied microcycle conidiation of *Penicillium urticae*. The fungus produced conidia because of nutritional stimuli as well as temperature cycles. Pitt and Poole (1981) reported that calcium was the key factor responsible for submerged conidiation of *Penicillium notatum*. The role of calcium has been extensively investigated for *Penicillium cyclopium* (Ugalde and Pitt, 1983a; Roncal *et al.*, 1993), but not for *Penicillium* spp. with potential as biocontrol agents.

Nutritional and environmental conditions during submerged fermentation not only determine the occurrence and extent of sporulation, but also the quality of the spores produced. For example, it has been shown for the mycoherbicide *Colletotrichum truncatum* that conidia produced at C:N ratio 10:1 germinated more rapidly and incited more disease in hemp sesbania seedlings than conidia produced at higher C:N ratios (Schisler *et al.*, 1990). The nutritional environment during submerged culture of *Trichoderma harzianum* significantly influenced the ability of spores to withstand drying and retain viability during storage (Harman *et al.*, 1991; Jin *et al.*, 1991). Studies by Lane *et al.* (1991 a, b) demonstrated that blastospores of entomogenous fungi produced in nitrogen-limitation conditions survived longer and were more virulent than carbon-limited blastospores. Jackson *et al.* (1997) found that concentrations of glucose and casaminoacids in the culture medium determined the production of desiccation tolerant blastospores of *Paecilomyces fumosoroseus*.

Spore production of *P.oxalicum* in Petri dishes has been evaluated elsewhere (Pascual *et al.*, 1997), but no studies of the potential for mass-production of any of the antagonists used in this project, either by solid or liquid fermentation have been attempted previously.

1.2.3. Characteristics of submerged spores: Hydrophobicity

When produced in aerial or submerged cultures, fungal spores probably have different characteristics, one of them being surface hydrophobicity. Surface hydrophobicity is a property conferred in many cases by small proteins called hydrophobins, which assemble as protein films and arise at the surface of emergent fungal structures, such as aerial hyphae, fruit bodies and air-borne

spores. Hydrophobins arrange forming rodlet layers, which, according to Wessels (1996), are universally present on hydrophobic surfaces of fungi. However, this pattern of hydrophobin rodlets was not observed on conidia of *Botrytis* spp. (Doss *et al.*, 1997), which adhered *via* hydrophobic interactions (Doss *et al.*, 1993). These rodlets have been observed on the surface of spores of *Penicillium* spp. by using the freeze-etching technique (Hess *et al.*, 1968). Submerged hyphae of *Schizophyllum commune* excrete all the hydrophobins they make into the culture medium while in aerial structures all are deposited in the walls as insoluble complexes (Wessels, 1992). It has also been reported that no rodlet layer is formed on submerged conidia of *Aspergillus nidulans* (Stringer and Timberlake, 1995), and a hydrophobin-like protein was found in higher amounts in aerial than in submerged conidia of *Trichoderma harzianum* (Muñoz *et al.*, 1995). In the ectomycorrhizal basidiomycete *Pisolithus tinctorius* the mRNAs for hydrophobins were barely detectable in mycelium grown in liquid culture and highly accumulated in aerial hyphae (Tagu *et al.*, 1996).

Surface hydrophobicity has been shown to be involved in attachment of fungal spores to hydrophobic artificial and natural substrates. This has been shown for plant pathogens, such as *Phyllosticta ampelicida* (Kuo and Hoch, 1996), *Botrytis cinerea* (Doss *et al*, 1993), *Uromyces appendiculatus* (Terhune and Hoch, 1993) and *Colletotrichum musae* (Sela-Buurlage *et al.*, 1991), and entomopathogenic fungi, such as *Beauveria bassiana* (Hegedus *et al.*, 1992) and *Nomuraea rileyi* or *Metarrhizium anisopliae* (Boucias *et al.*, 1988). According to Wessels (1996) hydrophobins have a biological role in processes of formation of aerial hyphae, fruit-body formation, formation of conidia, pathogenesis and symbiosis. Talbot *et*

al. (1996) demonstrated that a gene encoding a hydrophobin involved in attachment to plant surfaces was necessary for infection-related development of the rice-blast fungus *Magnaporthe grisea*, while Stringer and Timberlake (1993) showed that cerato-ulmin, a toxin involved in dutch elm disease, is a fungal hydrophobin.

Smith *et al.* (1998) have studied hydrophobicity of conidia of the mycoparasite *Coniothyrium minitans*, as a factor involved in non-specific attachment to surfaces. However, no other studies on hydrophobicity of fungal BCAs for plant pathogen control have been reported. This may be an important aspect if interactions between the biocontrol agent and the host plant are involved in the biocontrol, especially if hydrophobicity differs between aerial and submerged conidia.

1.2.4. Formulation of biological control agents: Seed coating.

By means of an adequate formulation, different characteristics of the BCA can be improved, such as survival during storage, growth in the environment and biocontrol ability. Biopolymers such as xanthan gum or alginate are remarkable for their viscosity, which limit heat transfer when inocula are dried. They give useful pseudoplastic characteristics to inocula, which immediately recover their viscosity when applied to soil or to plants. In the field, the microorganisms are protected until the polymer structure has been totally degraded (Mugnier and Jung, 1985). Conidia of *P.oxalicum* produced on PDA were encapsulated in an alginate-clay matrix (Fravel *et al.*, 1985). For other fungi, such as *Coniothyrium minitans*, *Gliocladium roseum*, *Trichoderma harzianum* and *T.viride* it was found that the actual growth in soil was

very limited from alginate pellets (Magan and Whipps, 1988). However, improvements can be made by additions to the formulation. Knudsen *et al.* (1991) formulated mycelial biomass of *T.harzianum* in alginate pellets with wheat bran, and after drying, pellets were immersed in aqueous solutions of PEG 8000. In this way a greater proliferation of hyphae of *T.harzianum* in soil was obtained. Daigle and Cotty (1992) produced conidia of *Alternaria cassiae* with alginate pellets, and showed that addition of nutrients in the formulation increased sporulation of the fungus. Fravel *et al.* (1995) found that two alginate prill formulations of *Talaromyces flavus* with organic carriers consistently enhanced biocontrol ability against *Verticillium dahliae.*

Seed treatment is an economical means to deliver biocontrol agents, compared to application to soil, because relatively small amounts of inoculum are needed. Different BCAs have been formulated with seed coating methods. *T.hamatum* was applied to seeds in water with an aqueous binder for the control of seed rot (Harman *et al.*, 1981). Taylor *et al.* (1991) developed a liquid coating formulation containing a suspension of an aqueous binder, a solid particulate material and *T.harzianum*, and found that efficacy of the *T.harzianum* applied with either liquid coating or powder coating was enhanced compared to that of a slurry application of *T.harzianum* in a binder. Biocontrol of *Fusarium* wilt of radish has also been achieved by seed treatment with *P.fluorescens* WCS374 in a methyl cellulose coating and radish seeds coated in this way are commercialised under the name of "BioCoat" (Leeman *et al.*, 1995). Alginate has also been used for delivery of *P.fluorescens* with sugar-beet seeds (Russo *et al.*, 1996). Industrial processes have also been used in the formulation of BCAs with seeds. McQuilken *et al.* (1990) used a commercial seed coating process for the application of *Pythium oligandrum* to sugar-beet, while more

recently, Cliquet and Scheffer (1996) showed the feasibility of biocontrol of seedling diseases by *Trichoderma* spp. applied onto seeds through an industrial film-coating process. *C.minitans* has been applied using a fluidized-bed film-coating process on to sunflower seeds for the control of *Sclerotinia sclerotiorum*. This treatment increased seed germination in agar plate tests, but failed to increase survival of seedlings in a glasshouse chamber test (McQuilken *et al.*, 1997).

P.oxalicum has been applied to seeds for the biocontrol of damping-off of pea, but the method of application was by shaking the seeds with the dry conidia, without any formulation (Windels, 1981). It has also been applied as a dry treatment to chickpea seeds for the control of seed rot and preemergence damping-off (Kaiser and Hannan, 1984; Trapero-Casas *et al.*, 1990). In the present study, different formulations have been assayed on tomato seeds to test the efficacy of *P.oxalicum* as a growth promoting agent.

1.3. THE ECOLOGICAL APPROACH IN BIOCONTROL

From an ecological point of view, the pathosystem usually represented by the disease triangle, is transformed into a disease square by the addition of the biological control agent (Fig 1.1), where six possible paths of interaction can occur, and which will determine the success of the biocontrol.

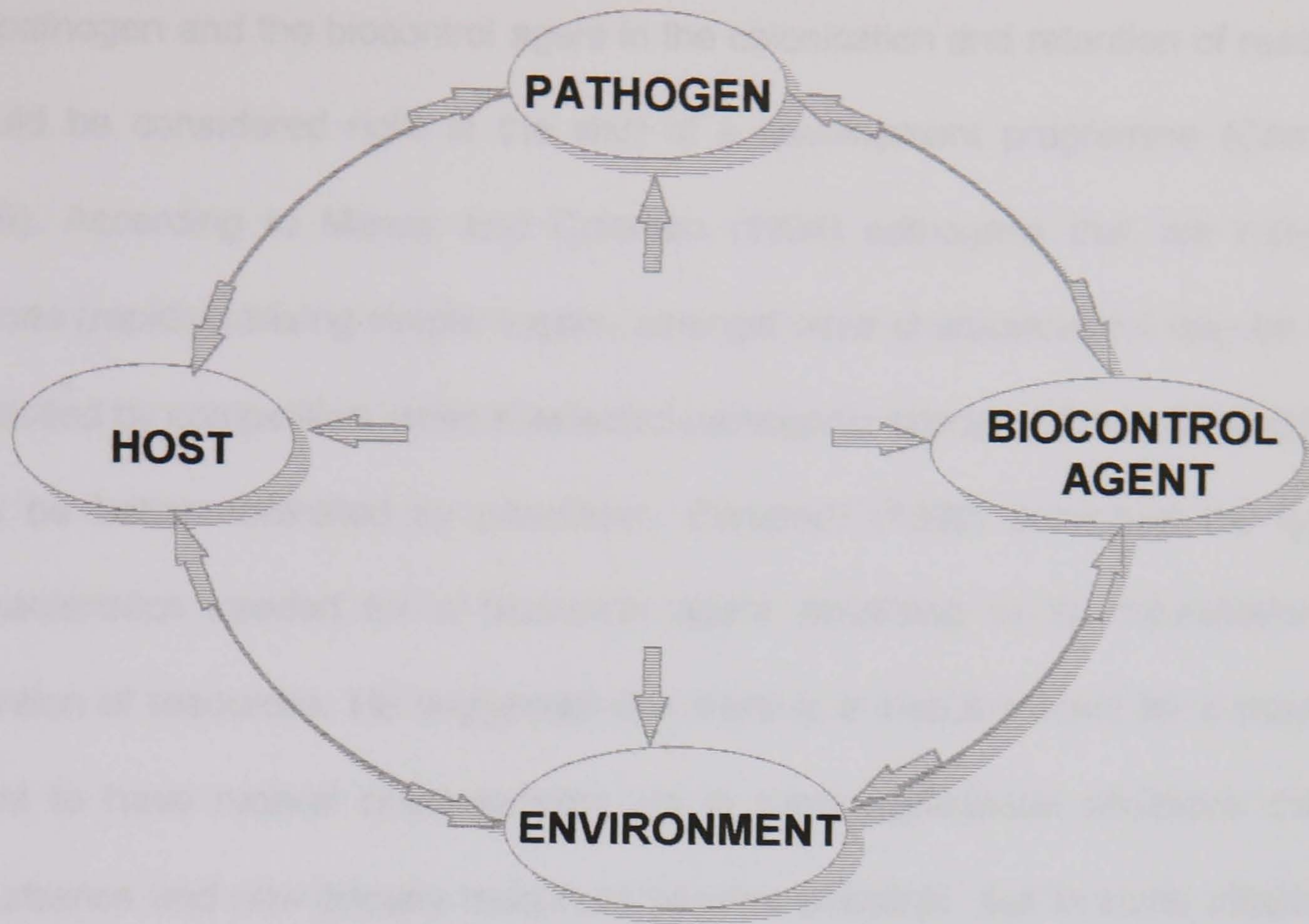


Figure 1.1. Interactions between factors involved in a biological control system.

Research in biocontrol has been focused largely in the interactions between the BCA and the pathogen, paying less attention to interactions between the BCA and the environment, which are of essential importance. The environment represents both biotic and abiotic factors, and the former include not only the microbial community but also other types of living organisms. In this respect, it is interesting that interactions between BCAs and fauna, an aspect in the disease square has been largely unexplored (Whipps, 1997).

When studying the interactions between the BCA and the pathogen, studies have largely focused on determining the mode of action, as explained earlier. However, not enough attention has been paid, in some cases, to establishing which antagonist could be most suitable from an ecological perspective. The possible strategies of both

the pathogen and the biocontrol agent in the colonisation and retention of resources should be considered right at the start of a development programme (Campbell, 1989). According to Marois and Coleman (1994) pathogens that are r-selected species (rapidly utilising simple sugars, amongst other characteristics) may be better controlled by competition, while K-selected pathogenic species (slowly utilising lignin) may be better controlled by parasitism. Campbell (1989) described the type of characteristics needed for a biocontrol agent according to its colonisation and retention of resources. He suggested that there is a frequent need for a biocontrol agent to have ruderal characteristics, as in many agricultural situations there is disturbance and new primary resources become available. But in some situations it may be better to have a more competitive species when the BCA is required to operate against a pathogen which has already invaded the host tissue. Also, a BCA may have to tolerate environmental stresses, especially for use on leaves or in dry climates where soil moisture deficits may be great.

Interactions between the biocontrol agent, the pathogen and the environment will determine the composition of the microbial community on the host plant. The outcome of these interactions will be determined by the competitive ability of every microorganism involved and the prevailing environmental conditions. A more detailed analysis of fungal competition will be discussed in the next section.

1.3.1. Fungal competition

Competitive interactions amongst fungal species are poorly understood. This is at least partly because fungi do not form easily definable populations, which results in methodological difficulties in investigating competition (Wardle, 1993). Therefore,

information about the role of competition in organising fungal communities is conspicuously absent from the ongoing general ecological debate (Shearer, 1995). Studies have been carried out *in vitro* and little agreement is usually found with the interactions which occur in more natural conditions. Magan and Lacey (1984a) established an Index of Dominance based on different interactions between fungi in culture media, varying from mutual intermingling to dominance by inhibition at a distance, but later found that the most competitive species on culture media did not always compete well on wheat grains (Magan and Lacey, 1985). However, in some cases good correlation has been found between cultural studies and field studies, as was found for wood decay fungi (Rayner and Boddy, 1988).

Other studies on fungal competition have used the de Wit (1960) replacement series design, which is a common tool in plant ecology. In this type of experimental design, the proportions of the competing species are varied while the overall density of the community is maintained at a constant level. The assumption is made that growth or some other parameter of species success will be proportional to the species proportion in mixtures. The data, when plotted against proportion, will result in a linear curve if the species have not affected each other. If negative interactions (competition) occur, the shape of the curve(s) will not be linear. Adee *et al.* (1990) showed, using this approach, that *Pyrenophora tritici-repentis* was a better competitor than *Septoria nodorum*, and they pointed out that the de Wit replacement series is a useful tool for quantitatively examining competitive interactions between plant pathogens. Wilson and Lindow (1994) assessed levels of coexistence between *P.syringae* and nonpathogenic epiphytic species by using the replacement series. They found that the level of coexistence was inversely correlated with the ecological

similarity of the strains, which was estimated with niche overlap indices (NOIs) derived from *in vitro* carbon source utilisation profiles (NOI is the proportion of the carbon compounds utilised by one species that are also utilised by the competing species). They hypothesised (Wilson *et al.*, 1995) that the effectiveness of a biocontrol strain as a pre-emptive biocontrol agent of an epiphytic phytopathogenic bacterium is proportional to the ecological similarity between the biocontrol agent and the target pathogen in the phyllosphere of the host plant. NOIs have been used in studies on interactions between *Fusarium* spp. producing maize ear rot and other fungal colonist of maize grains. It was found that NOIs varied depending on the a_w and temperature (Marín *et al.*, 1998). Therefore NOIs can be a useful tool in determining the levels of coexistence of microorganisms in different environmental conditions.

1.3.2. Ecological constraints in soil and the rhizosphere

The rhizosphere is composed of the various cell layers of the root itself (endorhizosphere), the area surrounding the root (ectorhizosphere) and the root surface (rhizoplane) (Lynch, 1990). The rhizosphere is a buffered milieu compared to aerial plant surfaces, and can be manipulated to improve the ecological competence of antagonists, and therefore to obtain improved biocontrol. Characteristics of the bulk soil are different to those of the rhizosphere. The environmental parameters are much more variable and nutrients are much more scarce. The bulk soil is thus a complex and competitive environment for microorganisms. Soil factors and biological components can vary markedly even within a single field. Moisture may vary from waterlogged conditions, where oxygen is deficient, to extreme dryness, temperature may range from 0°C to greater than 30°C, and the pH may vary from pH 5 to pH 8.

Levels of organic matter, available nutrients and ionic balance may also differ. Soil fungi, while lower in numbers than bacteria, may contribute even more to the total soil biomass. These, together with the soil fauna, make up an extremely diverse, densely populated and highly competitive community in this microenvironment.

The high level of competition in soil results in fungi being exposed to fungistasis, an exogenous dormancy imposed upon propagules of microorganisms (Harman and Lumsden, 1990). Not only nutrient competition, but also inhibitory substances may be responsible for fungistasis (Lockwood and Filonow, 1981). Failure of microorganisms such as *Trichoderma*, *Gliocladium*, or *Pseudomonas* to control soil-borne pathogens has been attributed to a lack of competitive ability in soil or a narrow environmental range (e.g. soil type, pH) for growth (Powell and Jutsum, 1993).

Harman and Lumsden (1990) have suggested that an improvement of the ecological competence of BCAs in soil could be achieved by modification of seed treatment systems. This could work by modifying the soil micro-environment by entrapping propagules of antagonists within pellets formed with an alginate matrix, together with a bulking agent. The bulking agent may provide a food base for rapid and non-competitive growth of the antagonist in soil. This approach has been used, amongst other organisms, with fermenter biomass of *Trichoderma* and *Gliocladium* (Fravel *et al*, 1985; Lewis and Papavizas, 1987).

Manipulation of the soil environment can also be a means of improving ecological competence of the BCA. Modifications of the soil pH, partial sterilisation, and addition of organic substrates, are all strategies aimed at providing the BCA with a more

conducive environment for growth. Unfortunately, to date none of these strategies has been used extensively to control soil-borne plant pathogens successfully.

An adequate strain selection considering ecological criteria is essential to get the biocontrol agent to work under the environmental conditions prone to the development of the disease. An example of this approach was that of Douglas and Deacon (1994), who proposed that variation in tolerating water stress by *Idriella bolleyi* strains could be used to select those with potential for biocontrol of different pathogens of cereals, such as *Fusarium culmorum*, a pathogen in dry conditions, or *Gaeumannomyces graminis*, which induces disease in wetter conditions.

In the present study, growth of *P.oxalicum* was studied under similar conditions to those found in seedbeds, to characterise the colonisation capability of the fungus under different conditions of water availability. As described by De Cal *et al.* (1995) *P.oxalicum* has to be applied in seedbeds to get effective treatment against *Fusarium oxysporum* f.sp.*lycopersici*.

1.3.3. Ecological constraints in the phyllosphere

The aerial surfaces of plants, particularly leaves, are often referred to as the phyllosphere. Andrews (1992) has suggested that biological control has been less successful in the phyllosphere than in the rhizosphere for a number of reasons. These include the surface features of leaves and roots, microclimate, nutrients and patterns of colonisation. The topography of roots is smooth compared to aerial organs, where hills, peaks, valleys, and craters are the common surface features. With regard to the microclimate, the root lives in a more stable environment than the

leaf and also exerts considerably more control over its environment than does the leaf. The phyllosphere is a dynamic environment with cyclic and non-cyclic environmental variables, including temperature, relative humidity, dew, rain, wind and radiation; moreover, substantial variation can occur with time and space even on a scale appropriate to microorganisms. Nutrients in the phyllosphere, unlike the rhizosphere, originate mostly from sources other than the plant, and it is generally agreed that nutrients are frequently limiting in this niche, although there may be temporary patches of excess carbon compounds. In general, the leaf is a much more dynamic environment than the root. Failure of several microorganisms such as *Sporobolomyces roseus* or *Pseudomonas fluorescens* to control foliar pathogens has been attributed to a low tolerance to UV light and desiccation, making the maintenance of an active population on new tissue difficult (Powell and Jutsum, 1993). Moisture has been shown to have an effect on the suppression of *P.syringae* by *Aureobasidium pullulans* (McCormack *et al.*, 1995).

Water availability is one of the most important limiting environmental factors for microbial growth in the phyllosphere. Several parameters describe water relations on aerial plant surfaces. The duration of leaf surface wetness is an important parameter, and it is commonly used in epidemiological studies in plant pathology. Conditions alternate between a surfeit of free water, which may be important in redistributing cells of BCAs, and drought, which is made worse by low atmospheric humidity (Dickinson, 1986). The irregular provision of surface water on leaves results in intermittent growth of microorganisms, and poses problems of survival during dry periods (Blakeman and Fokkema, 1982). Bacterial populations are less adapted to

daily changes in humidity than phyllosphere yeasts and filamentous fungi (Fokkema and Schippers, 1986).

Fungi consistently found in the phyllosphere have been shown to have hyphal tips tolerant to drying. This tolerance permits resumption of growth with very little lag period (Park, 1982). The adaptation of fungal antagonists to rapid changes between wet and dry periods may be a key factor in field performance. Köhl *et al.* (1995 a, b) have shown that this is essential for antagonists aimed at suppression of sporulation of *B.cinerea* in necrotic tissue. They showed that some antagonists, highly efficient under continuously wet conditions, were of low to moderate efficiency when leaf wetness periods had been interrupted 16 h after application of the antagonist. The most efficient antagonist, *Ulocladium atrum*, survived dry periods and consistently reduced sporulation of naturally occurring *B.cinerea*.

According to Griffin, D.M. (1981) the conceptual framework concerning water that exists in the various subdisciplines related to microbial ecology differs considerably and a thermodynamic approach is needed for the analysis of the role of water in microbial activity. Water moves along gradients of potential energy; in the absence of a pumping mechanism water will be available to an organism only if it can so adjust the potential energy of its existing water that the appropriate gradient is produced. Water potential can be defined as the amount of work that must be done per unit quantity of pure water in order to transport reversibly and isothermally an infinitesimal quantity of water from a pool of pure water at atmospheric pressure to a point in the system under consideration at the same elevation. Water potential is given by the formula:

$$\Psi = \frac{(\mu_w - \mu_w^o)}{V_w}$$

where Ψ is the water potential (per unit volume), μ_w is the chemical potential of water, μ_w^o is the chemical potential of water in standard conditions and V_w is the partial molal volume of water. Water potential will be determined by the sum of its components, which are: pressure potential (Ψ_p, the potential of water per unit volume as affected by external pressure), solute (or osmotic) potential (Ψ_s, the potential of water per unit volume as affected by the presence of solutes), and matric potential (Ψ_m, the potential of water per unit volume as affected by the presence of a solid matrix). Thus,

$$\Psi_w = \Psi_p + \Psi_s + \Psi_m$$

The relationship between water potential and equilibrium relative humidity is,

$$\Psi_w = \frac{RT}{V_w} \mathit{ln} \frac{\%RH}{100}$$

where R is the gas constant, T the temperature and % RH the percentage relative humidity. This equation can be also expressed as:

$$\Psi_w = \frac{RT}{V_w} \mathit{ln}\, a_w$$

where a_w is the water activity (a_w) of a solution in equilibrium with an atmosphere of the corresponding equilibrium relative humidity. Soil microbiology studies use water potential, while for solid substrates, where solute potential is the major force, a_w is commonly used.

Water potential of pure water is 0, corresponding to an atmosphere with 100 % relative humidity and to a water activity value of 1. Small reductions in atmospheric relative humidity result in big reductions of water potential. Thus, for example water potential in an atmosphere at 99.6 % relative humidity is -0.54 MPa, and the value at 95 % is -7.0 MPa (Table 1.2). The water potential of phylloplane microbes will be at some intermediate value between that of the boundary layer and the leaf, and will be continuously variable. In spatial terms, water potentials will be lower on exposed leaves at the periphery of a canopy than on sheltered leaves within, and the highest humidities are typically recorded in the shaded, dense regions of a crop (Andrews, 1992).

Table 1.2. Water activity, equilibrium relative humidity (ERH) and water potentials at 25°C. Taken from Magan (1997)

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

Microbial cells can absorb water only if their internal water potential is less than that of the environment. To reduce the internal water potential, microorganisms accumulate osmotically active compounds and reduce in this way the imbalance across the membrane when they are exposed to an environment of low water potential. Any solute retained within a cell contributes to its osmotic status, acting as an osmoregulator (Brown, 1976). However, only some solutes accumulate without interfering with cellular functions. Compounds which are able to accumulate and protect enzyme activity are called compatible solutes (Brown and Simpson, 1972). The number of molecules that can act as compatible solutes is relatively reduced and the same compounds can be found in organisms widely separated phylogenetically (Yancey, 1982). In fungi compatible solutes are generally polyhydroxyalcohols, with glycerol being the most important in many fungal groups, and proline in oomycetes (Griffin, D.M., 1981). Proline and other amino acids may also have a role in fungi such as *Fusarium solani* or *Sclerotium rolfsii*, indigenous to saline soils (El-Abyad *et al.*, 1994). High concentrations of compatible solutes enable enzyme systems to function under conditions of water or sometimes temperature stress (Brown, 1978).

In conditions of low water availability cells can suffer dehydration and membranes can be damaged (Leslie *et al.*, 1994). Trehalose can help to prevent this damage by replacing water in dehydrated phospholipid membranes. Replacement of water with trehalose would inhibit transition of the liquid crystalline phase to the gel phase and in doing so preserve membranes (Crowe *et al.*, 1984; Van Laere, 1989; Leslie *et al.*, 1994). Although trehalose does not act in osmotic adjustment, it is metabolically compatible with the fungal cell and has been referred as a compatible solute (Rudolph *et al.*, 1993).

Using different carbon sources and water stress conditions it has recently been shown that it is possible to channel elevated concentrations of polyols (particularly glycerol and erythritol) and trehalose into conidia of entomopathogenic fungi (Hallsworth and Magan, 1994a). Interestingly, such conidia tolerated lower water potentials and were more pathogenic than unmodified conidia over a range of water availabilities (Hallsworth and Magan, 1994b). To date, no research has been conducted using this approach to improve ecological competence and biocontrol of fungal plant pathogens in the phyllosphere.

1.4. HOST-PATHOGEN-BIOCONTROL SYSTEMS STUDIED

1.4.1. Rhizosphere system: *Lycopersicon esculentum* Mill. - *Fusarium oxysporum* Schlecht f.sp. *lycopersici* (Sacc.) Snyder et Hans. - *Penicillium oxalicum* Currie & Thom.

Tomato is one of the most important horticultural crops worldwide. Vascular wilt is one of the most important diseases affecting this crop. It can be induced by *Fusarium oxysporum* f.sp. *lycopersici* and by *Verticillium dahliae*. These two pathogens produce slightly different symptoms and induce disease under different environmental conditions.

Symptoms of the disease induced by *F.oxysporum* f.sp. *lycopersici* are leaf yellowing and necrosis, epinasty and wilting. The vascular system of diseased plants becomes characteristically brown (Tello and Lacasa, 1990). The pathogen has a saprophytic phase in plant debris and soil, and it can survive for a long time in the form of

chlamydospores. In the presence of a sensitive host, chlamydospores germinate and infect host tissues (Beckman, 1987).

The disease is controlled mainly by the use of resistant cultivars. The dominant gene I confers resistance to so-called race 1 of the pathogen, described in 1886. A new pathogenic race (race 2) appeared after years of intensive use of cultivars with the gene I (Alexander and Tucker, 1945). The gene I2 provided resistance against races 1 and 2. Race 2 replaced race 1 over a period of ten years (Tello and Lacasa, 1988). A new pathogenic race (race 3) has been found in locations outside of Europe, like Tunisia (El Manjoub, 1974) or America (Davis *et al.*, 1988; Valenzuela *et al.*, 1996). A new resistance gene (I3) has also been identified (Sarfatti *et al.*, 1991).

Cultural, physical and chemical methods are also used to control the disease by reduction of the amount of inoculum. Beckman (1987) reviewed cases of soil fumigation and use of systemic fungicides (especially benomyl) to control the disease. Chemical control by soil fumigation is efficient but presents problems of rapid reinfestation. Efficiency of chemical control with fungicides is low, produced by the high cost of treatments, and resistance can appear. Becker and Schwinn (1993) classified the status of chemical control of *Fusarium* spp. as moderate-unsatisfactory, making it necessary to search for other control methods. Physical methods have been tried, such as soil sterilisation by steaming (Westeijn, 1973), or solarization. A significant reduction in wilt incidence in susceptible tomato cultivars was obtained by Oliveira (1992), by soil solarization. Jarvis (1977) first suggested the possibility of using biological control. Marois and Mitchell (1981) and Marois *et al.* (1981) paid particular attention to microbial antagonists in soil, capable of preventing the

development of the pathogen in the first stages of soil reinfestation. Suppressive soils have been known for a long time, but have only carefully been investigated recently. In suppressive soils disease incidence remains low in spite of the presence of the pathogen, a susceptible host plant and climatic conditions favourable for disease development (Alabouvette, 1990).

Microorganisms responsible for this disease suppression are, amongst others, non-pathogenic *Fusarium oxysporum* isolates (Davis, 1968; Alabouvette *et al.*, 1985) and fluorescent pseudomonads (Scher and Baker, 1982; Lemanceau and Alabouvette, 1993). Both microorganisms have been used simultaneously to control fusarium diseases (Fuchs and Défago, 1991; Lemanceau and Alabouvette, 1991). Different modes of action have been proposed to explain biocontrol of the disease, observed not only in suppressive soils, but in other systems. These mechanisms include a greater capacity of the antagonistic microorganisms than the pathogen to compete for nutrients, especially carbon and iron (Scher and Baker, 1980; Couteaudier and Alabouvette, 1990; Lemanceau and Alabouvette, 1993), competition for root colonisation, and induction of resistance (Mandeel and Baker, 1991; Lemanceau and Alabouvette, 1993). Detoxification of fungal metabolites, such as fusaric acid could also be involved (Toyoda *et al.*, 1988; Lemanceau and Alabouvette, 1993). The production of lytic enzymes by antagonistic microorganisms can also be a mode of action (Mitchell and Alexander, 1961; Inbar and Chet, 1991). Other microorganisms reported as BCAs for *Fusarium* wilt of tomato are, for example, *Streptomyces* spp. (El-Abyad *et al.*, 1993; Elshaushonly *et al.*, 1996) or *Serratia plymuthica* (Frommel *et al.*, 1991). Cross-protection was attempted by inoculating plants with *Fusarium*

oxysporum f. sp. *dianthii*, but it was not effective under glasshouse conditions (Wymore and Baker, 1982).

De Cal *et al.* (1995) reported control of fusarium wilt of tomato using fungi known to produce lytic enzymes: *P.oxalicum*, *Penicillium purpurogenum* and *Aspergillus nidulans*. These species damaged hyphae of *Fusarium oxysporum* f.sp. *lycopersici in vitro* and reduced the numbers of microconidia in the soil. Treatments did not result in a reduction in either chlamydospores in soil or populations of the pathogen in the rhizosphere of tomato. It was concluded that the antagonist *P.oxalicum* must work by competition or induction of resistance in the host. *P.oxalicum* was the most effective agent of biocontrol, and it reduced disease severity in both non-autoclaved (20% decrease) and sterile soil. In sterile soil, *P.oxalicum* reduced disease with different levels of severity (27 % decrease at high levels (79 %) and 50 % decrease at low levels (62 %)). Disease control by *A.nidulans* and *P.purpurogenum* (55 % and 45 %, respectively) was only achieved when disease severity was low, and in sterile soil. Recently, De Cal *et al.* (1997a) have demonstrated induction of systemic resistance in tomato plants by *P.oxalicum*. Van Peer and Schippers (1992) also reported induction of resistance to *Fusarium* wilt of carnation by a *Pseudomonas* sp. For diseases with mobile infection courts like fusarium wilts it is agreed that induction of resistance is the best mechanism for a biocontrol agent (Baker and Paulitz, 1993). Induction of resistance has gained interest not only as a mechanism for biocontrol agents (Kúc, 1983), but also as a mode of action of chemicals (Kessmann *et al.*, 1994).

P.oxalicum has been reported to be a biocontrol agent against other diseases. Kommedahl and Windels (1978) showed that *P.oxalicum* was nearly as effective as

captan in protecting pea seedlings against root rot, caused by different fungal species. In subsequent studies they investigated growth of the antagonist and effects on rhizosphere organisms, and on factors affecting biocontrol efficacy (Windels and Kommedahl, 1978; Windels, 1981; Windels and Kommedahl, 1981; Windels and Kommedahl, 1982). Gintis and Benson (1987) also reported biological control of *Phytophthora* root-rot of azalea with *P.oxalicum.* It was also involved in biocontrol of *R.solani* (Huang and Kuhlman, 1991) and seed rot and preemergence damping-off caused by *P.ultimum*, under field and greenhouse conditions (Kaiser and Hannan, 1984; Trapero-Casas *et al.*, 1990). *P.oxalicum* has also been tested for the control of foliar diseases, such as bacterial leaf spot induced by *Xanthomonas campestris* pv. *vignaeradiatae* (Jindal and Thind, 1994) or *Colletotrichum corchori* on jute (Purkayastha and Bhattacharyya, 1982). It has been also cited as a pathogen on cucumber (Jarvis *et al.*, 1990), sweet corn (Halfonmeiri and Solel, 1990) or wheat (Ride and Barber, 1987), but in these cases it behaves normally as a weak pathogen. The isolate used in this work does not produce any visible damaging effect on tomato plants.

1.4.2. Phyllosphere system: *Prunus persicae* L. - *Monilinia laxa* (Aderh et Ruhl) Honey -*Epicoccum nigrum* Link/*Penicillium frequentans* Westling.

Prunus persicae is a stone fruit tree widely grown in temperate regions. The brown rot fungi are a group of fungal pathogens that cause considerable damage to cultivated fruit trees, particularly apples, pears and stone fruits, in the temperate regions of the world. *Monilinia fructigena* and *Monilinia laxa* are the two brown rot species in Europe. These two pathogens induce one of the most important diseases on peaches, producing fruit rot, blossom, twig and leaf blight and stem cankers (Byrde and Willets, 1977). In Spain *M.laxa* is the most important species causing peach twig blight

(Sampayo and Palazón, 1971). This pathogen is also important in other European countries, such as Switzerland, where an increased occurrence of sweet cherry blossom blight, caused by *M.laxa*, has been found recently (Tamm, 1994).

Primary infections are generally produced in spring. Twigs are infected by inoculum from mycelium or conidia from buds, flowers or rotted fruits, or from overwintering structures. The infected tissues are seen as brown, collapsed areas. These gradually extend up and down the twig. Gum accumulates on the surface of infected tissues. Stem cankers develop usually from blighted twigs or fruit spurs, cankers can heal up or continue to develop for several years, becoming a source of spore inoculum. In extreme cases, when damage is severe, the disease can cause death of the tree (Byrde and Willets, 1977; Biggs and Northover, 1985).

Chemical control of the disease started with the use of inorganic products, such as sulfur compounds. In the 1950s other fungicides like captan, dichloran, thiram, maneb and dichlone improved disease control. An effective chemical control was achieved with the use of benzimidazoles (Ramsdell *et al.*, 1970; Zehr, 1982). However, fungicide resistance was soon detected and has been reported frequently (Tate *et al.*, 1974; Zehr, 1982; Guizzardi *et al.*, 1995). Unfortunately, carbendazim-resistant isolates, are likely to persist in the population (Sanoamuang and Gaunt, 1995). Other fungicides have been introduced, with different modes of action, like dicarboximides or inhibitors of ergosterol synthesis, but fungicide resistance has again appeared in some cases (Katan and Shabi, 1981; Penrose *et al*, 1985; Elmer and Gaunt, 1993). Thus, new products are being sought to control *Monilinia* spp. (Latorre, 1986; Osorio *et al.*, 1993).

Biological control may contribute to an effective control system in an integrated disease management programme. Use of fungal antagonists to control *Monilinia* was first attempted by Khasanov (1962). Melgarejo and M-Sagasta (1984) and Melgarejo *et al.* (1986) observed *in vitro* antagonism by *Aspergillus flavus, Epicoccum nigrum, Penicillium frequentans* and *Penicillium purpurogenum* against *M.laxa*, and disease control in the field. The antagonists were isolated from healthy peach twigs and they were part of the resident epiphytic micoflora.

Disease control in the field by *P.purpurogenum* has been studied by Larena (1993). De Cal *et al.* (1990) using *P.frequentans* obtained different levels of disease control depending on environmental conditions and formulation of the biocontrol treatment. It was established that a minimum population size of the antagonist necessary to get successful biocontrol was 10^3 cfu g^{-1} twig weight. Colonisation of peach twigs by *P.frequentans* can be favoured in integrated control programmes. It has been shown that chemical treatments depress populations of other saprophytic fungi such as *Alternaria* or *Cladosporium* spp., resulting in this *Penicillium* spp. becoming dominant on twigs when pesticides were applied most frequently (De Cal and Melgarejo, 1992). Integrated control with captan was successful especially when weather conditions prevented effective control by *P.frequentans*. A relationship was established between the production of antifungal substances by the antagonist and the biocontrol of the pathogen (De Cal *et al.*, 1988).

Madrigal *et al.* (1994) investigated the biocontrol of *M.laxa* by *E.nigrum*, alone or in combination with captan in four field trials. Biocontrol obtained after application of the antagonist was variable each year, depending on the relative disease severity in the

first 2-3 weeks after infection and the climatic conditions. Mean relative humidity in the 2-3-week period after inoculation with *M.laxa* was 55-60 % in years with better disease control, while it was 40-30 % in years with poor disease control. This points to the possible dependence of *E.nigrum* on high humidity conditions.

E. nigrum (≡*E.purpurascens*) is one of the species consistently found in the phyllosphere flora of plants, grouped as "common primary saprophytes", together with *Alternaria* spp., *Aureobasidium pullulans*, *B.cinerea* and *Cladosporium* spp. (Park, 1982). The number of plant species from which *E.nigrum* has been isolated is large. Apart from peach twigs it has been found, for example, in mango flowers (Johnson *et al.*, 1991), as an endophyte in cherries also colonised by *M.laxa* (Tamm, 1994), in the phyllosphere of cereals (Magan and Lacey, 1986), bean and rapeseed (Inglis and Boland, 1992) and guava (Pandey *et al.*, 1993). Common primary saprophytes colonise leaves during senescence, and grow as active saprophytes within the dead leaf (Hudson, 1971). They may also infect living leaves to form latent or non-pathogenic infections. In some cases *E.nigrum* has been shown to be a pathogen on maize (Macek and Zupan, 1993) and a post-harvest pathogen (Bruton *et al.*, 1993). As a BCA, *E.nigrum* has been tested for control of several diseases. It was shown to be more effective than other epiphytes for reducing the pathogenic activity of *Leucostoma cincta* on peach bark tissues (Royse and Ries, 1978; Biggs and Alm, 1991). It was one of the best antagonists tested against *Sclerotinia sclerotiorum* on bean (Boland and Inglis, 1988), and it was able to reduce the disease in the greenhouse and in the field (Zhou and Reeleder, 1989, 1990) by reducing the growth of the pathogen in necrotic tissue (Zhou and Reeleder, 1991). It was antagonistic to *Colletotrichum gloeosporioides* (Pandey *et al.*, 1993), *B.cinerea* (Peng and Sutton,

1991) and wound pathogens on stems of *Picea abies* (Zimmerman *et al.*, 1995). It has also been tested as an antagonist of soil-borne pathogens (Brown *et al.*, 1987; Dhiman, 1993). Antagonism of *E.nigrum* has been associated frequently with antibiosis (Brown *et al.*, 1987; Madrigal *et al.*, 1991; Zhou *et al.*, 1991). In other cases, parasitism (Campbell, 1956) or competition for nutrients (Zhou *et al.*, 1991) have been suggested.

M.laxa is also important as a post-harvest pathogen. Together with *M.fructicola* and *M.fructigena* they constitute one of the major groups of decay pathogens of stone fruits (Melgarejo *et al.*, 1997). The importance of *M.laxa* as a post-harvest pathogen of peaches is increasing, and in some cases is viewed as the main problem after harvest (Foschi *et al.*, 1994).

Infections that cause post-harvest brown rot originate from preharvest quiescent or latent infections, or from the germination of conidia on the surface of ripe fruit, followed without pause by penetration and decay (Melgarejo *et al.*, 1997). Application of synthetic fungicides after harvest has been the main means of control of post-harvest diseases. However, the risk of fungicide use to human health and the environment, and the appearance of fungicide-resistant lines of *Monilinia*, makes it necessary for a search for alternative control methods. Combination of hot water and ethanol has been recently tested for the control of brown rot, with results sometimes similar to those obtained with fungicide treatments (Margosan *et al.*, 1997). The first investigation on post-harvest control of brown rot by microorganisms was made by Pusey and Wilson (1984). They found that the B-3 strain of *Bacillus subtilis* controlled brown rot of peaches, nectarines, apricots and plums. The biocontrol of B-3 was

effective during low temperature storage of fruit, and application of the bacterium was shown to be compatible with various kinds of waxes commonly used in the application of fungicides (Pusey *et al.*, 1986). Production of B-3 was scaled up using a low cost medium. Its efficacy, tested using simulated peach-packing lines, was equal to benomyl in controlling brown rot (Pusey *et al.*, 1988). *Bacillus subtilis* has been patented for the control of brown rot of fruit. However, to my knowledge, the biocontrol agent has not been registered and commercialised yet.

Utkhede and Sholberg (1986) also obtained some *B.subtilis* isolates which controlled rot of cherries caused by *A.alternata* and *M.fructicola*. Other bacteria, such as *P.corrugata* and *P.cepacia* (Smilanik *et al.*, 1993), or endophytic bacteria (Pratella *et al.*, 1993) have been shown to control brown rot of stone fruits. Fungi isolated by Melgarejo *et al.* (1986) (*E.nigrum*, *P.purpurogenum* and *P.frequentans*) together with an isolate of *Trichoderma pseudokoningii* were tested by Foschi *et al.* (1994) against *M.laxa* on peaches, with *E.nigrum* being the most effective. *E.nigrum* was also tested for post-harvest control of *B.cinerea*, *M.fructigena* and *P.expansum* (Falconi and Mendgen, 1994).

In the present study attempts were made to improve biocontrol of peach twig blight by *P.frequentans* and *E.nigrum* by ecophysiological manipulation, to increase their tolerance to conditions of low relative humidity, and perhaps improve competence in the field for the first time. *E.nigrum* produced under different water stress conditions was also tested as a post-harvest BCA for brown rot of cherries.

1.5. OBJECTIVES OF THIS WORK

The main aims of this work were three-fold:

a) To investigate methods of production for the biocontrol agents studied (Phase I)

b) To characterise the inoculum types produced, paying special attention to the ecological competence of the inocula (Phase II)

c) To analyse their efficacy in controlling the diseases (Phase III)

More specifically the objectives were to:

Rhizosphere pathosystem:

1: Develop a method for the induction of submerged conidiation of *P.oxalicum*

2: Examine the competence of *P.oxalicum* to grow in soil under different conditions of water availability and nutrients

3: Study surface properties (hydrophobicity and appearance under the SEM) of *P.oxalicum* spores produced by aerial and submerged cultivation, as well as their adhesion to tomato roots

4: Examine survival of aerial and submerged spores of *P.oxalicum* after storage in different conditions

5: Determine the minimum effective concentration of *P.oxalicum* to control *Fusarium* wilt of tomato

6: Assess the ability of different types of inoculum of *P.oxalicum* (mycelium and aerial and submerged spores) to control the disease

7: Develop methods to formulate *P.oxalicum* with tomato seeds and to assess its ability to promote growth

Phyllosphere pathosystem:

8: Develop a method for the production of spores of *E.nigrum* by solid substrate fermentation

9: Characterise growth and competitive ability (determined by Niche Overlap Index) of *E.nigrum* produced under unstressed or water stress conditions

10: Study the temporal changes and accumulation of compatible solutes and glucose by *E.nigrum* and *P.frequentans* grown under unstressed or water stress conditions, in culture media, and by solid substrate fermentation (only *E.nigrum*)

11: Determine turgor pressure of *E.nigrum* mycelium produced under unstressed or water stress conditions

12: Study survival of *E.nigrum* spores produced under unstressed or water stress conditions

13: Improve biocontrol of peach twig blight by physiological manipulation of *E.nigrum* and *P.frequentans*. The fungi were grown under water stress conditions to produce inoculants more adapted to shortage of water in the phyllosphere

14: Test the ability of *E.nigrum* produced under unstressed or water stress conditions to control brown rot of cherries

The structure of this thesis is represented in a flow chart in Fig. 1.2.

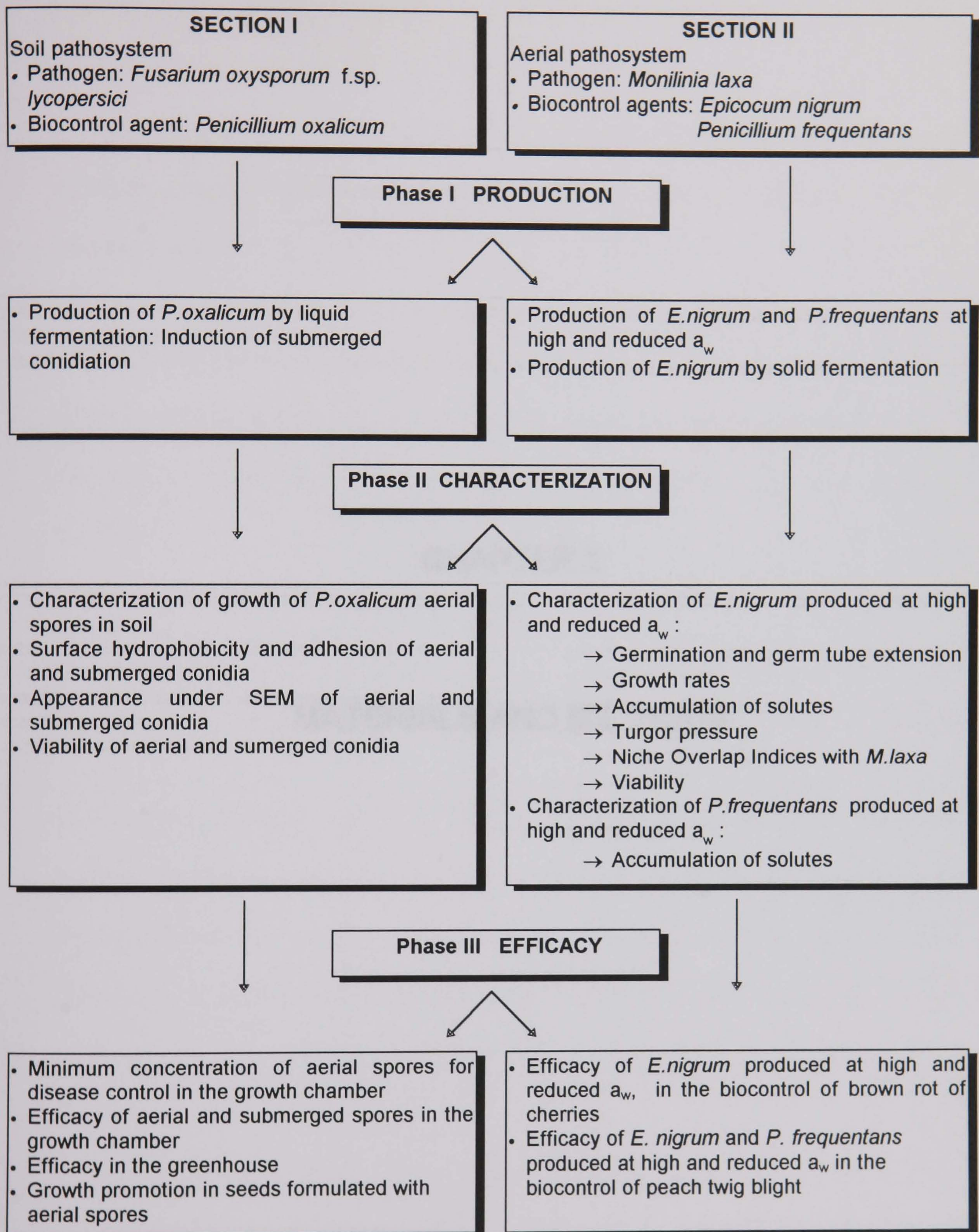


Figure 1.2. Flow chart showing the different Sections and Phases of work carried out in this thesis

CHAPTER 2

MATERIALS AND METHODS

2.1. CULTURE MEDIA

All culture media are detailed here except for those used in the experiments on induction of submerged conidiation of *Penicillium oxalicum*, which are detailed in Section 2.4.2.

(a) Carnation Leaf Agar: Prepared by placing 5-6 sterile pieces (5 mm^2) of carnation leaves in Petri plates and pouring water agar in the plates. Sterilisation was with 2.5 megarads of gamma irradiation from a Cobalt 60 source (Nelson *et al.*, 1983).

(b) Czapek Dox Agar (Difco)

(c) Czapek Dox Broth (Difco)

(d) *Fusarium* selective medium: Based on *Fusarium* selective medium described by Nash and Snyder (1962), containing per litre: 15 g peptone, 1 g KH_2PO_4, 0.5 g Mg SO_47H_2O and 20 g agar. After autoclaving the following was added per litre: 1g PCNB, 1g streptomycin sulphate, 12 ml neomycin (from a stock solution containing 10 g l^{-1}), and 625 µl benomyl (from a stock solution containing 10 g l^{-1})

(e) Hoagland Agar: Based on Hoagland n° 2 solution (Hoagland and Arnon, 1950), containing: $(NH_4)H_2PO_4$ (1mM), KNO_3 (6mM), $Ca(NO_3)_24H_2O$ (4mM), $MgSO_4$ (2mM), 1 ml l^{-1} of a micronutrient solution containing per 100 ml: 0.286 g H_3BO_3, 0.181 g $MnCl_2$, 0.2845 g $MnCl4H_2O$, 0.022 g $ZnSO_47H_2O$, 0.008 g $CuSO_45H_2O$, and 0.002 $H_6Mo_7O_{24}H_2O$; and 1 ml l^{-1} of an iron solution containing per 100 ml: 0.391 g $C_4H_6O_6$ and 0.4899 g $FeCl_36H_2O$.

(f) *Penicillium oxalicum* selective medium: As the *Fusarium* selective medium including 58.4 g l^{-1} NaCl (prior to autoclaving) instead of benomyl.

(g) Potato Dextrose Agar prepared with fresh potatoes (PDAP): Prepared as recommended by Booth (1971) with slight modifications. Two hundred grams of scrubbed and diced potatoes were boiled in distilled water for 1 h and then passed through a fine cheese cloth, squeezing through as much pulp as possible. Agar (Pronadisa) (20 g) was added, boiled until dissolved and removed from the heat. Dextrose (Panreac) (20 g) was then added and stirred until dissolved. Distilled water was added to make up to 1 l.

(h) Potato Dextrose Agar (Difco) (PDA)

(i) Potato Dextrose Agar (Difco) modified with glycerol to give a water activity (a_w) value of 0.98 (PDA98) and 0.95 (PDA95): To prepare these media, a 1.1 molal (m) and 2.7 m solution of glycerol respectively was used instead of water.

(j) Potato Dextrose Broth (Difco) (PDB)

(k) Potato Dextrose Broth (Difco) modified with glycerol to give 0.98 and 0.95 a_w (PDB98 and PDB95 respectively)

(l) Potato Dextrose Agar (Difco) modified with polyethylene glycol 200 (PEG 200) to give 0.99, 0.984 and 0.950 a_w (PDA99, PDA984 and PDA950 respectively). A 0.5, 0.7 or 1.25 m solution of PEG 200 respectively was used instead of water.

(m) One tenth strength Potato Dextrose Agar (Difco) (1/10 PDA): PDA 10 fold diluted plus 15 g agar l^{-1}

(n) One tenth strength 1/10 Potato Dextrose Agar (Difco) modified with PEG 200 to give 0.99, 0.984 and 0.95 a_w (1/10PDA99, 1/10PDA984 and 1/10PDA95 respectively)

(o) Minimal Medium (MM): Containing per litre: 1 g K_2HPO_4, 0.5 g $MgSO_47H_2O$, 0.5 g NaCl and 1 ml of a minor-element solution (containing per litre: 100 mg $Na_2B_4O_710H_2O$, 70 mg $ZnSO_47H_2O$, 50 mg $FeSO_47H_2O$, 10 mg $(NH_4)_6Mo_7O_{24}4H_2O$, 10 mg $MnSO_44H_2O$ and 10 mg $CuSO_45H_2O$).

(p) Minimal Medium modified with PEG 200 to give 0.99, 0.984 and 0.95 a_w (MM99, MM984 and MM95 respectively)

(q) Potato Salt Agar (PSA): Potato Dextrose Agar (Oxoid) amended with 7.5 % NaCl

(r) Water Agar: Containing per litre 20 g agar (Pronadisa)

(s) Water agar modified with PEG 200 and PEG 300 to give 0.935 a_w: Solutions of PEG 200 (1.25 m) and PEG 300 (1 m) were mixed (1:1 v:v). This mixed solution was used instead of water in the preparation of the medium (WA935)

All media were prepared with distilled water and sterilised at 1 kg cm^{-2} and 120°C for 20 min. Solid media were cooled at 40-45°C after autoclaving and for the most experiments plated into 9 cm diameter Petri plates.

2.2. APPARATUS

Autoclave Mediclave (Selecta)

Centrifuges:

- Sorvall RC5B (Dupont Instrument)
- Clino (Orto)

High Performance Liquid Chromatography (HPLC) equipment (Gilson). The column used was a Hamilton HC-75 Ca^{2+} Form column, special for the separation of sugars/polyols, in conjunction with a refraction index detector (Gilson)

Environmental shaker Lab-line (Orbit)

Incubators (Selecta)

Microscopes:

- Fluorescence microscope (Nikon) equipped with an epifluorescence illuminator and a UV excitor filter
- Optical microscope with planapomatic optics (Zeiss)
- Scanning electron microscope (JEOL JSM T33OA)

Psychrometer (Wescor HT33 + C52 sample chamber)

Spectrophotometer CE 594 (CECIL Instruments Limited)

2.3. BIOLOGICAL MATERIAL

2.3.1. Fungal isolates

(a) Pathogens:

The monosporic isolate of *Monilinia laxa* (Aderh et Ruhl) Honey, strain ZA-1 (ATCC number: 66106), was collected from a commercial apricot orchard in Almonacid de la Sierra (Zaragoza, Spain). *M.laxa* cultures were stored on potato dextrose agar (PDAP) slants at 4°C. Every year the pathogen was reisolated from artificially infected peach twigs to prevent loss of pathogenicity, and new stock cultures were prepared. Reisolation was as described in Section 2.9.2. for the determination of the extent of *M.laxa* colonisation.

The monosporic isolate of *Fusarium oxysporum* Schlecht f.sp. *lycopersici* (Sacc.) Snyder et Hans, was kindly provided by Dr. J. Tello (Universidad de Almería, Spain). *F.oxysporum* f.sp. *lycopersici* cultures were stored in sand. The stock cultures were prepared as follows: the pathogen freshly isolated from infected tomatoes was grown on carnation agar Petri plates for 15 days. Plates were incubated at room temperature, in diffuse daylight from a north window to favour sporulation (Nelson *et al.*, 1983). A suspension of macroconidia (approx. $1x10^6$ macroconidia ml^{-1}) was made from these plates in water and decanted into test tubes containing sterile sand and 3% (v:v) oat flakes. Tubes were incubated in the darkness at 25°C for 7 days and stored at 4°C.

(b) Antagonists:

Penicillium frequentans Westling, isolate 909 and *Epicoccum nigrum* Link, isolate 282 were originally isolated from the phyllosphere of peach twigs (Melgarejo and M-Sagasta, 1984) at an experimental orchard in Madrid, Spain.

The *Penicillium oxalicum* Currie & Thom isolate was kindly provided by Dr. F. Reyes (Consejo Superior de Investigaciones Cientificas, Madrid, Spain).

Cultures of the three antagonists were stored in potato dextrose agar (PDAP) slants at 4°C. Every year the antagonists were reisolated from plant material and new stock cultures were prepared.

2.3.2. Plant material

Peach trees (*Prunus persica* L.) cv. Baby Gold were used for the field trials. The orchard was situated at CIT-INIA, Madrid, Spain. Trees were nine years old in 1995.

Tomato seeds (*Lycopersicon esculentum* Mill.) cv. Lorena and Precodor (S&G, Sandoz Seeds) were used. These varieties are susceptible to race 2 of *Fusarium oxysporum* f.sp. *lycopersici* and resistant to race 1.

2.4. PRODUCTION OF *Penicillium oxalicum* INOCULUM

2.4.1. Production on solid culture medium

Aerial spores of *P.oxalicum* were produced on Potato Dextrose Agar (PDAP) Petri plates. PDAP plates were inoculated by spreading a loop full of culture from the stock PDAP slants. Plates were incubated at 25°C for seven days. Spores were gently removed from the surface of each colony in sterile water. Spores produced by this method will be referred to as aerial spores.

2.4.2. Production in submerged culture: induction of submerged conidiation

(a) Media

Synthetic media:

1) Mannose medium: Containing per litre: 20 g mannose, 2 g arginine, 2 g glycine, 1g K_2HPO_4, 0.5g Mg SO_47H_2O, 0.5g NaCl, and 1ml of a trace element solution containing per litre: 100mg $Na_2B_4O_710H_2O$, 70 mg $ZnSO_47H_2O$, 50mg $FeSO_47H_2O$, 10mg $(NH_4)_6MoO_{24}4H_2O$ and 10mg $CuSO_45H_2O$. Other media tested were based on mannose medium with one of the following additions: polyethylene glycol 6000 (200 or 400 g l^{-1}), NaCl (50 or 100 g l^{-1}), trehalose (100 or 200 g l^{-1}) or glycerol (100 or 200 g l^{-1}).

2) Czapek Dox Broth: Containing per litre: 30 g sucrose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl and 0.01 g $FeSO_4$. Other media tested were based on Czapek medium with one of the following additions: polyethylene glycol 6000 (200 g l^{-1}), V8 (150 ml l^{-1}) or $CaCl_2$ (9mM).

3) Richard's medium: Containing per litre: 8 g sucrose, 10 g KNO_3, 5 g KH_2PO_4, 1.3 g $MgSO_4$, 20 mg $FeCl_3$. The same additions as in 2) were tested.

4) Medium F (Foster *et al.*, 1945): Containing per litre: 20 g sucrose, 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_47H_2O$ and 1 ml of the same trace element solution used in 1). Other media tested were based on medium F with one of the following additions: $CaCl_2$ (10, 20 or 40 mM) or 10 mM $CaCl_2$ and 10 mM $(NH_4)_3C_6H_5O_7$.

5) Morton's media (Morton *et al.*, 1958): Medium A contained per litre: 50 g glucose, 1g KH_2PO_4, 0.5 g $MgSO_47H_2O$, 2.3 g KNO_3 and 1ml of a trace element solution containing per litre: 225 mg $ZnSO_47H_2O$, 200 mg $FeSO_47H_2O$, 40 mg $(NH_4)_6MoO_{24}4H_2O$, 38 mg $CuSO_45H_2O$ and 24 mg $MnCl_2$. Medium B differed from medium A in nitrogen concentration (absent in medium B).

Complex media:

1) Malt extract medium: Containing: 20 g l^{-1} malt extract, 10 mM $CaCl_2$ and 10 mM $(NH_4)_3C_6H_5O_7$.

2) Sucrose and peptone medium: Containing: 20 g l^{-1} sucrose, 10 g l^{-1} peptone, 10 mM $CaCl_2$ and 10 mM $(NH_4)_3C_6H_5O_7$.

3) Maxwell and Lumsden medium (Maxwell and Lumsden, 1970): Containing per litre: 13.3 g glucose, 0.5 g yeast extract, 180 mg $MgSO_4$, 149 mg KCl, 1 g NH_4NO_3, 680 mg KH_2PO_4, 11.3 mg $ZnSO_4.7H_2O$, 13.9 mg $MnSO_4.H_2O$, 4 mg $FeCl_3$, 4 mg $CuSO_4$, 15.13 g $C_4H_4Na_2O_4$.

Complex media based on byproducts:

1) Molasses medium: Containing 20 g l^{-1} molasses (Sigma). Addition of 10 mM $CaCl_2$ and 10 mM $(NH_4)_3C_6H_5O_7$ was alternatively used.

2) Corn Steep liquor medium: Containing 20 g l^{-1} corn steep liquor (Sigma).

3) Brewer's yeast medium: Containing 20 g l^{-1} brewer's yeast (Yestamin, English Grains Healthcare, Derby, UK).

(b) Culture procedures

P.oxalicum was grown at 25°C in submerged culture in 250 ml flasks on an orbital shaker at 150 rpm in the dark; each flask contained 50 ml culture medium. Flasks were inoculated with 1 ml of a spore suspension ($1x10^7$ spores ml^{-1}) from PS agar plates. This general method was followed for all the media except for Morton's media. In the case of Morton's media the fungus was grown in medium A for 24 h before following one of the two following procedures: a) concentration of glucose was increased by adding 10 ml of a glucose solution (500 g l^{-1}) to each flask; b) the culture was filtered through Whatman No 1 filter paper and the mycelium was washed with sterile water and transferred to medium B, this method will be referred as Morton's method and it was the one used in all the subsequent experiments in this section.

The cultures were monitored microscopically for sporulation daily for a 7 d period. Three flasks were inoculated for each medium.

(c) Studies on Morton's method

Time course study: Quantification of sporulation was made as explained later at 2, 3 and 6 days after transference of mycelium to medium B.

Effect of age of mycelium at transference: The fungus was grown in medium A for 24 or 48 h before being transferred to medium B.

Effect of pH: The pH of media (medium A and medium B) was modified with 1N HCl or NaOH solutions before autoclaving to obtain the following levels: 4,5,6,7 and 8. After autoclaving the levels were: 4.06, 4.6, 5.72, 6.42 and 6.32 (medium A) and 3.89, 4.49, 5.77, 6.25 and 6.33 (medium B).

Effect of Calcium: Medium B was amended with $CaCl_2$ to give a concentration of 40 mM.

Effect of PEG: Medium B was amended with PEG 6000 at two rates: 200 and 400 gl^{-1}.

Effect of reduction in nitrogen concentration and calcium: In this experiment no transference to medium B was done. Medium A was adjusted to pH 6. The levels of KNO_3 were (in g l^{-1}): 2.3, 1.5, 1.0, and 0.5. The levels of Cl_2Ca were: 0 and 2.94 g l^{-1}. Flasks were coated with Sigmacote (Sigma) to prevent accretion on flask walls (Ugalde and Pitt, 1983b). Spores produced using this method will be referred to as submerged spores.

Quantification of spore production: Spore production was quantified seven days after inoculation or six days after transference to medium B. Cultures were homogenised in a blender operating at 4000 rpm for 2 min, made up to the original 50 ml with water and divided into two 25 ml samples. The first sample was filtered through Whatman No 1 filter paper and dried to constant weight at 100 C. The second sample was filtered through glass wool after adding a drop of Tween 80. The conidia were counted in a Thomas counting chamber and spore concentration (no. spores ml^{-1}) and sporulation index (Pitt and Poole, 1981) (millions of spores per milligram of culture dry weight) were determined.

(d) Spore production in modified PDB

The C:N ratio of PDB was modified by adding 16 or 37 g glucose l^{-1} (C:N=93 or 144, respectively). Modified PDB was used undiluted and 2 and 10 fold diluted. Medium A of Morton's method with reduced nitrogen (1.5 g KNO_3 l^{-1}, equivalent to C:N=93) was also included. Calcium was then added to all media (20 mM) and pH adjusted to 6. Culture procedures and quantification of spore production was as described for the studies on Morton's method.

(e) Statistical analysis: Experiments were with three replicates per treatment and carried out twice. Data from representative experiments are presented. The data were analysed by analysis of variance. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.5. CHARACTERISATION OF *Penicillium oxalicum* SPORES

2.5.1. Study on growth of *Penicillium oxalicum* inoculum in sterile soil

(a) Soil properties

The characteristics of the soil used in this study were: pH = 7.3; total organic matter = 45 %; initial water content 3.35 % and field capacity = 42.8 %. The soil was obtained from a nursery in Madrid, Spain.

Soil adsorption curves: To build the soil adsorption curves, different amounts of water were added to 10 g samples of soil and allowed to equilibrate for 24 h with regular mixing. Subsamples were then placed in an psychrometer (Wescor HT33 Psychrometer) and subsequently dried in an oven at 80°C for 24 h to determine actual water content.

(b) Soil inoculation and growth conditions

Ten g subsamples of soil were placed in test tubes and autoclaved for 1h at 1 kg cm^2 and 120°C; water losses during the sterilisation were taken into account when adjusting the soil water potential. Water potential of soil was adjusted by adding sterile distilled water to autoclaved soil to bring the final moisture content to a water potential extrapolated from the adsorption curve. Soil moistures were adjusted to give water contents of 45.3, 40, 28 and 19 %, equivalent to water potential values of 1.0, 1.5, 4.0 and 7.0 (-MPa) respectively.

The effect of nutrients on growth of *P.oxalicum* was studied at -1.0 MPa water potential. Water content was adjusted in this case by adding sterile nutrient solution to soil to bring the final moisture content to 45.3 %. The sterile nutrient solution

contained 0.8 % mannose and 0.08 % arginine; these nutrients were selected in a previous *in vitro* study by Pascual *et al.* (1997).

Soil in test tubes was allowed to equilibrate for 24 h in five separate hermetic containers with relative humidities of 99.3, 98.9, 97.1 and 95 % (equivalent to 1.0, 1.5, 4.0 and 7 (-MPa)); relative humidities were in equilibrium with glycerol solutions of the corresponding water activities. Each tube was then inoculated with 0.5 ml of a spore suspension of *P.oxalicum* containing 1×10^8 spores ml^{-1}, this was taken into account when calculating the water needed to adjust the water content. The closed containers were placed in an incubator at 22-28°C.

(c) Characterisation of *P.oxalicum* growth

Quantification of population: *P.oxalicum* populations were estimated at 1, 5, 10, 20, 30, 40, 50 and 60 days after inoculation. Three test tubes were taken from each container at each date, and a 1 g sample was taken from each tube. Samples were placed in 250 ml flasks containing 150 ml phosphate buffer (pH = 7) and shaken for 30 min at 150 rpm. After 100 and 1000-fold dilution three 0.1 ml aliquots per dilution were spread on Petri dishes containing PDA amended with 0.5 g l^{-1} streptomycin. Plates were incubated in the dark at 25ºC for five days before the colonies were counted. Plates containing less than 5 or more than 150 colonies were discarded (Lacey *et al.*, 1980). The number of colony forming units (cfu) g^{-1} of soil dry weight was estimated for each sample.

Microscopy study: A microscopy study was carried out at the same dates the population of *P.oxalicum* was estimated. Samples were taken as described above.

The procedure was as described by Couteaudier and Steinberg (1990). Each sample was incubated for two hours with 0.5 ml of a 0.3 % calcofluor (Sigma) solution. After incubation, 100 ml of phosphate buffer (0.06 M, pH = 7.6) were added to each sample and three ml were taken and filtered through a Millipore membrane (GSWP, 0.2 mm pore diameter). The filter was mounted with Entellan (Merck) and the preparation was observed using a Nikon microscope equipped with an epifluorescence illuminator and a UV excitor filter. Three photographs were taken per slide and the number of germinated spores out of 50 was determined per photograph to calculate percentage germination. Germ tube length of 25 spores was measured for each photograph.

Statistical analysis: The data were analysed by analysis of variance. Before analysis cfu data were transformed by a sqrt function. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.5.2. Surface hydrophobicity and adhesion of aerial and submerged spores

An adhesion test was used to determine spore hydrophobicity. Adhesion of spores to tomato roots was also studied. Spore hydrophobicity was also determined by a phase distribution assay.

(a) Adhesion test

The test is based on the assumption that there is a correlation between the hydrophobicity of the cells and their adherence to polystyrene (hydrophobic surface) (Mozes and Rouxhet, 1987).

Spores from PDA plates were gently dislodged in sterile distilled water and the suspension filtered through glass wool and centrifuged for 15 min at 4000 rpm. The pellet was resuspended in sterile distilled water and centrifuged again. This washing procedure was done twice. The concentration of the spore suspension was adjusted to $2x10^6$ spores ml^{-1}. Spores from submerged culture were obtained by filtering the flask cultures through glass wool. The suspension was centrifuged and the rest of the procedure was as described for aerial spores.

Spore attachment to hydrophobic and hydrophilic surfaces was determined. Polystyrene Petri plates (Sterilin) were used as the hydrophobic surface. Tissue culture Petri plates (Corning) were used as the hydrophilic surface. Both types of plates were 35 mm diameter. Spore suspensions were poured in the plates (3 ml per plate) and incubated at 25°C in the dark. At each sampling time the supernatant was removed and the plates were rinsed twice with 3 ml sterile distilled water. Spore concentration was determined used an haemacytometer and the percentage adhesion was calculated as described by Sela-Buurlage *et al.*, (1991):

$$\% \text{ adhesion} = (1-(n/m)) \times 100$$

were n = concentration of unbound conidia after incubation

m = concentration of conidia before incubation

Sampling was done at 0 and 22 h and at 0, 10, 30, 60 and 1,020 min in a replicate experiment. Three replicate plates were used at each sampling time per type of surface and type of conidia.

Sampling was done at 0 and 22 h and at 0, 10, 30, 60 and 1,020 min in a replicate experiment. For germ tubes sampling was done at 0 and 22 h. Three replicate plates were used at each sampling time per type of surface and type of conidia.

Adhesion to tomato roots was also determined. Tomato seedlings were produced in the growth chamber as described in Section 2.6.1. Roots were washed and 50 mg fragments were placed in test tubes together with 1 ml spore suspension. Incubation times were 0, 10, 30, 60 and 1,020 minutes, and the percentage adhesion was calculated at each sampling time as described for the adhesion to artificial surfaces.

(b) Phase distribution

The test determines the distribution ratio of cells between water and an organic phase. The organic phase was toluene. Spore suspensions were prepared at pH 3, 6 and 9 using Tris buffer (0.1 M). The spore concentration was such that the suspension had an absorbance of 0.6 at 600 nm. Four ml of the cell suspension were taken into a 25 ml glass universal bottle and an equivalent volume of toluene was added. After vigorous vortexing for 10 s the bottle was left to rest for 30 min during which the two phases separated completely. The absorbance of the aqueous phase was measured. A blank (buffer without cells mixed with toluene in the same manner) was also measured. Results are expressed as the proportion of the cells which were excluded from the aqueous phase (Mozes and Rouxhet, 1987):

$$100 \times (A_i - A_f)/A_i$$

where A_i and A_f are initial and net final optical densities of the aqueous phase.

Statistical analysis: Data on percentage adhesion were subjected to analysis of variance. Hydrophobicity indices from the phase distribution assay were transformed

by arcsin(sqrt(x/100)) before analysis of variance. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.5.3. Scanning electron microscopy of aerial and submerged spores

Agar blocks (0.5 cm diameter) were cut from the margins of PDA colonies. These blocks and pellets bearing submerged conidia (from culture in medium A with 1.5 KNO_3 g l^{-1}, as explained in Section 2.4.2.) were treated for observation under scanning electron microscope as follows:

The samples were fixed in a solution containing ethanol:glacial acetic acid: formaldehyde:water (50:10:35:5 % v:v) for 24 h. Dehydration was carried out in an ethanol series (70-80-90-100, 1.5 h each), followed by 2 h in acetone. Samples were dried through the critical point and coated with gold for observation (García-Arenal and M-Sagasta, 1980).

2.5.4. Viability of *Penicillium oxalicum* spores after storage

Aerial spores and submerged spores were tested. Aerial spores were gently dislodged from Petri plates in sterile water. Spores from submerged culture were obtained by filtering the flask cultures through two layers of cheese cloth. The resulting suspensions (about $5x10^7$ spores ml^{-1}) were dispensed into 1 ml eppendorf tubes, which were centrifuged at 4000 rpm for 15 min. The supernatant was discarded and the spores were either stored as wet spores or freeze-dried. The spores were stored at two temperatures: 4 and 25°C. Therefore the treatments were:

1. Wet spores at 4°C
2. Wet spores at 25°C

3. Freeze-dried spores at 4°C

4. Freeze-dried spores at 25°C

Sampling was done after 0, 4, 8, 12, 16, 21 and 27 weeks. At each sampling date three replicate tubes were taken per treatment, 1 ml sterile water was added to each tube and spore concentration was adjusted to about $1x10^7$ spores/ml. Suspensions were plated onto PDA Petri plates (100 µl per plate) and two replicate plates were used per sample. Plates were incubated at 25°C for about 16 h for wet spores and 20 h for freeze-dried spores. After incubation, plates were covered with lactophenol cotton blue. The number of germinated spores out of 50 were counted in three different sectors in each plate and percentage germination was calculated.

Statistical analysis: Data were subjected to analysis of variance. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.6. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum*

2.6.1. Minimum concentration of aerial conidia for disease control in growth chamber studies

The system used was as described by De Cal *et al* (1997b). Tomato seeds cv. Lorena were used. Tomato seeds were sown in trays containing an autoclaved (1 h at 1 kg cm^2 and 120°C) mixture of vermiculite:peat (1:1, v:v) and maintained in a chamber at 22-28°C with fluorescent light (100µE m^{-2} s^{-1}, 16 h photoperiod) and 80-100 % relative humidity for about three weeks, when seedlings had 2-4 true leaves.

Tomato seedlings were then transplanted into 100 ml flasks containing 125 ml sterile Hoagland nº 2 solution (Hoagland and Arnon, 1950). Seedlings were held by a plastic screen attached to the flask neck. Tomato seedlings in seedbeds were treated 7 days before transplanting, by watering with spore suspensions of *P.oxalicum* (60 ml were applied per litre substrate). These suspensions were prepared in water from PDAP plates at the following concentrations: $1x10^8$, 10^7, 10^6, 10^5 and 10^4 spores ml^{-1}, giving concentrations of $6x10^6$, 10^5, 10^4, 10^3 and 10^2 spores ml^{-1} substratum respectively.

Flasks were inoculated with a microconidial suspension of *Fusarium oxysporum* f. sp. *lycopersici* in water just before transplanting. The final concentration of the pathogen in the flasks was 10^4 microconidia ml^{-1}. This suspension was obtained as follows: 250 ml flasks containing 150 ml of Czapek Dox Broth were inoculated with three agar blocks (1 cm diameter) from the actively growing margins of 7-day-old PDA cultures. The flasks were incubated for 5 days at 25°C in a rotatory shaker at 150 rpm, and the culture was filtered through glass wool.

Five replicate flasks, each containing four plants, were used per treatment. The flasks were placed in a randomised block design in a growth chamber at 22-28°C with fluorescent light (100µE m^{-2} s^{-1}, 16 h photoperiod) and 80-100 % relative humidity for about three weeks. When any flask consumed the initial nutrient solution, fresh solution was added to every flask to give a final volume of 100 ml and the consumption of solution was recorded. Uninoculated plants always consumed the nutrient solution before the rest of plants, usually at 8 days after transplanting. This process was repeated each time that any flask consumed the nutrient solution, usually every 3-5 days. Disease induced by *Fusarium oxysporum* f.sp.*lycopersici* was

assessed by the disease index described by De Cal *et al.* (1995) each time that the flasks were replenished. The disease index was as follows:

1: healthy plant, all leaves were green

2: lower leaves yellow

3: lower leaves dead and some upper leaves yellow

4: upper leaves wilted, lower leaves dead

5: dead plant

Disease index was determined at 13, 18, 21 and 25 days after transplanting. The number of leaves was determined at the end of the experiment, and the consumption of nutrient solution (ml plant^{-1} day^{-1}) calculated for each treatment.

Populations of pathogen and antagonist in the rhizosphere of tomato were estimated at the end of the experiment. No estimation of populations inside the roots was made, because it is known that *P.oxalicum* does not penetrate the roots (De Cal, personal communication). Three plants per treatment were taken. Roots were weighed and placed in 250 ml flasks with 150 ml phosphate buffer (pH = 7). Flasks were shaken for 30 minutes at 150 rpm and 10 and 100-fold dilutions were made. Aliquots (100 ml) from undiluted and diluted suspensions were spread onto Petri plates containing selective media for antagonist and pathogen. Three Petri dishes were used per dilution. Plates were incubated in the dark at 25°C for 5 days before the colonies were counted. The number of colony forming units (cfu) g^{-1} fresh root weight was estimated for each plant. The experiment was carried out twice.

Statistical analysis: Data were subjected to analysis of variance. Population density data were subjected to Ln transformation. When significant differences were found, means were compared by least Significant Difference (LSD) test ($P=0.05$).

2.6.2. Efficacy of aerial and submerged spores in growth chamber studies

The system used was as explained in Section 2.6.1. Tomato seeds cv. Lorena and Precodor were used. Treatments applied were:

1: Conidial suspension of *P.oxalicum* from PDAP cultures in sterile water

2: Conidial suspension of *P.oxalicum* from PDAP cultures in Morton´s medium (Medium A with 1.5 g KNO_3 l^{-1} and 20mM Ca^{2+}, see Section 2.4.2)

3: Conidial suspension of *P.oxalicum* from submerged culture in sterile water. Cultures were filtered through cheesecloth to remove pellets, centrifuged (10000 rpm, 20 min) and spores resuspended in water

4: Mycelium from submerged cultivation: to obtain this mycelium 250 ml flasks with 150 ml PDB were inoculated with 1ml of a spore suspension ($1x10^7$ spores ml^{-1}) from 7-d-old PDA cultures. Flasks were incubated at 25°C in a shaker at 150 rpm. Then the pellets were separated and homogenised with 150 ml water in a blender for 15 s.

5: Liquid culture from submerged cultivation: Submerged cultures of *P.oxalicum* were centrifuged and the supernatant was applied (60 ml l^{-1} substratum).

6: *P.oxalicum* untreated seedlings

Treatment 4 was only applied to cv. Lorena. Concentration of spore suspensions was approx. $1x10^8$ spores ml^{-1} ($6x10^6$ spores ml^{-1} substratum) with the exception of treatment 3 to cv. Precodor, which was applied at $1x10^6$ spores ml^{-1} ($6x10^4$ spores ml^{-1} substratum). Concentration of the mycelial suspension was not determined. Plants

inoculated and uninoculated with *Fusarium oxysporum* f.sp. *lycopersici* were included for each treatment. Inoculation was as described in 2.6.1.

At the end of the experiment populations of pathogen and antagonist were estimated as described in 2.6.1. Percentage disease severity and consumption of nutrient solution (ml plant^{-1} day^{-1}) were determined for each treatment. Percentage disease severity was calculated by comparison with untreated and uninoculated plants.

Statistical analysis: Data were subjected to analysis of variance. Population density data were subjected to Log_{10} transformation. Percentage disease severity was converted to Ln (1/(1-x)) when necessary to perform analysis of variance. When significant differences were found, means were compared by least Significant Difference (LSD) test (P=0.05).

2.6.3. Efficacy of *Penicillium oxalicum* in greenhouse experiments

(a) Plant material

Seeds of tomato cv. Lorena were sown in an autoclaved mixture of vermiculite:peat (1:1 v:v) in trays and maintained in a culture chamber at 22-28°C and 80-100 % humidity with a 16-h light period for three weeks. The trays were covered with aluminium foil for the first 3-4 days to favour germination, and watered regularly. The trays were maintained in the culture chamber for two weeks and then in a greenhouse for one week at 20-30°C before transplanting, for the plants to get adapted to greenhouse conditions. Seedlings (2-4 true leaf stage) were then transplanted into pots (20x20x9 cm, five plants per pot) containing sterile peat (autoclaved for 1 h at 1 kg cm^{-2} and 120°C) and kept in the greenhouse. Peat was obtained from Gebr. BRILL substrate GmbH&Co. KG, Germany.

(b) Inoculation with *Fusarium oxysporum* f.sp. *lycopersici*

After transplanting seedlings were inoculated by applying 10 ml of a suspension of microconidia of the pathogen to the radicular system. This suspension was obtained as explained in Section 2.6.1 and adjusted to 10^6 conidia ml^{-1} with Czapek Dox Broth. The final concentration was $1x10^4$ conidia ml^{-1} peat.

(c) Disease assessment and estimation of populations of *Fusarium oxysporum* f.sp. *lycopersici* and *Penicillium oxalicum*

Disease development and severity was assessed using the following parameters:

(i) Disease index as explained in Section 2.6.1.

This parameter was measured at 12, 24, 36, 50 and 64 days after inoculation with the pathogen.

(ii) Number of plants from which the pathogen was isolated. It was determined for wilted plants on each date of evaluation and for the rest of the plants at the end of the experiment. The plants were incubated in a humid chamber for three days and the presence/absence of the pathogen was recorded.

(iii) Reduction in plant growth. It was determined at the end of the experiment by recording weights of roots and aerial part of plants, and number of leaves.

Populations of pathogen and antagonist in the rhizosphere of tomato were estimated one day after transplanting and at the end of the experiment (64 days after transplanting) as described in Section 2.6.1.

(d) Treatments applied

Seedlings were treated with *P.oxalicum* one week before transplanting. The treatments consisted of conidia of the antagonist suspended in sterile water + 0.1 % Tween 80. Seedlings were watered with the suspensions (60 ml per litre substrate). The antagonist was produced on solid substrate (maize grits) as explained below, or in submerged culture, at different values of water activity, giving the following treatments:

- Treatment 1: Solid substrate a_w= 0.98
- Treatment 2: Solid substrate a_w= 0.95
- Treatment 3: Liquid medium a_w=0.998
- Treatment 4: Liquid medium a_w=0.98
- Treatment 5: Liquid medium a_w=0.95

The concentration of the spore suspensions in treatments 1-5 was 10^6 spores ml^{-1}, giving a final concentration of $6x10^4$ spores ml^{-1} substratum.

- Treatment 6: Control treatment 1: Spores from PDA at 10^6 spores ml^{-1} ($6x10^4$ spores ml^{-1} substratum).
- Treatment 7: Control treatment 2: Spores from PDA at 10^8 spores ml^{-1} ($6x10^6$ spores ml^{-1} substratum). This treatment reduces the disease till a 50 % level (De Cal *et al.*, 1995).
- Treatment 8: Control treatment 3: Plants inoculated with the pathogen but untreated with the antagonist.
- Treatment 9: Control treatment 4: Plants uninoculated and untreated.

For the production of conidia from solid substrates, 400 g of maize grits were placed in beakers and water content was adjusted prior to sterilisation to obtain a_w values of 0.998, 0.98 and 0.95, according to the water adsorption curve. Water content was adjusted again after sterilisation. Beakers were placed in sterile hermetic containers

together with glycerol solutions of the corresponding water activities and left to equilibrate overnight at 4°C. Each beaker was then inoculated with 0.8 ml of a spore suspension of *P.oxalicum* in sterile water + 0.1 % Tween 80 containing $1x10^8$ spores ml^{-1}, from 7-day old PSA cultures; a sterile spatula was used to get an even inoculation of the maize grits. The containers were incubated for 9 days in a culture chamber at 25°C and the beakers were shaken regularly to obtain a homogeneous culture. After the incubation period the contents of the beakers were placed in 250 ml flasks with sterile water + 0.1 % Tween 80 and shaken to dislodge conidia. The suspension was filtered through glass wool and adjusted to 10^6 spores ml^{-1}.

For the production of submerged conidia from liquid media, 2l flasks containing 500 ml of medium A (see Morton's method, Section 2.4.2) were inoculated with 1 ml of a spore suspension of *P.oxalicum* in water + Tween 80 from 7-day old PSA plates containing 10^8 spores ml^{-1}. Flasks were incubated at 25°C in a rotary shaker at 150 rpm for 24 h and then the biomass was transferred to flasks containing 500 ml of medium B (see Morton's method, Section 2.4.2) amended with Ca_2Cl and modified with glycerol to obtain a_w values of 0.998 (unmodified), 0.98 and 0.95. After 9 days incubation in the same conditions, spores were extracted as described in Section 2.4.2. The suspension was centrifuged, the supernatant discarded and the spores were resuspended in sterile water + 0.1 % Tween 80 to obtain 10^6 spores ml^{-1}.

Conidia from 7-day old PDA cultures were gently dislodged in sterile water + Tween 80 and the suspension was filtered though glass wool and adjusted to 10^8 or 10^6 spores ml^{-1}.

Each treatment was applied to 40 plants, corresponding to 8 pots. Pots were placed in a randomised block design.

Statistical analysis: Data of colony forming units were transformed by Ln. All data were subjected to analysis of variance. When significant differences were observed, means were compared by least significant difference (LSD) testing at $P = 0.05$.

2.6.4. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion

(a) Plant material

Tomato seeds cv. Lorena and Precodor were used. Seeds were surface sterilised by immersion in a 0.5 % sodium hypochlorite solution for 30 min. Seeds were then washed twice with distilled sterile water.

(b) Formulation of seeds with aerial spores

A spore suspension *P.oxalicum* was prepared in water from PDAP plates at 1×10^8 spores ml^{-1}. Seeds were formulated with *P.oxalicum* using different methods, giving the following treatments:

1. Seeds formulated with sodium alginate: A 3 % sodium alginate (BDH) solution was prepared in distilled water. The solution was stirred overnight at room temperature and was autoclaved. Spore suspension and sodium alginate solution were mixed, giving a concentration of 2.4 % alginate. Tomato seeds coated with alginate containing *P.oxalicum* were prepared by putting the seeds into the sodium alginate/cell suspension for 1 h prior to transfer into 0.1 M calcium chloride solution.

Seeds were left in the calcium chloride solution for 30 min for gel strengthening and were washed twice in sterile distilled water.

2. Seeds formulated with alginate and nutrients: The method was as in 1, but the sodium alginate solution was made in Morton`s medium with 1.5 g KNO_3 l^{-1} (see Section 2.4.2)

3. Seeds formulated with methyl cellulose: A 2 % methyl cellulose (Sigma) solution was prepared in distilled water and autoclaved. Spore suspension and methyl cellulose were mixed (1:1). Tomato seeds coated with methyl cellulose containing *P.oxalicum* were prepared by putting the seeds into the methyl cellulose/cell suspension for 30 min.

4. Seeds formulated with methyl cellulose and nutrients: the method was as in 3, but the methyl cellulose solution was prepared in Morton´s medium

5. Control treatment 1: Seeds were not formulated, but only immersed in the spore suspension for 30 min (seed bath).

6. Control treatment 2: Untreated seeds

Seeds coated with either sodium alginate or methyl cellulose were left drying overnight in the flow cabinet.

(c) Effect of *P.oxalicum* formulated with seeds on plant growth

After drying in the flow cabinet seeds were placed in either water agar or Hoagland agar Petri dishes, which were sealed with parafilm. Four seeds were placed per Petri plate, and three replicate plates were used per treatment and tomato cultivar. Petri plates were incubated in a chamber at 22-28°C with fluorescent light (100μE m^{-2} s^{-1}, 16 h photoperiod). Plates were evaluated every 2-3 days and the following

parameters were recorded: 1) germination, 2) presence of cotyledons, 3) presence of leaves. At the end of the experiment fresh weight of plants was recorded.

(d) *P.oxalicum* populations in formulated seeds

Number of colony forming units (CFU) per seed was determined for formulated seeds before and after drying. For seeds immersed in the spore suspension CFU per seed was also determined. Seeds formulated with alginate were immersed in 5 ml, 1 % sodium citrate, pH 6.5 for 1 h to dissolve the alginate gel. Seeds formulated with methyl cellulose or treated by immersion in the spore suspension were immersed in 5 ml water. Seeds were then vortexed for 1 min, the suspension diluted and plated on PDA Petri plates. Dilutions were 10^{-1} and 10^{-2} for treatments 1,2,3 and 4 and 10^{-2} and 10^{-3} for treatment 5.

2.7. PRODUCTION OF *Epicoccum nigrum* AND *Penicillium frequentans*

2.7.1. Production in culture media

E.nigrum and *P.frequentans* were produced in culture media at standard and reduced a_w. For the production at standard a_w (0.996) PDA plates were inoculated with the antagonists and incubated at 25°C in the darkness for 7 days. Agar plugs from the actively growing margins of these colonies were used to inoculate PDB flasks (250 ml flasks containing 150 ml PDB, three plugs per flask). For *E.nigrum* PDA plates were also inoculated with the 7-d-old PDA cultures. *E.nigrum* spores were produced mainly from plates, whereas mycelium was produced for the most part from flask cultures. Plates and flasks were incubated at 25°C in the darkness for 10 days. Flasks were not shaken.

For the production at reduced a_w the same method was used but media were PDA98 and PDB98 for *E.nigrum* and PDA95 and PDB95 for *P.frequentans*. Cultures produced at 0.996 a_w will be referred to as *E.nigrum*996 and *P.frequentans*996. Cultures produced at reduced a_w will be referred to as *E.nigrum*98 and *P.frequentans*95.

2.7.2. Production of *Epicoccum nigrum* by solid substrate fermentation

(a) Solid substrates

Maize grits and wheat grains (cv. Rendeveau and Brigadier) were used as solid substrates. The sorption curves of these substrates were constructed by psychrometry, using a Wescor HT33 psycrometer as explained in Section 2.5.1.

(b) Solid fermentation conditions

Samples of 20 g of the substrates were placed in plastic bags especially designed for solid fermentation studies (Cuero *et al.*, 1985) and sterilised by autoclaving at 1 kg cm^{-2} and 120°C for 1 h. The water content of the solid substrates was then adjusted with sterile water to different values of water activity, according to the adsorption curves. The loss/gain of water during autoclaving was taken into account when adjusting the water content. Bags were placed in hermetic plastic containers together with sterile glycerol solutions of the equivalent water activity to guarantee the maintenance of the water activity of the solid substrates, and left to equilibrate overnight at 4°C. Bags were then inoculated with 1 ml of a spore suspension of *E.nigrum* ($0.5x10^6$ spores/ml), prepared by dislodging the spores from 7-d-old PDA Petri dishes in water. The water added with the inoculum was also taken into account when adjusting the water content of the substrates. The plastic containers were placed in an incubator at 25°C.

For maize grits and wheat grains cv. Rendeveau the a_w values tested were 0.996, 0.98 and 0.96, and water was used to adjust the water content. For wheat grains cv. Brigadier the a_w values used were 0.996 and 0.98. Water was used to adjust the water content to 0.996 a_w. To get 0.98 a_w two different methods were used: a) water was added as in the case of 0.996 a_w, and b) a mixture of water:glycerol (0.99 a_w) was added at a ratio 3ml/10g wheat grain.

(c) Spore extraction

Spore production was quantified only for the wheat substrates, as the fungus did not sporulate on maize grits. Samples were taken at 7, 14, 18 and 23 days after inoculation for cv. Rendeveau and 7, 14, 21 and 28 days for cv. Brigadier. Three bags were taken per water activity level at each sampling time. The contents of the bags was placed into 250 ml flasks and 50 ml water + 0.05 % Tween 80 was added. Flasks were placed in an orbital shaker at 350 rpm for 30 min, then the liquid was filtered through cheese cloth. Water was added again and the process was repeated until the grain appeared clean of spores. Spore concentration of the suspensions obtained was quantified using an haematocytometer and spore yield (no. spores g^{-1} fresh substrate) was calculated.

2.8. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans*

2.8.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions

*E.nigrum*996 and *E.nigrum*98 spores were gently dislodged in water or in a 0.935 a_w solution (1:1 mixture of a 1.25 m PEG200 solution and a 1 m PEG300 solution).

Spore suspensions were adjusted to $1x10^6$ spores ml^{-1} and 0.1 ml of each suspension was plated on WA or WA935. Plates were incubated at 25°C in the darkness for 4 and 6 h (WA plates) and for 8, 12 and 23 h (WA935 plates). At each sampling time three replicate plates per spore type were taken and covered with lactophenol cotton blue. The number of germinated spores out of 50 were counted in three different sectors in each plate and percentage germination was calculated. Germ tube length was determined for 25 spores in each of three sectors of every plate.

Statistical analysis: Data were subjected to analysis of variance. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.8.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w

E. nigrum spores were produced at 0.996 and 0.98 a_w, as explained in Section 2.7.1. Growth rates of the two types of inoculum were determined at 10, 17, 25 and 37°C. Culture media used were PDA, PDA99, PDA984, PDA95, 1/10PDA, 1/10PDA99, 1/10PDA984, 1/10PDA95, MM, MM99, MM984 and MM95.

Plates were centrally inoculated with 0.5 cm diameter disks from the actively growing margins of *E. nigrum* colonies grown at 0.996 or 0.98 a_w. Plates were incubated in the dark and colony diameter was measured every 2-3 days. Two perpendicular diameters were measured per plate and five replicate plates were used per treatment. Growth rates were determined using regression analysis at the lineal growth phase.

Statistical analysis: Growth rate data were subjected to analysis of variance. When significant differences were found means were compared by an LSD test (P=0.05).

2.8.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans*

(a) Equipment

Solutes were analysed and quantified by high performance liquid chromatography (HPLC) with a Gilson equipment. The column used was a Hamilton HC-75 Ca^{2+} Form column, specifically for the separation of sugars/polyols; using a Refraction Index detector. The mobile phase was acetonitrile:water (40:60). The maximum pressure was 1 psi and the flow rate 1 ml min^{-1}. The solutes were quantified with Gilson software adapted to the HPLC equipment. Peak areas were integrated by the software and amounts were obtained according to calibration curves.

(b) Calibration curves

To construct calibration curves, standard solutions were prepared by dissolving known amounts of trehalose, glucose, glycerol, erythritol, arabitol and mannitol in the mobile phase. The concentrations used were 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg ml^{-1}.

(c) Types of cultures analysed

1: *E.nigrum* and *P.frequentans* produced in culture media: *E.nigrum* (996 and 98) and *P.frequentans* (996 and 95) (see Section 2.7.1.) were analysed. PDB, PDB98 and PDB95 cultures were decanted to separate out the mycelium and biomass was washed with Analar water to eliminate glycerol coming from culture medium. Mycelium and spores of *P.frequentans* were collected from flask cultures. For

E.nigrum mycelium was mainly produced in flask cultures and spores from Petri dishes; they were analysed separately. Spores of *E.nigrum* were gently dislodged in Analar water. All the material was then centrifuged (15 min at 4000rpm), the supernatant discarded and the pellets freeze-dried overnight and kept at room temperature till use. Three replicate flasks were used for each fungus. For *E.nigrum* 12 Petri dishes were used, and the spores from four replicate plates were combined for analysis.

2: *E.nigrum* and *P.frequentans* produced in culture media and homogenised: The protocol was as described in 1, but the biomass from flask cultures was homogenised after washing (in a commercial blender for 30 s) and filtered through two layers of cheesecloth. Spores of *E.nigrum* from PDA and PDA98 cultures were gently dislodged in Analar water and added to the homogenate before filtration. To prepare the samples for quantification the filtrate was centrifuged, the supernatant discarded and the biomass freeze-dried overnight and kept at room temperature till used. Three replicate flasks were used for each fungus. For *E.nigrum* the spores from four Petri dishes were mixed with the contents of each replicate flask (this proportion is used when applying the fungus in the field trial (Section 2.9.2.).

3: Time-course study of solute accumulation in *E.nigrum* and *P.frequentans* produced in culture media: The general protocol was as described in 1. Three replicate flasks and 12 Petri dishes (for *E.nigrum*) were used for analysis at 5, 10, 15 and 20 days after incubation.

4: *E.nigrum* spores produced in solid substrates: Spores were produced as explained in Section 2.7.2. The spore suspensions obtained were centrifuged (20 min at 10000 rpm followed by 15 min at 4000 rpm) and the pellets freeze dried.

5: Concentration of solutes was also determined in the washings from the extraction of spores of *E.nigrum* produced by solid fermentation.

(d) Sample preparation

Sample preparation was done according to the protocol developed by Hallsworth and Magan (1997) for optimum extraction of polyols and trehalose. Samples of twenty mg of freeze-dried material were mixed with 1ml Analar water in a 2 ml eppendorf tube and sonicated for 2 minutes using a Soniprep 150 (Fisons), at 28 μm amplitude. After immersion in a boiling water bath for 5.5 min, the samples were allowed to cool and 667 μl acetonitrile were added to each sample to get the same proportion of acetonitrile:water as in the mobile phase. The tubes were left overnight at 4°C and then centrifuged for 10 min at 13000 rpm. The supernatant was filtered through 0.2 μm filters and injected onto the HPLC for quantification of solutes. Amounts in ppm were obtained from the calibration curves and transformed to mg g^{-1} freeze dried biomass.

Statistical analysis: Data were subjected to analysis of variance. When significant differences were observed means were compared by least significant difference (LSD) testing at $P = 0.05$.

2.8.4. Determination of turgor pressure in *Epicoccum nigrum* mycelium

E.nigrum mycelium was produced in flask cultures at 0.996 and 0.98 a_w as explained in Section 2.7.1. Turgor pressure was determined by thermocouple psychrometry using a Wescor HT33 psychrometer.

A small portion of mycelium was cut from the edge of the colony, washed once with deionised water and the surface water removed using a tissue. The mycelium was placed in a chamber and the total water potential of the mycelium (Ψ) was determined. The chamber containing the mycelium was then wrapped with aluminium foil and immersed in liquid nitrogen for 1 min. After coming to room temperature the solute water potential or osmotic potential of the cytoplasm (Ψ_Π) was then measured from the equation

$$\Psi = \Psi_\Pi + \Psi_p$$ (Thompson *et al.*, 1985)

where Ψ is the water potential, Ψ_Π the solute potential and Ψ_p the turgor potential. The latter is reduced to zero after freezing, due to rupturing of the hyphae as a result of ice-formation in the cell. Consequently after freeze thawing in liquid nitrogen, the Ψ which is determined is equal to Ψ_Π since Ψ_p is zero. For the intact hyphae, Ψ and Ψ_Π can be inserted in the above equation and Ψ_p calculated.

Three replicate flask cultures were used per water activity and three measurements were taken per replicate culture. The experiment was carried out twice.

2.8.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa*

The experiment was carried out in Biolog (GN MicroPlates) plates to evaluate the number of carbon sources utilised by *E.nigrum*996, *E.nigrum*98 and *M.laxa*. *M.laxa* was grown for 7 days in PDAP Petri plates. Spores of each fungus were suspended in sterile water or sterile water modified to 0.985 a_w (0.75 m PEG200 solution). Spore suspensions were washed by centrifugation (15 min at 4000 rpm) three times, adjusted to $1x10^6$ spores ml^{-1}, and added to each of the test wells (10 μl per well). The BIOLOG plates were incubated in the darkness at 25°C for two weeks or four weeks (0.985 a_w) prior to examination for utilisation of carbon sources.

The Niche Overlap Index (NOI) was determined by dividing the number of carbon sources in common between *M.laxa* and *E.nigrum* (produced at standard or reduced a_w) by the total number of carbon sources utilised by each one (Wilson and Lindow, 1994). The NOI ratios were compared for the two a_w values tested.

2.8.6. Viability of *Epicoccum nigrum* spores after storage

*E.nigrum*996 and *E.nigrum* 98 spores were tested. Spores were gently dislodged from Petri plates in water and the suspension dispensed into 1 ml eppendorf tubes. The rest of the protocol was as explained in Section 2.5.4 for *P.oxalicum* spores. The incubation times of PDA plates were approximately 8 h for wet spores and 20 h for freeze-dried spores.

2.9. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans*

2.9.1. Efficacy of *Epicoccum nigrum* as a post-harvest BCA for brown rot of fruit

The test was carried out using cherries as plant material. *E.nigrum*996 and *E.nigrum*98 spores were tested. Spores were gently dislodged from Petri plates in sterile distilled water and the suspensions adjusted to about $1x16^6$ spores ml^{-1}. Cherries were surface sterilised as described by Sauer and Burroghs (1986) by immersion in 0.5 % sodium hypochloride for 5 min, followed by immersion in 70 % ethanol for 1 min, followed by two washes with sterile distilled water. After drying in the flow cabinet, a 20 μm drop of the spore suspension of the antagonist was placed on each fruit, at the insertion point of the peduncle, left to dry and a 20 μl drop of the pathogen (*Monilinia laxa*) applied on it. The *M.laxa* spore suspension was obtained from 7-d-old PDAP cultures by gently dislodging the spores in Czapek Dox Broth. The suspension was adjusted to $1x10^5$ spores ml^{-1}. The drops were left to dry and the fruits were placed in sterile Petri plates (5 fruits per plate) without a lid. Cherries inoculated with the pathogen and untreated were used as controls.

Biocontrol activity was tested at three relative humidities: 33, 75 and 100 %. Control of relative humidity was achieved by using hermetic containers with sterile water (100 %), a saturated NaCl solution (75 %) or a saturated $MgCl_26H_2O$ (33 %) (Winston and Bates, 1960). Six replicate plates were used per treatment. Plates were randomly placed in a rack and racks placed in the hermetic containers, which were incubated at 25°C. Cherries were assessed at 3, 7 and 10 days after the experiment was set up.

The percentage of fruit covered by *M.laxa* mycelium was estimated as the disease percentage.

Statistical analysis: Percentage disease data were transformed by asin(sqrt(x/100)) and then subjected to analysis of variance. Means were compared by a Least Significant Difference test (P = 0.05) when significant effects were found.

2.9.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans*

(a) Inoculation with *Monilinia laxa*

Peach twigs were artificially inoculated with *M.laxa* to get an uniform infection. The inoculation method was as described by Melgarejo *et al.* (1986): A small incision (3-4 mm long, 1 mm wide, 0.5 mm deep approximately) was made in the bark, 2-3 cm from the base of the shoot. Inoculum of *M.laxa* was applied to the wound, the inoculum consisted of disks of 1 cm diameter from 7-day-old PDA cultures. To prevent desiccation of the inoculum it was protected by wrapping in moist cotton wool and aluminium foil. The inoculum was removed after five days, when infection had been successful.

(b) Disease assessment

Once twigs were infected with the pathogen disease development and severity were assessed during the trial using the following parameters:

(i) Arbitrary index rating the appearance of the twig as follows:

0: healthy twig

1: partially healthy twig, leaves slightly wilted

2: leaves yellow and wilted

3: twig slightly wilted, necrosis starts in leaves

4: twig wilted, leaves start to fall

5: completely wilted twig, without leaves

(ii) Length of the lesion induced by *M.laxa* in the shoots.

(iii) Extent of *M.laxa* colonisation on shoots. It was determined at the end of the experiment by cutting shoots into 5 cm pieces from the inoculation point to the end of the shoot. Pieces were surface sterilised as described by Sauer and Burroughs (1986) and plated on PDA amended with 0.5 g l^{-1} streptomycin. After incubation in the dark at 25°C for 7 days, the presence or absence of the pathogen in each piece was recorded. The 5-cm piece from which the pathogen was recovered and was the farthest away from the inoculation point indicated the extent of fungal colonisation in each shoot.

These parameters were measured at 7, 17, 26, 35 and 44 days after inoculation with the pathogen, except the extent of colonisation that was determined at the end of the experiment. The experiment was concluded when growth of shoots and pathogen stopped.

(c) Treatments applied in the field trial

Seven shoots of approximately the same diameter (0.5 cm) and length (10-15 cm) on each of six trees were exposed to each treatment.

Treatments consisted of the following preparations:

Treatment 1: conidia + mycelium of *E.nigrum*996 + nutrients 1: After incubation PDB996 was decanted to separate out the mycelium, which was homogenised with the nutrient solution (in a commercial blender for 30 s); conidia were gently dislodged from the PDA996 cultures in the nutrient solution and mixed with the homogenised mycelium. The fungal suspension was filtered through two layers of sterile cheesecloth and was adjusted to 10^6 conidia (plus mycelial fragments) per ml by adding nutrient solution. The nutrient solution consisted of lactose (20 g l^{-1}), KNO_3 (10 g l^{-1}) prepared in 0.06 % Nu-Film-17, a commercial preparation of 96 % di-menthane from Miller Chemical and Fertiliser Co., Hanover, PA, USA. This nutrient mix enhances the growth and sporulation of *E.nigrum* but not that of *M.laxa* (De Cal *et al.*, 1993).

Treatment 2: conidia + mycelium of *E.nigrum*98 + nutrients 1: The preparation was obtained as described in treatment 1, but with *E.nigrum*98 cultures.

Treatment 3: conidia + mycelium of *E.nigrum*996. The preparation was obtained as described in treatment 1, but in this case a 0.06 % Nu-Film-17 was used instead of the nutrient solution.

Treatment 4: conidia + mycelium of *E.nigrum*98: The preparation was obtained as in treatment 1, but with *E.nigrum*98 cultures and a 0.06 % Nu-Film-17 solution was used instead of the nutrient solution.

Treatment 5: conidia + mycelium of *P.frequentans*996 + nutrients 2. The preparation was obtained from *P.frequentans*996 flask cultures, which were homogenised and filtered as described for treatment 1. The fungal suspension was adjusted to 10^8 conidia (plus mycelial fragments) per ml by adding nutrient solution. In this case the nutrient solution consisted of malt extract (10 g l^{-1}) and yeast extract (3 g l^{-1}) prepared in 0.1 % Tween 80.

Treatment 6: conidia + mycelium of *P.frequentans*95 + nutrients 2. The preparation was obtained as in treatment 5, but with *P.frequentans*95 cultures.

Treatment 7: captan (1.3 g a.i. l^{-1}).

Treatment 8: untreated control.

Treatments were first applied the day before inoculation with *M.laxa*, and applications were then repeated four times at 7-day intervals. Treatments were applied by spraying to run-off, at sunset to favour the development of the antagonists. The orchard did not receive any other treatment during the experiment.

Statistical analysis: Results from each lesion length assessment and extent of colonisation were analysed independently by contrast with the F test at 0.05 significance level (Snedecor and Cochran, 1980).

CHAPTER 3

RESULTS

I: STUDIES ON *Penicillium oxalicum* FOR BIOCONTROL OF *Fusarium* WILT OF TOMATO IN THE RHIZOSPHERE

3.1. PRODUCTION OF *Penicillium oxalicum* INOCULUM: INDUCTION OF SUBMERGED CONIDIATION

3.1.1. Spore production in different media

Complex synthetic media and those based on by-products were not suitable to induce submerged sporulation of *P.oxalicum*. In all cases the fungus grew, but only formed mycelial pellets. This same behaviour was observed for synthetic media with the exception of Morton's media. However, by following Morton's method the fungus grew in medium "A" forming vegetative mycelial pellets, with conidiophores and conidia being observed 24 h after transference to nitrogen-free medium "B".

3.1.2. Studies using Morton's method

Time course study: Spore concentration and sporulation index increased from the second to the sixth day after transference to medium "B" as shown in Fig. 3.1. There was a significant increase in both sporulation index and spore concentration between days 3 and 6.

Effect of age of mycelium at transference: The difference in spore concentration between the two treatments (24 and 48 h growth in medium "A" prior to transference) was not significant. On the other hand the sporulation index was higher in the 24 h treatment (Fig. 3.1).

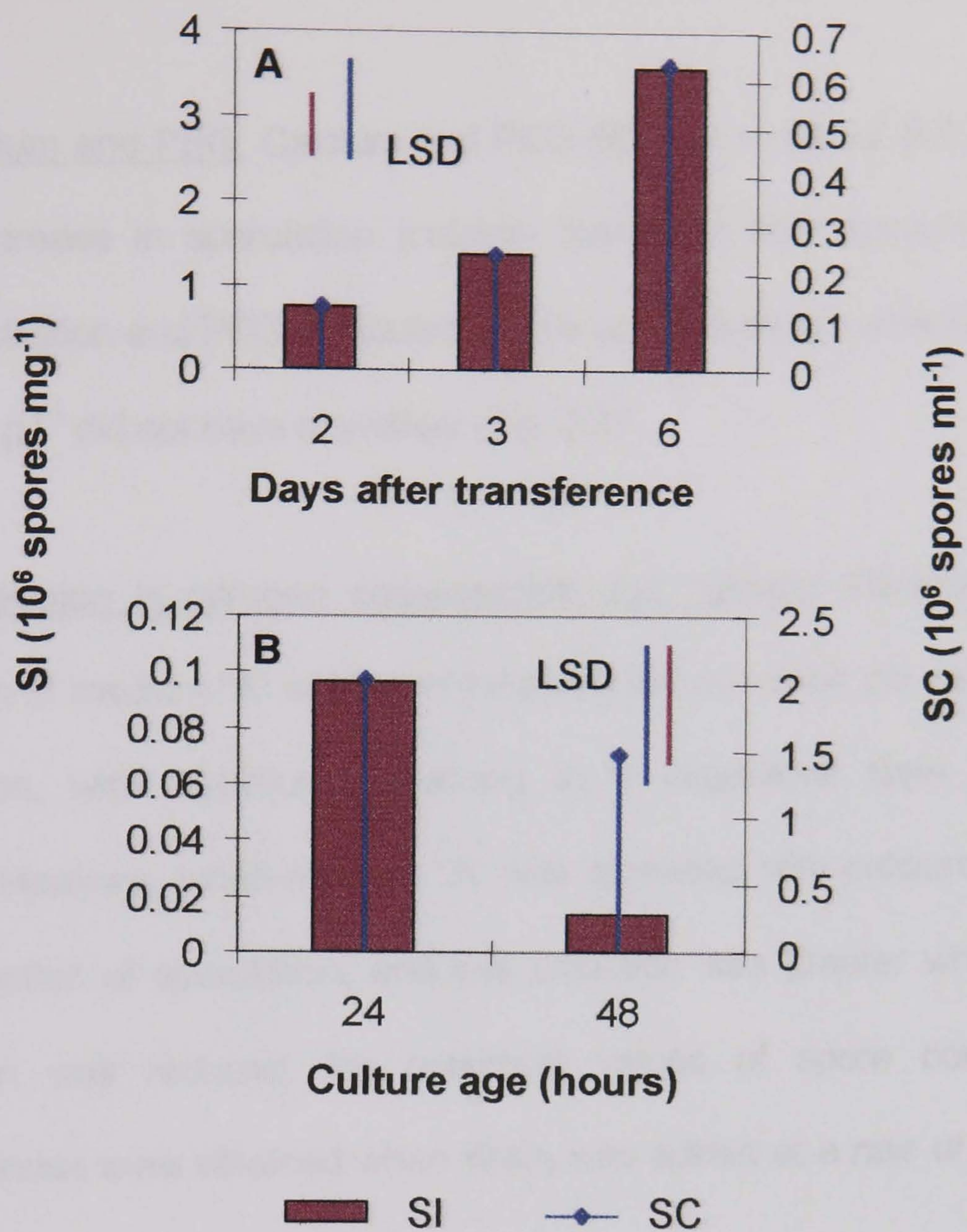


Figure 3.1. A: Time course development of sporulation of *P.oxalicum* in submerged culture. B: Effect of age of mycelium at transference on sporulation of *P.oxalicum* in submerged culture. Morton's method was used. Medium pH was 5. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration.

Effect of pH: Spore concentration was maximum at pH=6. However, there were no significant differences in the values of sporulation indices at pH range 4-8 (Fig. 3.2).

Effect of calcium and PEG: Calcium and PEG 6000 at a rate of 400 g l^{-1} induced a significant increase in sporulation (calcium increased both sporulation index and spore concentration and PEG increased spore concentration), whereas PEG 6000 at a rate of 200 g l^{-1} did not have any effect (Fig. 3.2).

Effect of reduction in nitrogen concentration and calcium: Reduction of nitrogen concentration in medium "A" of Morton's method did not result *per se* in an induction of sporulation, with mycelium remaining in a vegetative state throughout the experiment. However, when medium "A" was amended with calcium (20 mM) there was an induction of sporulation, and this induction was greater when the nitrogen concentration was reduced; the maximum values of spore concentration and sporulation index were obtained when KNO_3 was added at a rate of 1-1.5 g l^{-1} (Fig. 3.3).

3.1.3. Sporulation in modified Potato Dextrose Broth (PDB)

Sporulation of *P.oxalicum* was observed in PDB with modified C:N ratios, but in all cases the levels of sporulation were lower than in Morton's medium (C:N=93) (Table 3.1). Comparisons amongst the different PDB media indicated that the highest spore concentrations were obtained in undiluted PDB with a C:N ratio of 144, and when diluted 2-fold, and when modified to a C:N ratio of 93 and also diluted by 50%. When PDB was diluted 1:10, spore concentration was significantly lower, for both C:N ratios tested. The highest sporulation index was also observed in the case of Morton's medium modified to a C:N ratio of 93.

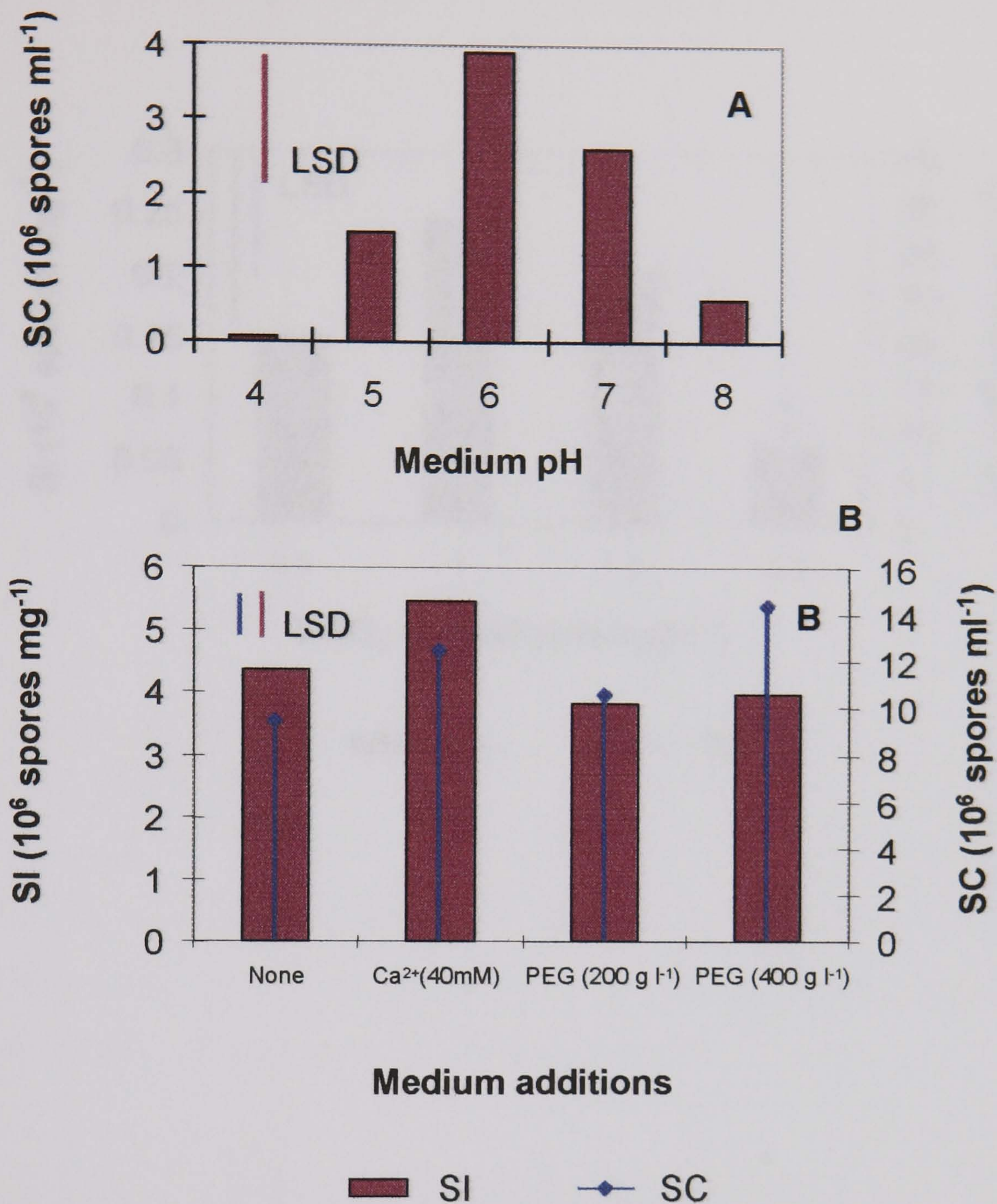


Figure 3.2. Effect of pH (A) and calcium or PEG addition (B) on sporulation of *P.oxalicum* in submerged culture. Morton's method was used. Medium pH was 5 in B. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration

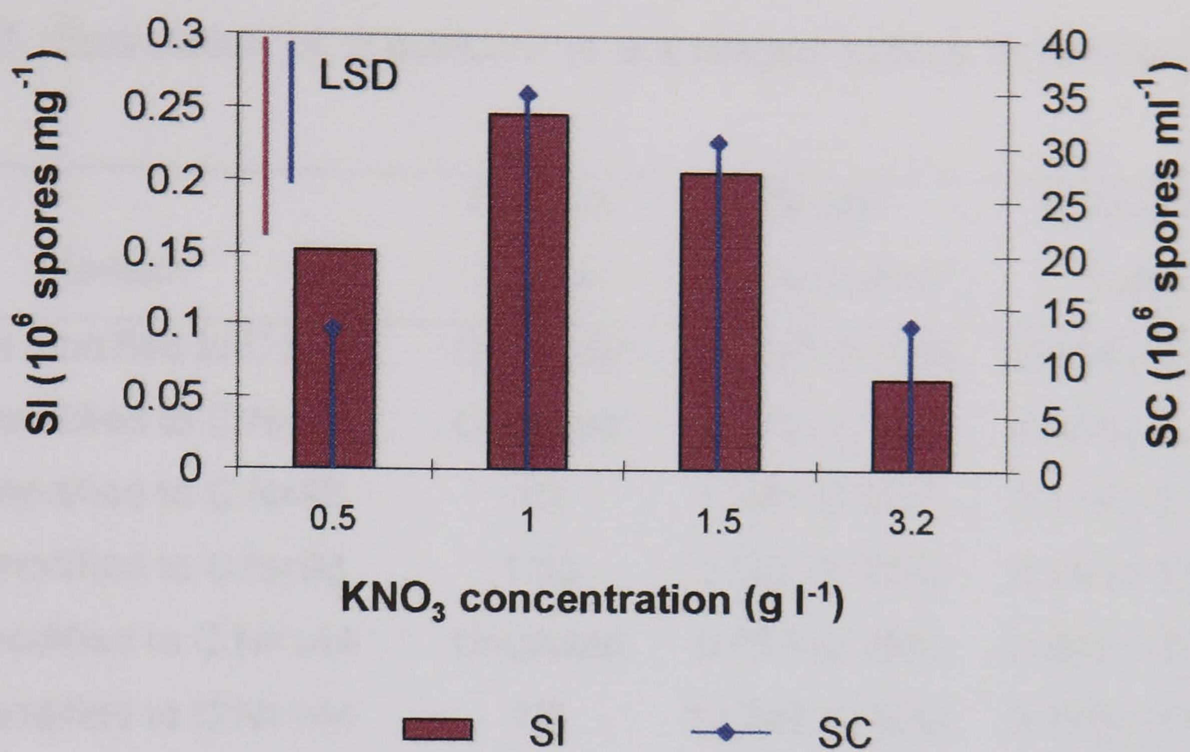


Figure 3.3. Effect of reduction in KNO_3 concentration in Morton's medium amended with Cl_2Ca (2.94 g l^{-1}) on sporulation of *P.oxalicum* in submerged culture. Data are means of three replicates. SI: Sporulation index. SC: Spore concentration.

For PDB media the highest sporulation indices were obtained when the medium was diluted 2 or 10-fold. The lowest sporulation indices were for undiluted PDB.

Table 3.1. Sporulation of *P.oxalicum* in submerged culture in different culture media [a]

Medium[b]	Medium dilution	Spore concentration[c]	Sporulation index[d]
Morton's modified to C:N=93	Undiluted	62.960 (3.919)	0.554 (-0.761)
PDB modified to C:N=93	Undiluted	6.170 (1.787)	0.038 (-3.313)
PDB modified to C:N=93	1/2	7.980 (2.067)	0.119 (-2.138)
PDB modified to C:N=93	1/10	2.027 (0.705)	0.140 (-1.967)
PDB modified to C:N=144	Undiluted	9.733 (2.256)	0.063 (-2.782)
PDB modified to C:N=144	1/2	12.345 (2.509)	0.184 (-1.699)
PDB modified to C:N=144	1/10	2.207 (0.775)	0.146 (-1.945)
LSD		(0.716)	(0.643)

[a] Data are means of three replicates. Data in brackets are transformed by Ln. LSD are for transformed data. [b] All media were amended with $CaCl_2$ (20 mM) and adjusted to pH=6. [c] 10^6 spores ml^{-1}. [d] 10^6 spores mg^{-1}.

3.2. CHARACTERISATION OF *Penicillium oxalicum* SPORES

3.2.1. Study on growth of *Penicillium oxalicum* inoculum in soil

(a) Soil characteristics

Soil adsorption curves were constructed for the relationship between water potential and moisture content and for water potential and the amounts of added water (Fig. 3.4).

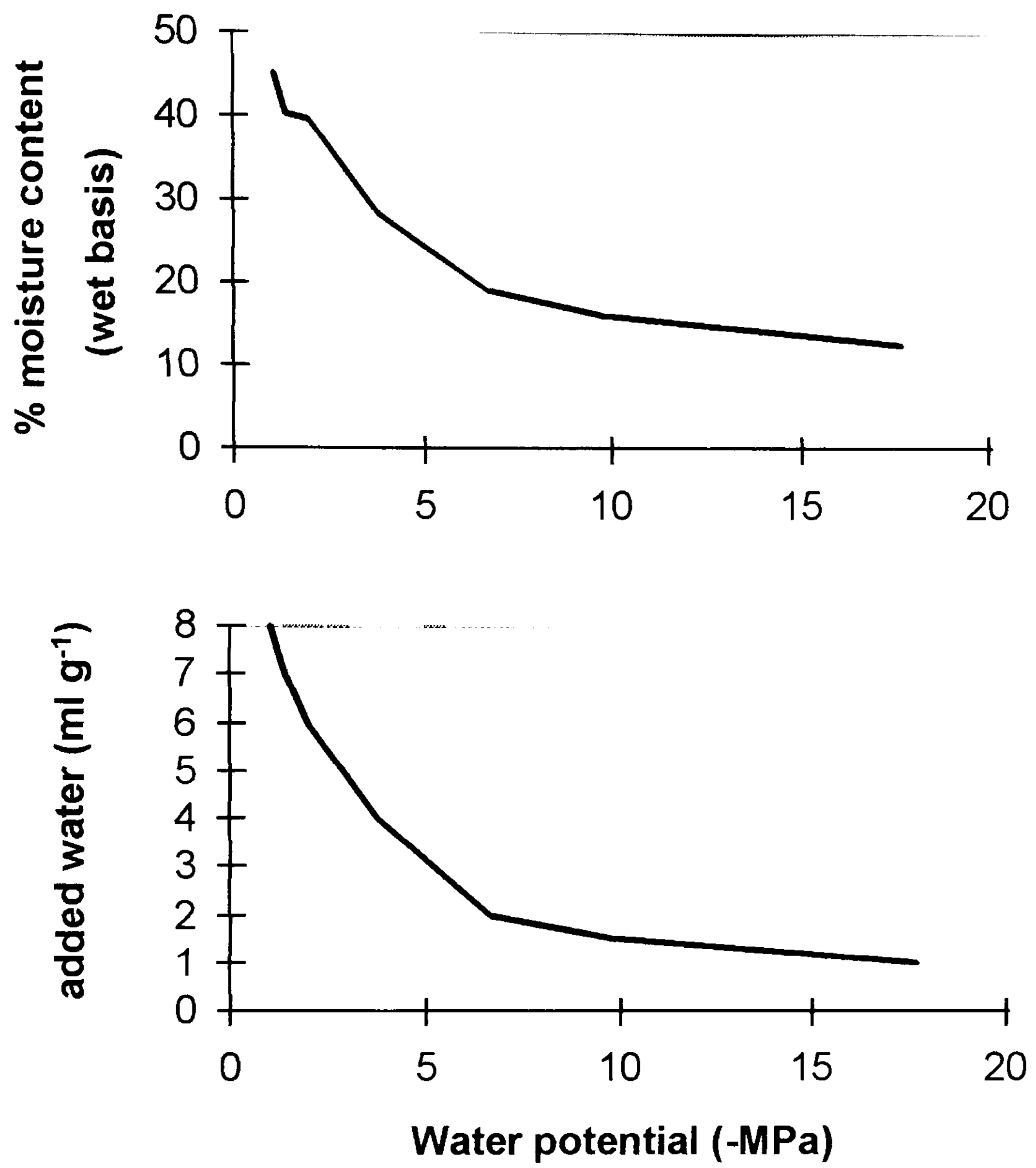


Figure 3.4. Soil adsorption curves at 25°C

(b) Quantification of populations of *P.oxalicum* in soil

Data of temporal changes in populations of *P.oxalicum* (colony forming units (cfu)) in soil are shown in Fig. 3.5. Evolution of *P.oxalicum* populations were affected by both soil water potential and nutrient addition (0.8 % mannose and 0.08 % arginine).

After 24 h incubation there were no differences between treatments. The effect of soil water potential was already observed at day 5 and this was maintained throughout the experiment: the lower the water potential the higher the cfu number. Populations showed a decreasing trend when water potential was -1.0 MPa and it increased slightly at -1.5 MPa. Increase in populations were higher for lower water potentials, until the 20-30th day and then the numbers isolated levelled off with no linear trend.

The effect of nutrients on populations of *P.oxalicum* was first observed 20 days after incubation: cfu values were higher in treatments where nutrients were added. At the end of the experiment this effect was equivalent to that obtained under the driest conditions tested (-7.0 MPa).

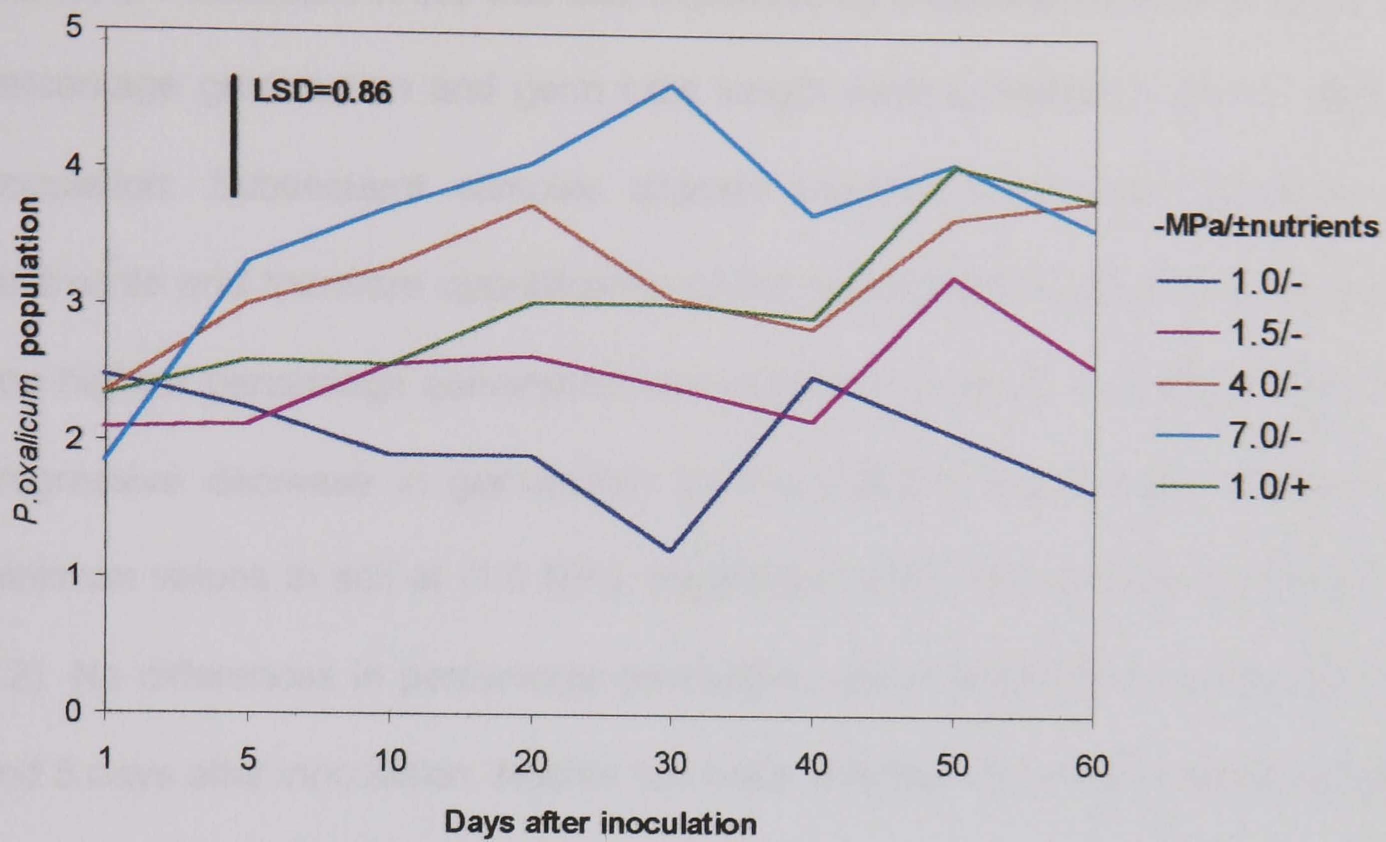


Figure 3.5. Effect of soil water potential (-MPa) and nutrient addition on evolution of *P.oxalicum* population (sqrt (10^6 cfu g^{-1} dry soil)) for 60 days. Data are means of three replicates.

(c) Microscopy study

Growth of *P.oxalicum* in soil was also monitored by a fluorescence microscopy study. Percentage germination and germ tube length were estimated 1 and 5 days after inoculation. Subsequent samples showed mycelial mats and fragments in all treatments and therefore quantification of the parameters studied was not possible. The highest percentage germination was obtained in soil at -7.0 MPa. There was a progressive decrease in germination as the water potential was increased, with minimum values in soil at -1.0 MPa, regardless of the presence of nutrients (Table 3.2). No differences in percentage germination were found when comparing data 1 and 5 days after inoculation. Neither soil water potential nor nutrient addition had any effect on germ tube length. On the other hand, germ tube length increased slightly from the first day (21μm) until the 5th day (28μm).

Table 3.2. Effect of soil water potential (Ψ_w) and nutrient addition on percentage germination of *Penicillium oxalicum* 5 days after inoculation [a].

	Ψ_w (-MPa)				
	1.0	1.5	4.0	7.0	LSD
Without nutrients	32.83	68.00	77.33	83.50	
With nutrients	45.67	-	-	-	15.31

[a] Data are means of three replicates. - not tested

3.2.2. Surface hydrophobicity and adhesion of aerial and submerged spores

(a) Adhesion tests

No remarkable changes in adhesion were observed when comparing treatments after 1 or 22 h incubation. In both cases the percentage adhesion to the hydrophobic surface was higher for submerged than for aerial spores produced on PDA, while

that for the hydrophilic surface was similar for both spore types. Aerial spores showed similar adhesion to hydrophobic and hydrophilic surfaces. However, submerged spores adhered better to the hydrophobic surface (Table 3.3).

Table 3.3. Percentage adhesion of aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces [a]

	Incubation time (h)			
	1		22	
Spore Type	Hydrophobic	Hydrophilic	Hydrophobic	Hydrophilic
Aerial	8.67	19.67	14.33	0.00
Submerged	35.67	19.67	38.00	6.33
LSD = 15.25				

[a] Data are means of three replicates

When the experiment was carried out including intermediate incubation times it was observed that adhesion of submerged spores to the hydrophobic surface occurred very quickly: after 10 min the percentage was 52.34 %, significantly higher than that for the hydrophilic surface and for aerial spores for any surface (Table 3.4). This percentage increased with time until 1 h incubation. Aerial spores adhered more to the hydrophilic than to the hydrophobic surface, after 30 min incubation. Submerged spores always showed more adhesion than aerial spores to the hydrophobic surface, and less to the hydrophilic surface.

Adhesion of germ tubes from aerial and submerged spores was not very different when compared 1 and 22 h after incubation (Table 3.5). In general, there were no differences between the two types of germ tubes. Both showed similar adhesion percentages, which were higher on the hydrophobic surface.

Table 3.4. Percentage adhesion of aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces [a]

		Incubation time (min)				
Spore Type	Surface	0	10	30	60	1,020
Aerial	Hydrophobic	0.00	2.63	9.36	18.13	7.60
	Hydrophilic	3.22	15.50	33.63	52.05	81.29
Submerged	Hydrophobic	9.61	52.34	80.40	100.00	82.17
	Hydrophilic	15.80	33.24	30.54	31.73	41.46
LSD = 16.46						

[a] Data are means of three replicates

Table 3.5. Percentage adhesion of germ tubes from aerial and submerged *P.oxalicum* spores to hydrophobic and hydrophilic surfaces [a]

	Incubation time (h)			
	1		22	
Spore Type	Hydrophobic	Hydrophilic	Hydrophobic	Hydrophilic
Aerial	67.00	19.33	71.33	22.00
Submerged	51.00	5.67	67.67	0.00
LSD = 20.35				

[a] Data are means of three replicates

Both aerial and submerged spores adhered slightly to tomato roots after 1,020 min incubation (Table 3.6). Percentage adhesion was slightly higher for submerged spores.

Table 3.6. Percentage adhesion of aerial and submerged *P.oxalicum* spores to tomato roots [a]

Spore Type	Incubation time (min)				
	0	10	30	60	1,020
Aerial	14.69	9.99	0.00	9.99	33.19
Submerged	15.86	11.75	19.68	13.80	45.23
LSD = 17.13					

[a] Data are means of three replicates

(b) Phase distribution assay

Hydrophobicity indices calculated using this method are presented in Table 3.7 . The values indicate that both spore types are highly hydrophobic, but aerial spores are slightly more hydrophobic than submerged ones. For aerial spores hydrophobicity indices decreased with increasing pH values. For submerged spores the index decreased and then increased, for increasing pH values.

Table 3.7. Hydrophobicity indices for aerial and submerged *P.oxalicum* spores, calculated by the phase distribution method [a]

Spore Type	PH		
	3	6	9
Aerial	96.56 (1.3842)	96.11 (1.3728)	94.45 (1.3330)
Submerged	91.70 (1.2785)	84.94 (1.1742)	88.59 (1.2264)
LSD for transformed data = 0.0447			

[a] Data are means of three replicates. Data in brackets are transformed by asin(sqrt(x/100)).

3.2.3. Scanning electron microscopy of aerial and submerged spores

The appearance of aerial and submerged conidiophores and conidia was similar when observed by scanning electron microscopy (SEM) (Plate 3.1). Phialides are cylindrical with a distinctly tapering tip. Typical conidia of *P.oxalicum* are strongly ellipsoidal, smooth walled, and 4.5-6.5 x 3-4 μm in size (Raper and Thom, 1948). The isolate used in this study did not have such strongly ellipsoidal conidia, which were produced on solid media and in submerged culture. Submerged conidiophores and conidia appeared covered by a "filamentous net", which was absent in conidiophores and conidia from aerial produced spores.

3.2.4. Viability of *Penicilliun oxalicum* spores after storage

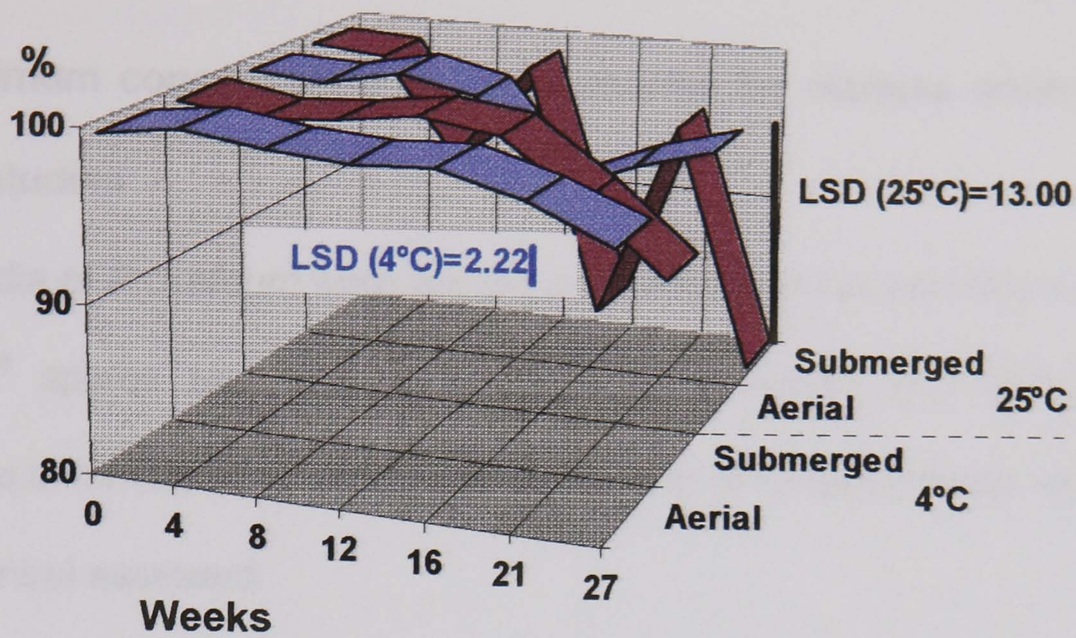
Viability of fresh *P.oxalicum* spores was high after 27 weeks storage at either 4 or 25°C (Fig. 3.6). Only on the last two sampling dates was there a decrease in viability in some cases. Data on submerged spores at 25°C showed abnormal variations in the last three sampling dates. Aerial spores survived slightly better than submerged spores after 27 weeks storage at either 4 or 25°C.

Freeze-drying severely affected the viability of *P.oxalicum* spores, which was reduced from 99.33 to 74 % for aerially produced spores and from 99.33 to 24 % for submerged spores. Viability of submerged spores was lower than that of aerial spores on all the dates tested (Fig. 3.6). There was also a significant decrease in viability with time for both spore types, which was slightly faster at 25°C.

1

2

Plate 3.1. Aspect of phialides and conidia of *P.oxalicum* observed by scanning electron microscopy. 1: from aerial culture, 2: from submerged culture.



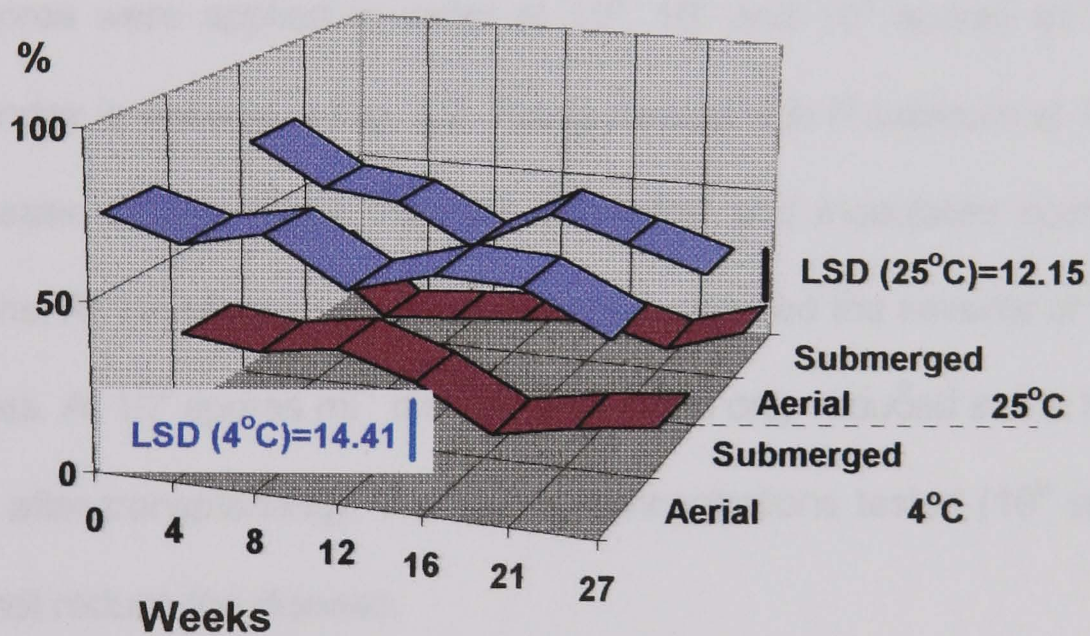


Figure 3.6. Viability of fresh and freeze-dried *P.oxalicum* spores stored at 4 or 25°C. Data are means of three replicates and indicate percentage germination on Potato Dextrose Agar (PDA). Incubation times were 16 and 20 h for fresh and freeze-dried spores, respectively.

3.3. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum*

3.3.1. Minimum concentration of aerial conidia for disease control in growth chamber studies

Aerial conidia of *P.oxalicum* were applied in seedbeds at concentrations ranging from 10^4 to 10^8 spores ml^{-1}. Populations of both pathogen and antagonist in the rhizosphere of tomato were determined at the end of the experiment, and the level of disease control assessed.

(a) Disease control

Disease induced by *F.oxysporum* f.sp. *lycopersici* was reduced by *P.oxalicum* when aerial spores were applied in water at 10^8, 10^7 and 10^6 spores ml^{-1}. Evolution of disease index is showed in Fig. 3.7. Plants treated with *P.oxalicum* at 10^6 spores ml^{-1} gave disease indices lower than the untreated and inoculated control, in all the evaluations. At 10^7 spores ml^{-1} *P.oxalicum* also reduced the severity of the disease in most cases. At 10^8 spores ml^{-1} disease index was only reduced in the first evaluation (13 days after transplanting). The lowest concentrations tested (10^5 and 10^4 spores ml^{-1}) did not reduce the disease.

The pathogen produced a reduction in the number of leaves of tomato plants (Table 3.8). The number of leaves on plants treated with *P.oxalicum* at 10^8 spores ml^{-1} was not significantly ($P=0.05$) different from that of untreated and inoculated plants. However, the number of leaves in plants treated with any other concentration of the antagonist was significantly higher than that of untreated and inoculated plants.

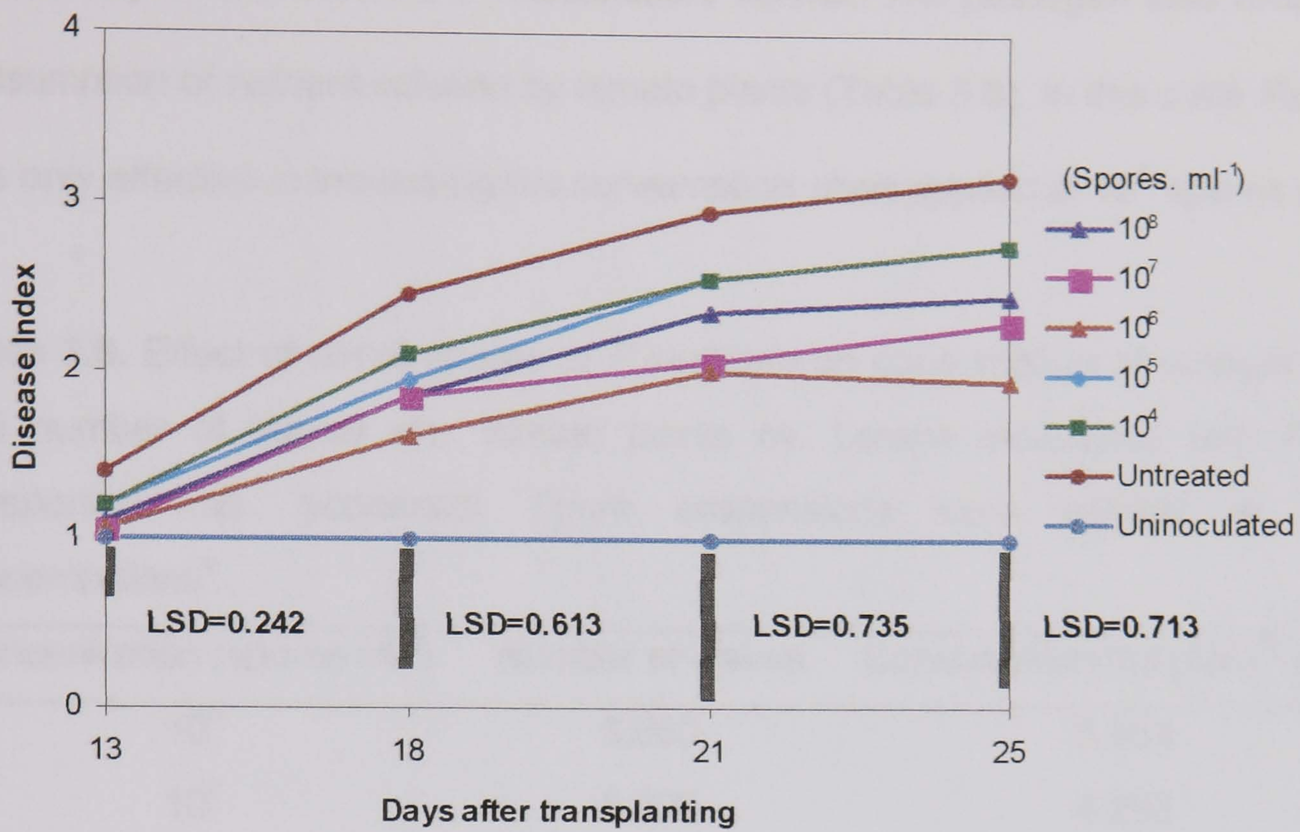


Figure 3.7. Evolution of disease index in tomato plants cv. Lorena inoculated with *F.oxysporum* f.sp. *lycopersici* and treated with aerial spores of *P.oxalicum* at different concentrations. Data are means of five replicates in all cases

The number of leaves in plants treated with *P.oxalicum* at 10^6, 10^5 and 10^4 was not significantly different from the uninoculated control. The pathogen also reduced the consumption of nutrient solution by tomato plants (Table 3.8). In this case *P.oxalicum* was only effective in increasing the consumption when applied at 10^7 spores ml^{-1}.

Table 3.8. Effect of aerial spores of *P.oxalicum* on consumption of nutrient solution and number of leaves on tomato plants cv. Lorena inoculated with *Fusarium oxysporum* f.sp. *lycopersici*. Spore suspensions were applied at different concentrations[a].

Concentration (spores ml^{-1})	Number of leaves	Consumption (ml $plant^{-1}$ day^{-1})
10^8	6.650	3.954
10^7	6.800	4.293
10^6	7.200	4.184
10^5	7.050	4.174
10^4	6.950	3.995
Untreated and inoculated	6.100	3.776
Uninoculated (no pathogen)	7.400	4.892
LSD	0.571	0.472

[a] Data are means of five replicates

(b) Populations of *P.oxalicum* and *F.oxysporum* f.sp. *lycopersici*

Populations of *P.oxalicum* at the end of the experiment were not significantly different in the rhizosphere of plants treated with the antagonist at 10^8 and 10^7 spores ml^{-1}. For lower treatment concentrations, populations of *P.oxalicum* were progressively smaller (Fig. 3.8). Populations of *F.oxysporum* f.sp. *lycopersici* were not affected by the presence of the antagonist, except when *P.oxalicum* was applied at 10^6 spores ml^{-1}. This treatment reduced the populations of the pathogen compared to the untreated control.

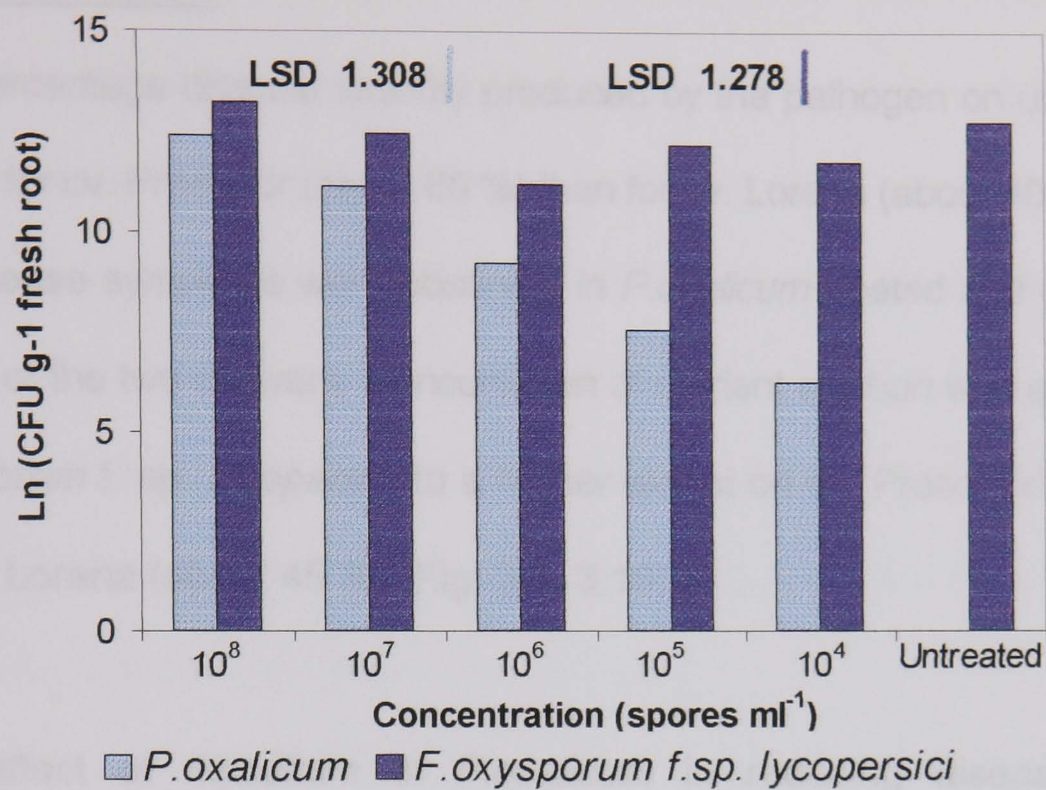


Figure 3.8. Populations of *P.oxalicum* and *F.oxysporum* f.sp. *lycopersici* in the rhizosphere of tomato plants reated with *P.oxalicum* aerial spores at different concentrations. Data are means of three replicates

3.3.2. Efficacy of aerial and submerged spores in growth chamber studies

(a) Disease control

The percentage disease severity produced by the pathogen on untreated plants was higher for cv. Precodor (about 65 %) than for cv. Lorena (about 40 %) (Fig. 3.9, 3.10). No disease symptoms were observed in *P.oxalicum* treated and uninoculated plants in any of the two cultivars. Consumption of nutrient solution was again reduced by *F. oxysporum* f. sp. *lycopersici* to a higher extent on cv. Precodor (about 65 %) than on cv. Lorena (about 45 %) (Fig. 3.9, 3.10).

The effect of mycelium of *P.oxalicum* in reducing disease caused by *F. oxysporum* f. sp. *lycopersici* was tested on tomato plants cv. Lorena (treatment 4 in Fig. 3.9.). This type of inoculum was unable to reduce disease severity or to increase the consumption of nutrient solution in plants inoculated with the pathogen.

P.oxalicum applied as aerial spores in water was able to reduce disease induced by *F. oxysporum* f. sp. *lycopersici* in one of the three repetitions with tomato plants cv. Lorena (treatment 1 in Fig. 3.9, 3.10). However, this treatment was always effective reducing percentage disease severity in cv. Precodor (treatment 1 in Fig. 3.10). When aerial spores were applied in Morton´s medium (treatment 2 in Fig. 3.9, 3.10) similar results were obtained regarding disease severity. However, this treatment was also able to increase the consumption of nutrient solution by inoculated plants in some of the repetitions in both cultivars tested.

Submerged spores of *P.oxalicum* (treatment 3 in Fig. 3.9, 3.10) were less effective than aerial ones. This treatment was able to increase the consumption of nutrient solution of inoculated plants cv. Lorena in one of the three repetitions, and it reduced disease severity in one of the two repetitions in cv. Precodor. The liquid from submerged cultivation (treatment 5 in Fig. 3.10) produced in general inverse results to those obtained with the submerged spores of the antagonist. It was able to reduce disease severity and increase the consumption of nutrient solution in inoculated plants cv. Lorena when the treatment with submerged spores was ineffective. Similar behaviour was also observed for cv. Precodor.

(b) Populations of *P. oxalicum* and *F. oxysporum* f. sp. *lycopersici*

No significant (P=0.05) differences between treatments were found in populations of *F.oxysporum* f.sp. *lycopersici* in the rhizosphere of tomato plants (cv. Lorena and Precodor) at the end of the experiment (Table 3.9). Populations ranged between 16.60×10^4 and 9.48×10^4 cfu g^{-1} fresh root weight in cv. Lorena.

For cv. Precodor populations of the pathogen were smaller than for cv. Lorena and ranged between 24.38×10^3 and 0.02×10^3 cfu g^{-1} fresh root weight, with a much higher variation between the replicates.

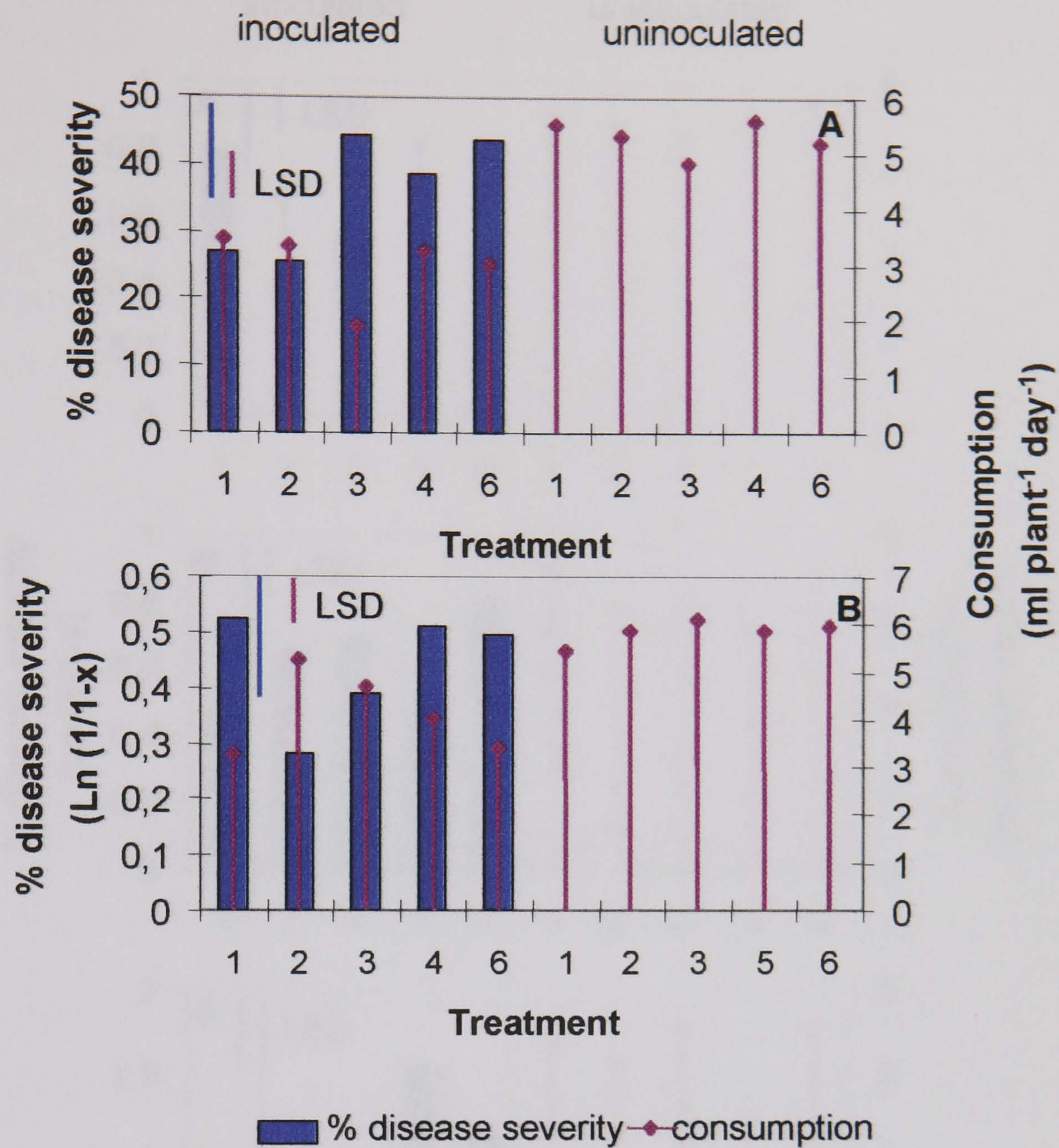


Figure 3.9. Effect of *P.oxalicum* treatments on disease severity and consumption of nutrient solution in tomato plants cv. Lorena inoculated or uninoculated with *F.oxysporum* f.sp. *lycopersici*. Data are means of five replicates. A and B are repetitions. Key to treatments: 1: aerial spores in water, 2: aerial spores in Morton's medium, 3: submerged spores, 4: mycelium, 6: untreated control

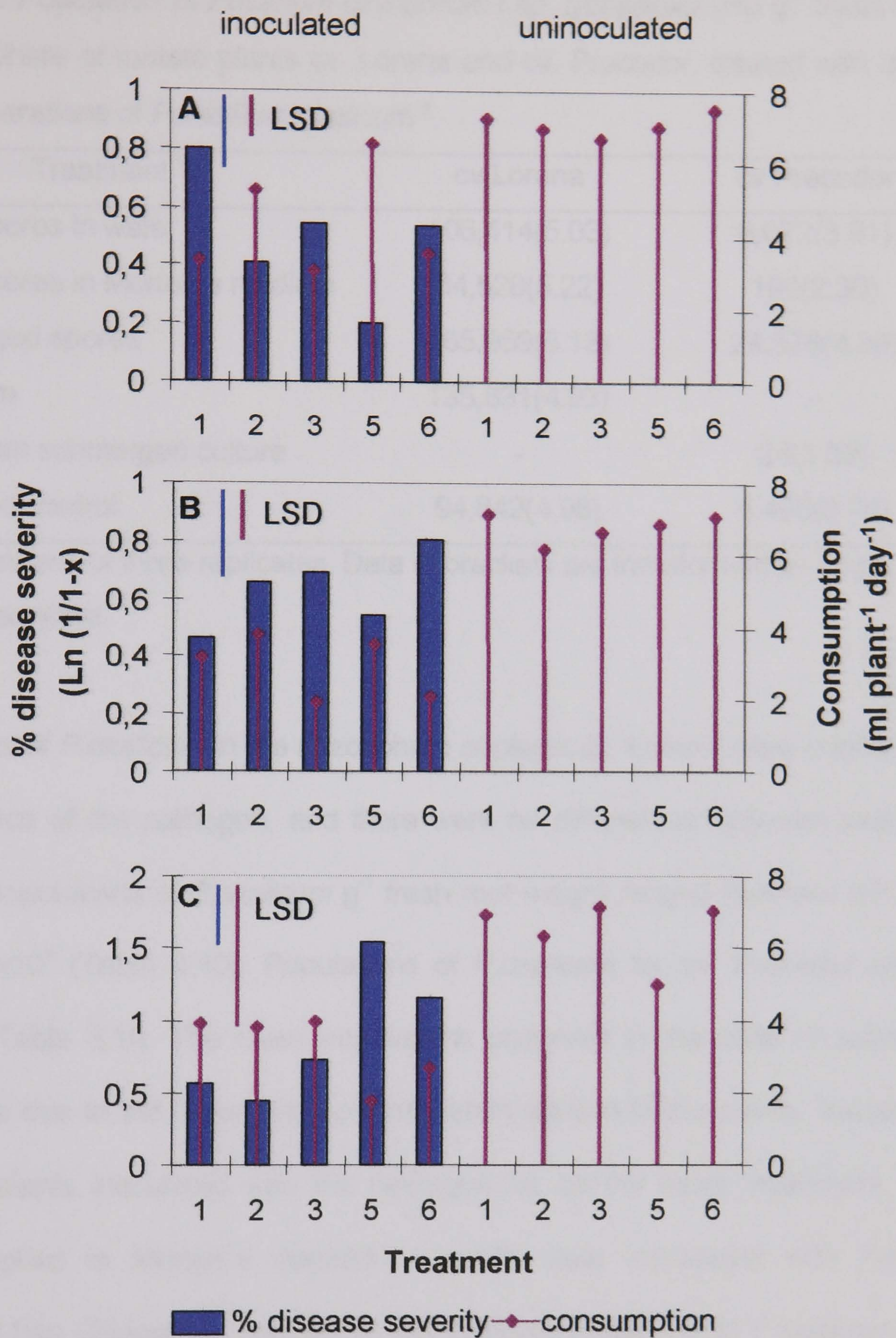


Figure 3.10. Effect of *P.oxalicum* treatments on disease severity and consumption of nutrient solution in tomato plants cv. Lorena (A) and cv. Precodor (B and C) inoculated or uninoculated with *F.oxysporum* f.sp. *lycopersici*. Data are means of five replicates. Key to treatments: 1: aerial spores in water, 2: aerial spores in Morton's medium, 3: submerged spores, 5: liquid from submerged cultivation, 6: untreated control

Table 3.9. Population of *Fusarium oxysporum* f.sp. *lycopersici* (cfu g^{-1} fresh root) in the rhizosphere of tomato plants cv. Lorena and cv. Precodor, treated with different spore preparations of *Penicillium oxalicum* [a].

Treatment [b]	cv.Lorena	cv.Precodor
1: aerial spores in water	106,414(5.03)	8,072(3.91)
2: aerial spores in Morton`s medium	84,528(5.22)	199(2.30)
3: submerged spores	165,959(5.13)	24,378(4.39)
4: mycelium	135,831(4.93)	-
5: liquid from submerged culture	-	24(1.38)
6: untreated control	94,842(4.98)	5,495(3.74)

[a]Data are means of three replicates. Data in brackets are transformed by Log_{10}.

- : non determined.

Populations of *P.oxalicum* in the rhizosphere of plants cv. Lorena were unaffected by the presence of the pathogen, and there were no differences between treatments. The total populations of *P.oxalicum* g^{-1} fresh root weight ranged between 9.55 x 10^4 and 3.61 x10^4 (Table 3.10). Populations of *P.oxalicum* for cv. Precodor are also shown in Table 3.10. The lower populations observed in the case of submerged spores was due to the lower initial concentration applied to the plants. Values were higher in plants inoculated with the pathogen for all the three treatments. Aerial spores applied in Morton`s medium to plants later inoculated with *Fusarium oxysporum* f.sp. *lycopersici*, resulted in the highest populations of *P.oxalicum* in the tomato rhizosphere.

Table 3.10. Populations of *Penicillium oxalicum* (cfu g^{-1} fresh root) in the rhizosphere of tomato plants cv. Lorena and cv. Precodor inoculated (I) or uninoculated (U) with *Fusarium oxysporum* f.sp. *lycopersici*, and treated with different spore preparations of the antagonist[a]

	cv. Lorena		cv. Precodor	
Treatment[b]	I	U	I	U
1	36,057(4.557)	75,336(4.877)	1,114,294(6.047)	598,412(5.777)
2	81,283(4.910)	37,411(4.633)	2,041,738(6.310)	758,578(5.880)
3	95,499(4.980)	42,954(4.903)	54,075(4.733)	13,183(4.120)
4	16,711(4.223)	79,983(4.573)	-	-
LSD	NS		0.477	

[a]Data are means of three replicates. Data in brackets are transformed by Log_{10}. LSD is for transformed data. [b] 1: aerial spores in water, 2: aerial spores in Morton`s medium, 3: submerged spores, 4: mycelium. - : non determined. NS: non significant.

3.3.3. Efficacy of *Penicillium oxalicum* in greenhouse experiments

(a) Disease control

Fig. 3.11, 3.12 and 3.13 show the development of disease induced by *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants treated with different preparations of *Penicillium oxalicum*, expressed by the disease index. Fig. 3.11 shows the results from treatment with spores produced in a solid substrate (maize grits). Fig. 3.12 shows the results from treatments with spores produced in liquid medium. Fig. 3.13 shows the results from treatments with spores produced on PDA plates. The general trend is the same in all three cases: disease developed slowly until the 24th day, then there was a marked increase in the disease index, followed by a more gradual increase until the end of the experiment. The disease index in treated plants was between disease indices in the untreated and the uninoculated

control, regardless of the production method, and the a_w of the medium used to grow the antagonist on. Uninoculated plants started showing disease symptoms by the 36th day and then developed parallel to the other treatments.

Values of disease indices for the last three dates of disease assessment are also shown in Table 3.11. On the 36th and 50th day disease indices in all the treatments were significantly smaller than in the untreated control, except for treatment 5 (liquid medium, 0.95 a_w) on the 36th day. However, at the end of the experiment only disease indices in treatments 1, 6 and 7 were significantly smaller than the disease index in untreated plants.

No differences were observed in the number of plants from which the pathogen was isolated at the end of the experiment. Similarly, no differences were observed either in the weight of roots or the aerial part of the plants. However, some differences were observed in the number of leaves of plants at the end of the experiment (Table 3.12). Plants treated with aerial spores from agar medium had less leaves than plants treated with *P.oxalicum* produced on solid grain based substrates. Treatments 1, 2, 4 and 5 resulted in plants having similar leaf number to the uninoculated control.

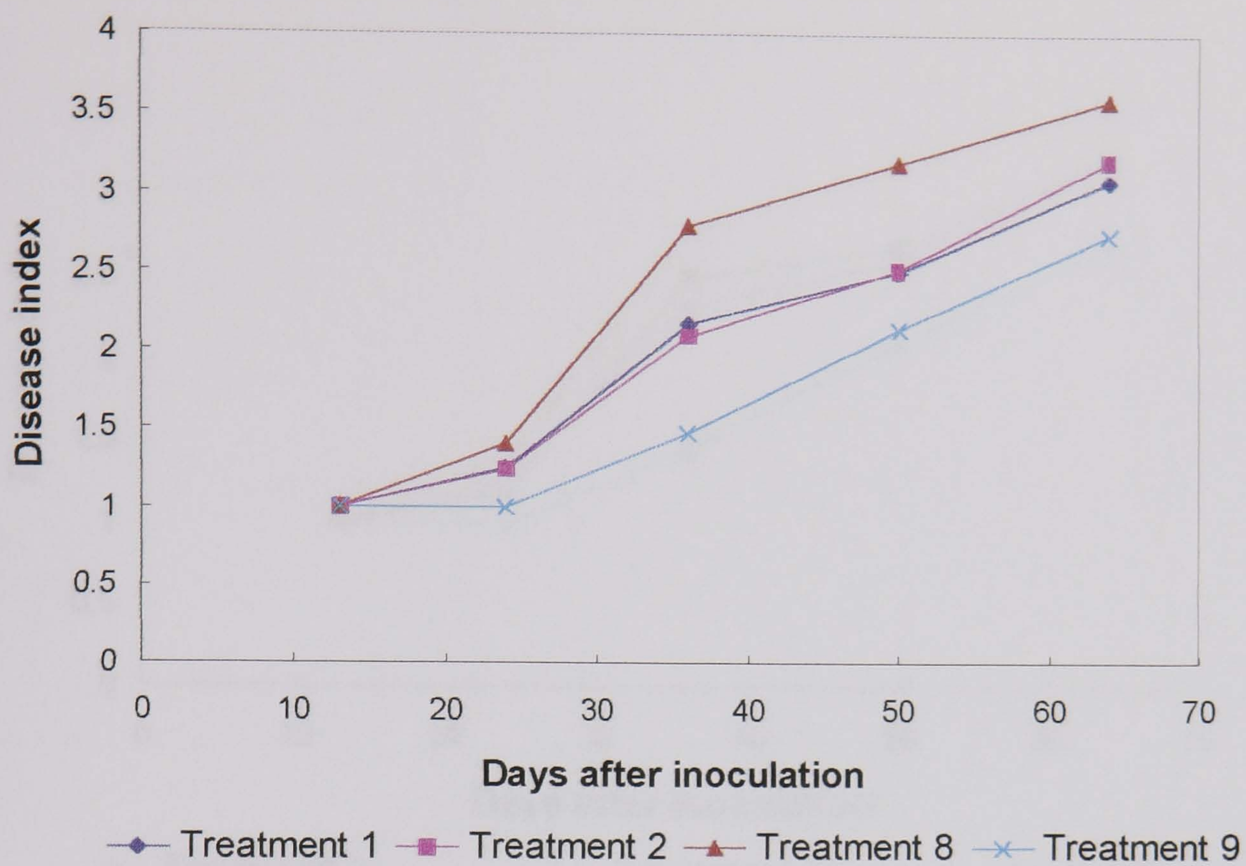


Figure 3.11. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced on solid substrate at a_w = 0.98 (Treatment 1) or a_w = 0.95 (Treatment 2).

Treatment 8: untreated control Treatment 9: uninoculated control

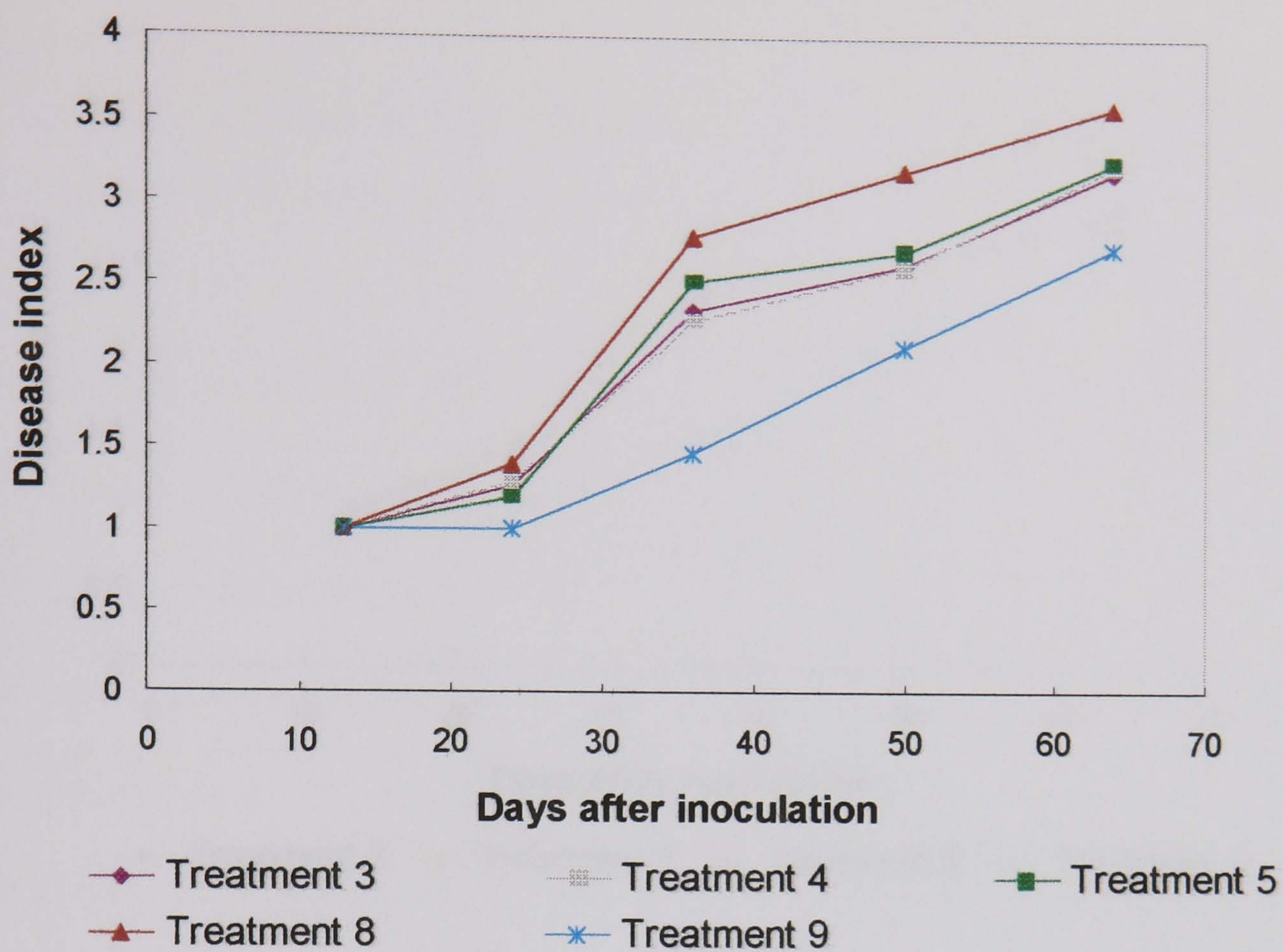


Figure 3.12. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced in liquid medium at a_w = 0.998 (Treatment 3), a_w = 0.98 (Treatment 4) or a_w = 0.95 (Treatment 5). Treatment 8: untreated control. Treatment 9 uninoculated control.

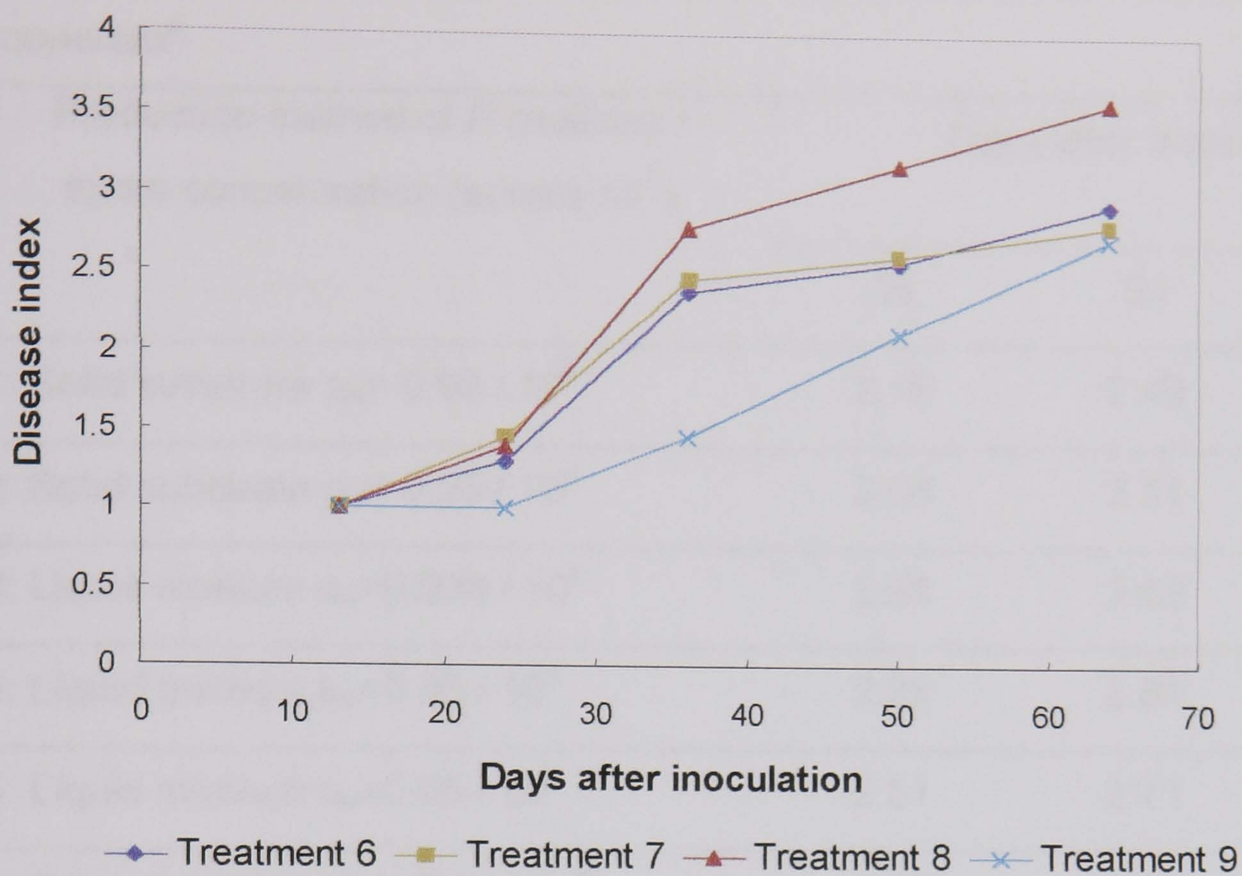


Figure 3.13. Temporal development of disease index in tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* and treated with *Penicillium oxalicum* produced on PDA plates and applied as a spore suspension at 10^6 spores ml^{-1} (Treatment 6) or 10^8 spores ml $^{-1}$ (Treatment 7). Treatments 8: untreated control. Treatement 9: uninoculated control.

Table 3.11. Disease indices in plants treated with different spore preparations of *P.oxalicum*, 36, 50 and 64 days after inoculation with *F.oxysporum* f.sp. *lycopersici*[a]

Production method of *P.oxalicum* / spore concentration (spores ml^{-1})	Days after inoculation		
	36	50	64
1: Solid substrate a_w= 0.98 / 10^6	2.16	2.49	3.07
2: Solid substrate a_w= 0.95 / 10^6	2.08	2.51	3.21
3: Liquid medium a_w=0.998 / 10^6	2.33	2.62	3.19
4: Liquid medium a_w=0.98 / 10^6	2.28	2.61	3.23
5: Liquid medium a_w=0.95 / 10^6	2.51	2.71	3.26
6: Control treatment 1: PDA / 10^6	2.38	2.57	2.94
7: Control treatment 2: PDA / 10^8	2.45	2.61	2.81
8: Untreated control	2.78	3.18	3.59
9: Uninoculated control	1.46	2.13	2.73
LSD	0.28	0.37	0.45

[a] Data are means of eight replicates.

Table 3.12. Number of leaves of tomato plants treated with different spore preparations of *P.oxalicum* 64 days after inoculation with *Fusarium oxysporum* f.sp. *lycopersici.*

Production method of *P.oxalicum* / spore concentration (spores ml^{-1})	Number of leaves
1: Solid substrate a_w= 0.98 / 10^6	14.887
2: Solid substrate a_w= 0.95 / 10^6	14.725
3: Liquid medium a_w=0.998 / 10^6	13.675
4: Liquid medium a_w=0.98 / 10^6	14.475
5: Liquid medium a_w=0.95 / 10^6	14.250
6: Control treatment 1: PDA / 10^6	13.363
7: Control treatment 2: PDA / 10^8	13.300
8: Untreated control	13.775
9: Uninoculated control	15.387
LSD	1.332

[a] Data are means of eight replicates.

(b) Populations of *P. oxalicum* and *F. oxysporum* f. sp. *lycopersici*

No significant differences between treatments were found in populations of *F.oxysporum* f. sp. *lycopersici* in the rhizosphere; numbers of cfu of the pathogen per gram fresh root weight decreased from 44.2 x 10^4 at the beginning of the experiment to 7.4 x 10^4 at the end of the experiment.

Table 3.13 shows the changes in populations of a range of *P.oxalicum* treatments, obtained from rich and unstressed, or stressed substrates (liquid, solid grain or culture media). One day after inoculation with the pathogen, the

P.oxalicum populations were significantly higher in treatment 7 (Control treatment 2: PDA / 10^8), where spores were initially applied at a rate 100-fold higher than in the rest of treatments. In treatments 4 (Liquid medium a_w=0.98 / 10^6) and 6 (Control treatment 1: PDA / 10^6) populations were significantly higher than in the other treatments (except treatment 7). Populations of *P.oxalicum* in all treatments decreased 64 days after inoculation. The biggest decrease was observed in treatment 7 (95-fold decrease); in the rest of treatments the populations decreased by a factor of 5-10. On this date differences in population sizes amongst treatments were smaller than in the first evaluation. The highest populations were in treatment 7 (Control treatment 2: PDA / 10^8) and 4 (Liquid medium a_w=0.98 / 10^6) and the lowest in treatment 3 (Liquid medium a_w=1 / 10^6).

Table 3.13. Populations of *P.oxalicum* (cfu g^{-1} fresh root) in the rhizosphere of tomato plants treated with different spore preparations of the antagonist, 1 and 64 days after inoculation with *F. oxysporum* f.sp. *lycopersici*[a]

Production method of *P.oxalicum* / spore concentration (spores ml^{-1})	Days after inoculation	
	1	64
1: Solid substrate a_w= 0.98 / 10^6	6,000 (8.64)	836 (6.55)
2: Solid substrate a_w= 0.95 / 10^6	13,000 (9.14)	1,845 (7.02)
3: Liquid medium a_w=0.998 / 10^6	3,000 (7.97)	313 (5.74)
4: Liquid medium a_w=0.98 / 10^6	20,083 (9.79)	2,676 (7.85)
5: Liquid medium a_w=0.95 / 10^6	3,917 (7.96)	721 (6.33)
6: Control treatment 1: PDA / 10^6	17,917 (9.78)	1,948 (7.20)
7: Control treatment 2: PDA / 10^8	392,500 (12.86)	4,118 (8.20)
LSD for transformed data = 1.23		

[a] Data are means of three replicates. Data in brackets are transformed by Ln.

3.3.4. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion

Aerial spores of *P.oxalicum* were formulated with tomato seeds, which were sown on Hoagland and water agar plates to test the effect of the BCA on growth. Formulations were made using sodium alginate and methyl cellulose, with or without nutrients. Populations of the antagonist on the seeds were determined.

(a) Growth promotion

The effect of *P.oxalicum* as a growth promoting agent was observed as a general rule in both the two cultivars studied (Lorena and Precodor) and in the two types of culture media used (water agar and Hoagland agar). However, significant effects were not found in all cases for all the parameters studied.

P.oxalicum inoculations resulted in a faster germination of tomato seeds of cv. Precodor when seeds were sown on Hoagland agar (Fig. 3.14). This effect was observed 4, 6, 8, 10 and 12 days after sowing. When the fungus was formulated with nutrients (formulations 2 and 4) germination was improved on the five dates compared to untreated control seeds. This was also observed in the standard treatment (seed bath). When formulations did not include nutrients, improved germination was only observed for the alginate formulation after the 8th day, and from the 10th day onwards in the case of the methyl cellulose formulation.

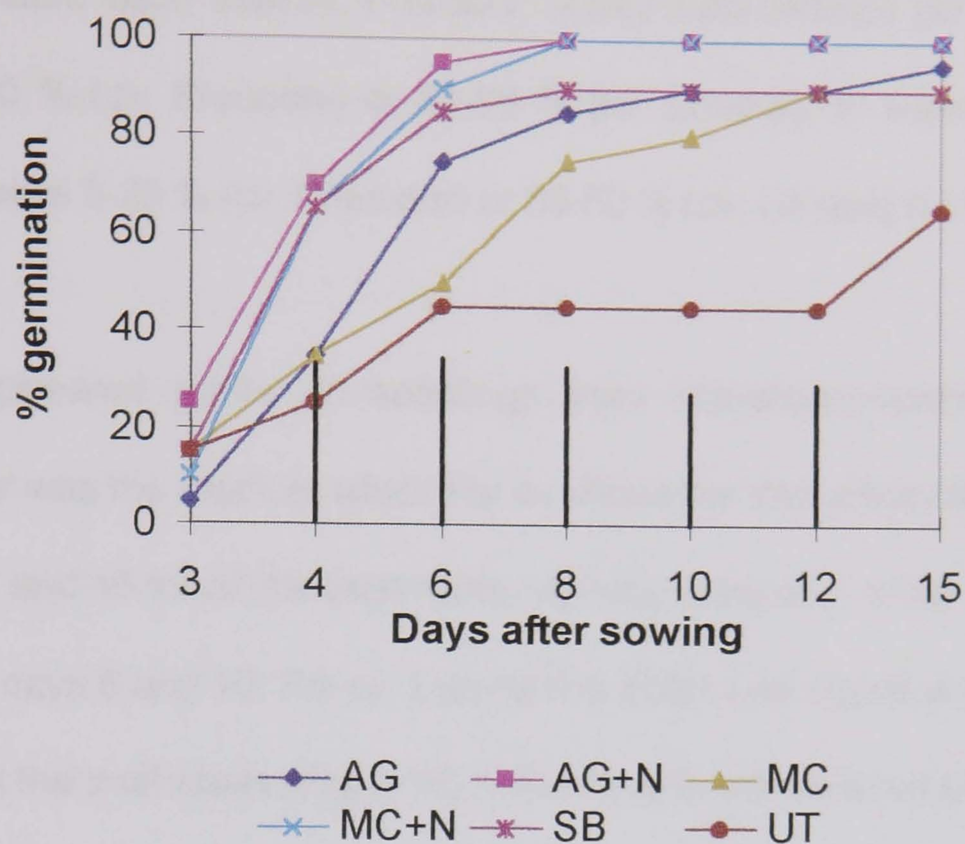


Figure 3.14. Percentage germination of tomato seeds cv. Precodor formulated with *P.oxalicum*, on Hoagland agar. Data are means of five replicates. Bars indicate Least Significant Differences (LSD) for each date. Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated.

Germination was faster when seeds were placed on water agar, and seeds cv. Lorena germinated faster than cv. Precodor: at day 3 percentage germination ranged between 50-60 % (cv. Precodor) or 68-95 % (cv. Lorena) on water agar, whereas these values were 5-25 % (cv. Precodor) or 30-50 % (cv. Lorena) on Hoagland agar.

Cotyledons appeared earlier in seedlings from *P.oxalicum*-treated seeds when Hoagland agar was the medium used. For cv. Precodor this effect was significant on days 8, 10, 12 and 15 for all the treatments, with the exception of the methyl cellulose formulation at days 8 and 10. For cv. Lorena this effect was significant on the 6th and 8th days for all the treatments (Fig. 3.15). When seeds were placed on water agar, no significant differences were observed between treatments.

Appearance of cotyledons was faster in seedlings cv. Lorena, with the percentage of seedlings bearing cotyledons on day 6 ranging from 15-50 % or 10-53.3 % (cv. Precodor on Hoagland or water agar, respectively) compared to 20-85 % or 46.7-75 % for cv. Lorena on Hoagland or water agar respectively.

The appearance of true leaves was faster after seed treatment with *P.oxalicum* for the two cultivars and culture media used (Fig. 3.16). For cv. Precodor on water agar this effect was significant on the 10th day for the alginate formulation and on the 12th and 15th days for all the treatments except the standard treatment (seed bath). On Hoagland agar on the 8th day only the formulation with alginate and nutrients gave a higher percentage leaf appearance; on day 10 only formulations with nutrients and the standard treatment resulted in a higher percentage of leaf appearance. On days 12 and 15 all treatments improved leaf appearance. For cv. Lorena on water agar the

appearance of leaves was faster only for alginate formulations and methyl cellulose without nutrients, on the 15th day. On Hoagland agar all treatments improved leaf emergence on the 8th and 10th days (Fig. 3.17).

Leaf emergence was faster on Hoagland agar: at day 10, 20-80 % of seedlings cv. Precodor had true leaves on this medium, whereas on water agar percentage ranged between 0-2 %. For cv. Lorena these values were 30-100 % (Hoagland agar) and 5-18.3 % (water agar).

The weight of tomato seedlings was increased by the *P.oxalicum* seed treatments for the two cultivars and culture media tested (Fig. 3.18). On water agar this effect was significant for all treatments with the exception of the standard treatment, for both cultivars. The presence of nutrients in the formulations did not have a significant effect on the weight of seedlings. On Hoagland agar all treatments resulted in a higher weight of seedlings, except the alginate formulation with cv. Precodor.

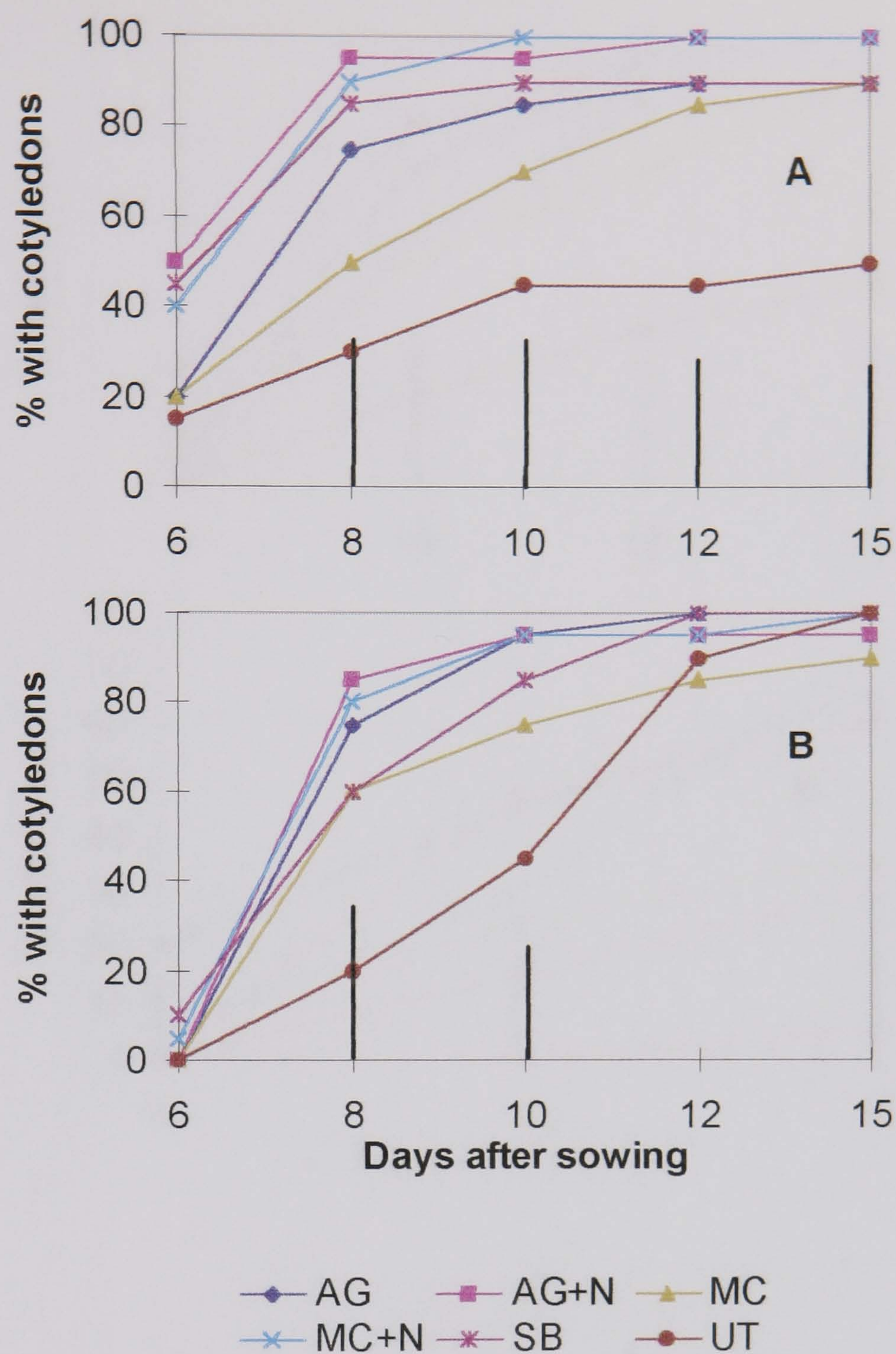


Figure 3.15. Percentage of tomato seeds cv. Precodor (A) and cv. Lorena (B) bearing cotyledons from seeds formulated with *P.oxalicum* and sown on Hoagland agar. Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated.

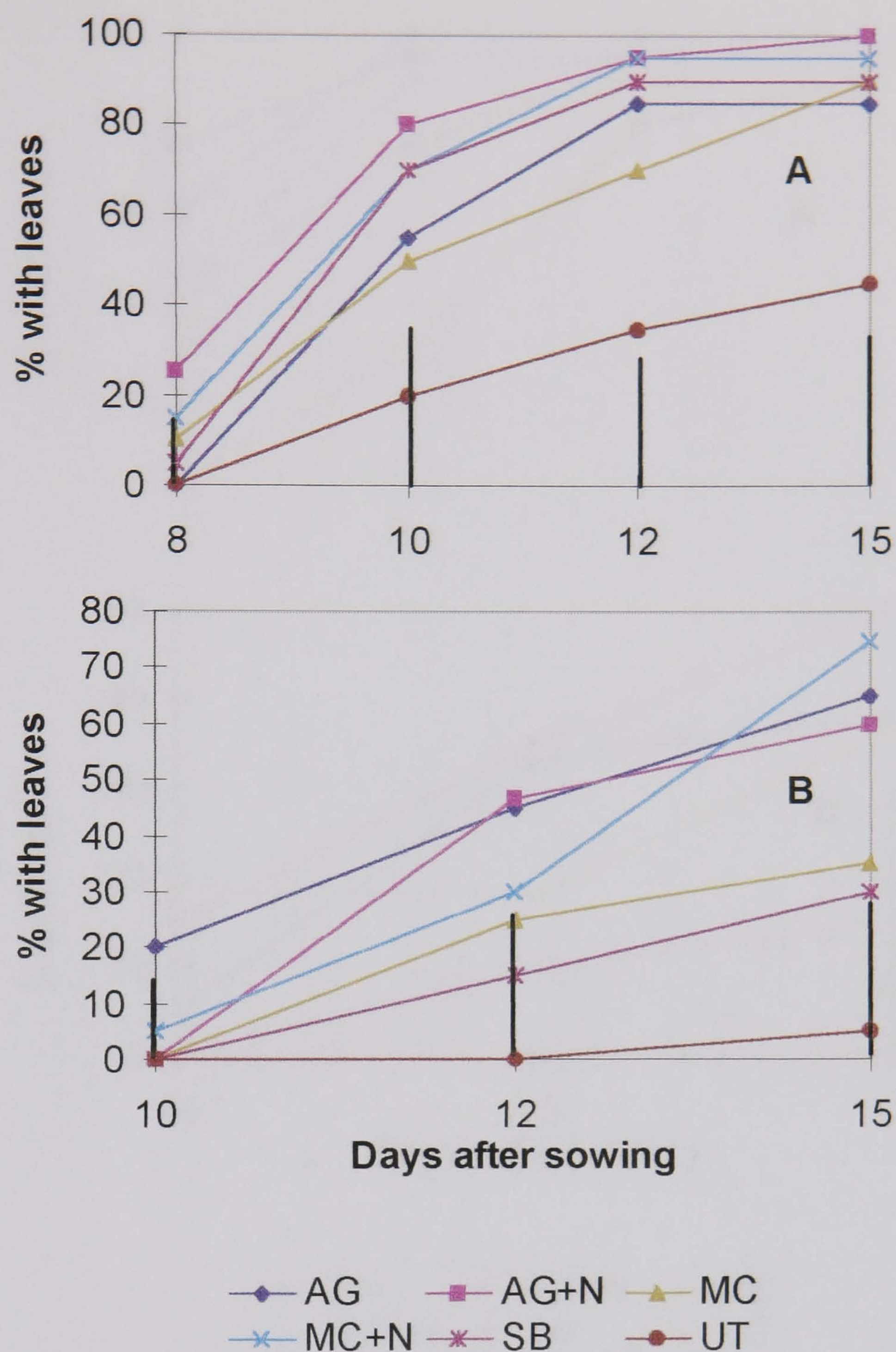


Figure 3.16. Percentage of tomato seeds cv. Precodor bearing leaves from seeds formulated with *P.oxalicum* and sown on Hoagland agar (A) or water agar (B). Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated.

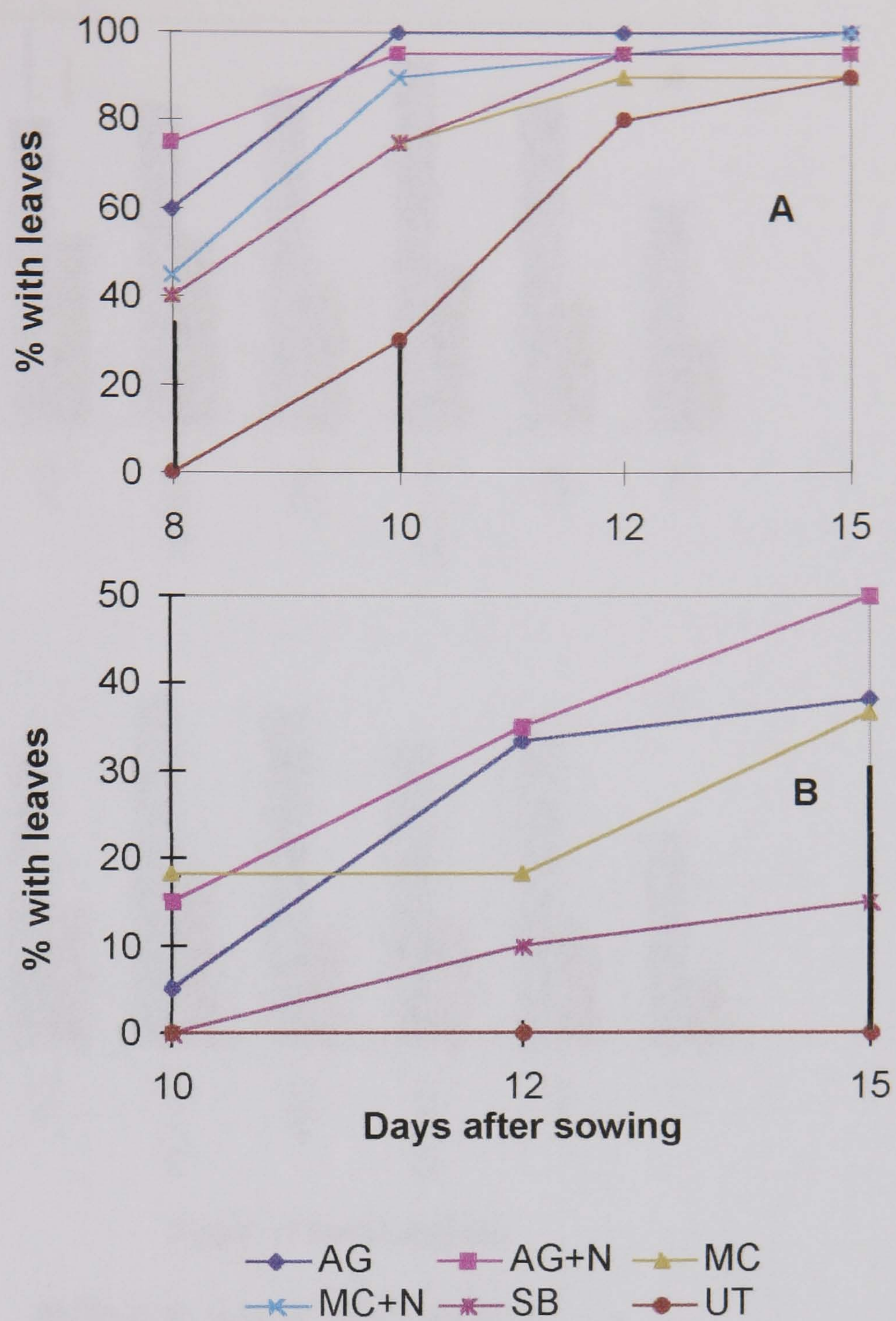


Figure 3.17. Percentage of tomato seeds cv. Lorena bearing leaves from seeds formulated with *P.oxalicum* and sown on Hoagland agar (A) or water agar (B). Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated.

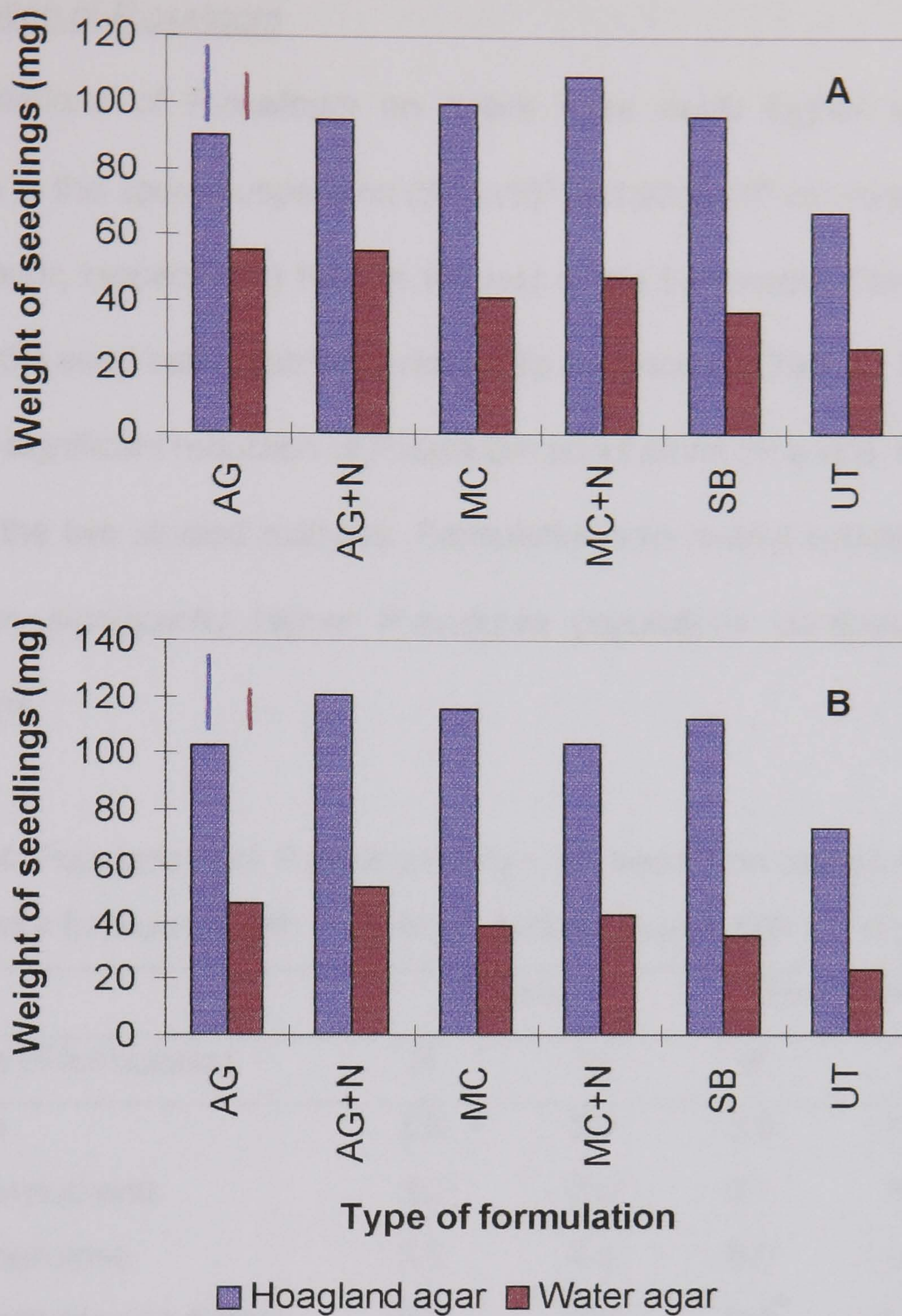


Figure 3.18. Weight of tomato seedlings cv. Precodor (A) and cv. Lorena (B) 15 days after sowing on Hoagland agar or water agar. Seeds were formulated with *P.oxalicum*. Data are means of five replicates. Bars indicate Least Significant Differences (LSD). Key to treatments: AG: Alginate, AG+N: Alginate+nutrients, MC: Methyl cellulose, MC+N: Methyl cellulose+nutrients, SB: Seed bath, UT: Untreated.

(b) Population of *P.oxalicum*

The populations of *P.oxalicum* on seeds were much higher when treated by immersion in the spore suspension ($67.1x10^4$ and $92.2x10^4$ cfu $seed^{-1}$ for cv. Lorena and Precodor, respectively) than in the rest of the treatments. Data were analysed excluding the seed bath treatment and results are shown in Table 3.14. Drying did not result in a significant reduction of *P.oxalicum* populations on seeds. Populations were similar in the two studied cultivars. Formulation with methyl cellulose and nutrients resulted in significantly higher *P.oxalicum* populations compared to the other formulations.

Table 3.14. Populations of *P.oxalicum* (cfu x 10^4 $seed^{-1}$) on tomato seeds cv. Lorena and Precodor formulated with *P.oxalicum* before (B) and after (A) drying [a]

	Lorena		Precodor		
Type of formulation	B	A	B	A	mean
1: Alginate	5.3	3.1	3.9	5.4	4.4
2: Alginate+nutrients	3.2	2.9	9.1	8.1	5.8
3: Methyl cellulose	6.3	4.4	6.0	5.6	5.6
4: Methyl cellulose+nutrients	6.2	10.4	6.4	11.1	8.5
			LSD for mean values = 2.6		

[a] Data are means of three replicates

3.4. PRODUCTION OF *Epicoccum nigrum* BY SOLID SUBSTRATE FERMENTATION

Sporulation of *E.nigrum* on maize grits was very poor and therefore quantification was not possible. Spore production on wheat grains cv. Rendeveau was affected by the a_w of the substratum (Table 3.15). Levels of sporulation increased with time for the three levels of a_w, but the increase was much greater when water was freely available (0.996 a_w). The maximum level of sporulation was obtained on the substrate at 0.996 a_w, 23 days after inoculation. At 0.96 a_w spore production was very low, with a maximum sporulation level of about $1x10^6$ spores g^{-1}, 23 days after inoculation (12 x lower than at 0.996 a_w). At 0.98 a_w about $4x10^6$ spores g^{-1} were produced 23 days after inoculation. However this was still 2.7 times less than at 0.996 a_w.

Table 3.15. Spore production by *E.nigrum* on wheat grain cv. Rendeveau at different levels of water activity (a_w), expressed as millions ($x10^6$) spores g^{-1} grain[a]

	Days after inoculation			
a_w	7	14	18	23
0.960	0.076 (-2.718)	0.292 (-1.333)	0.381 (-1.023)	0.943 (-0.113)
0.980	0.097 (-2.370)	1.131 (0.067)	2.813 (1.003)	4.181 (1.401)
0.996	0.175 (-1.785)	2.190 (0.740)	4.694 (1.526)	11.405 (2.425)

LSD for transformed data = 0.676

[a] Data are means of three replicates. Data in brackets are transformed by Ln

A second experiment was carried out in which the a_w of the wheat grain cv. Brigadier was manipulated in two ways. Firstly by the addition of water only, and secondly by addition of water/glycerol solutions (Table 3.16). In this experiment sporulation increased with time for the three treatments and the largest increase in this case was at 0.98 a_w, when adjusted with water/glycerol solutions. At day 7 spore yield was higher at 0.996 a_w than in the other two treatments. However, from day 14 onwards maximum spore yield was obtained at 0.980 a_w, adjusted with water/glycerol. Maximum yield was obtained on day 21 for this treatment (7.11×10^6 spores g^{-1} grain), about twice the yield obtained in the other two treatments.

Table 3.16. Spore production by *E.nigrum* on wheat grain cv. Brigadier at different levels of water activity (a_w), expressed as millions ($\times 10^6$) spores g^{-1} grain[a]

a_w	Days after inoculation		
	7	14	21
0.980	1.720 (0.528)	2.413 (0.876)	3.307 (1.169)
0.980[g]	2.317 (0.836)	3.720 (1.312)	7.113 (1.916)
0.996	2.620 (0.940)	2.757 (0.997)	4.357 (1.464)
LSD for transformed data = 0.384			

[a] Data are means of three replicates. Data were transformed by Ln.

[g] In this case water activity of the solid substrate was adjusted with a solution of glycerol in water.

3.5. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans*

3.5.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions

In media with freely available water (0.996 a_w) *E.nigrum*996 spores germinated faster than *E.nigrum*98 spores (Table 3.17 A). After 6h incubation percentage germination was similar for both inoculum types, and higher than 95%. Germination was much slower at reduced a_w (0.935), with *E.nigrum*996 spores also germinating faster than *E.nigrum*98 spores (Table 3.17 B). After 12 h incubation there was no statistically significant difference between the two spore types, and after 23 h, germination was higher than 95% in both cases.

However, growth of germ tubes showed a very different pattern compared to that for germination. At 0.996 a_w germ tube extension was similar for both types of spore after both 4 and 6 h incubation (Table 3.18 A). At 0.935 a_w germ tube length was similar for both spore types after 8 h incubation. However, after 12 h incubation, growth of germ tubes from *E.nigrum*98 spores were significantly longer than those from *E.nigrum*996 spores, and this difference was much greater after 23 h incubation (Table 3.18 B, Plate 3.2).

Table 3.17. Percentage germination of *E.nigrum* spores on water agar at 0.996 a_w (A) and 0.935 a_w (B) [a]

(A)

Type of inoculum	Incubation time (h)	
	4	6
*E.nigrum*996	87.333	98.000
*E.nigrum*98	83.000	97.333
	LSD = 3.958	

(B)

Type of inoculum	Incubation time (h)		
	8	12	23
*E.nigrum*996	51.333	82.000	98.667
*E.nigrum*98	32.667	80.000	95.333
	LSD = 7.464		

[a] Data are means of three replicates

Table 3.18. Germ tube extension (μm) from *E.nigrum* spores on water agar at 0.996 a_w (A) and 0.935 a_w (B) [a]

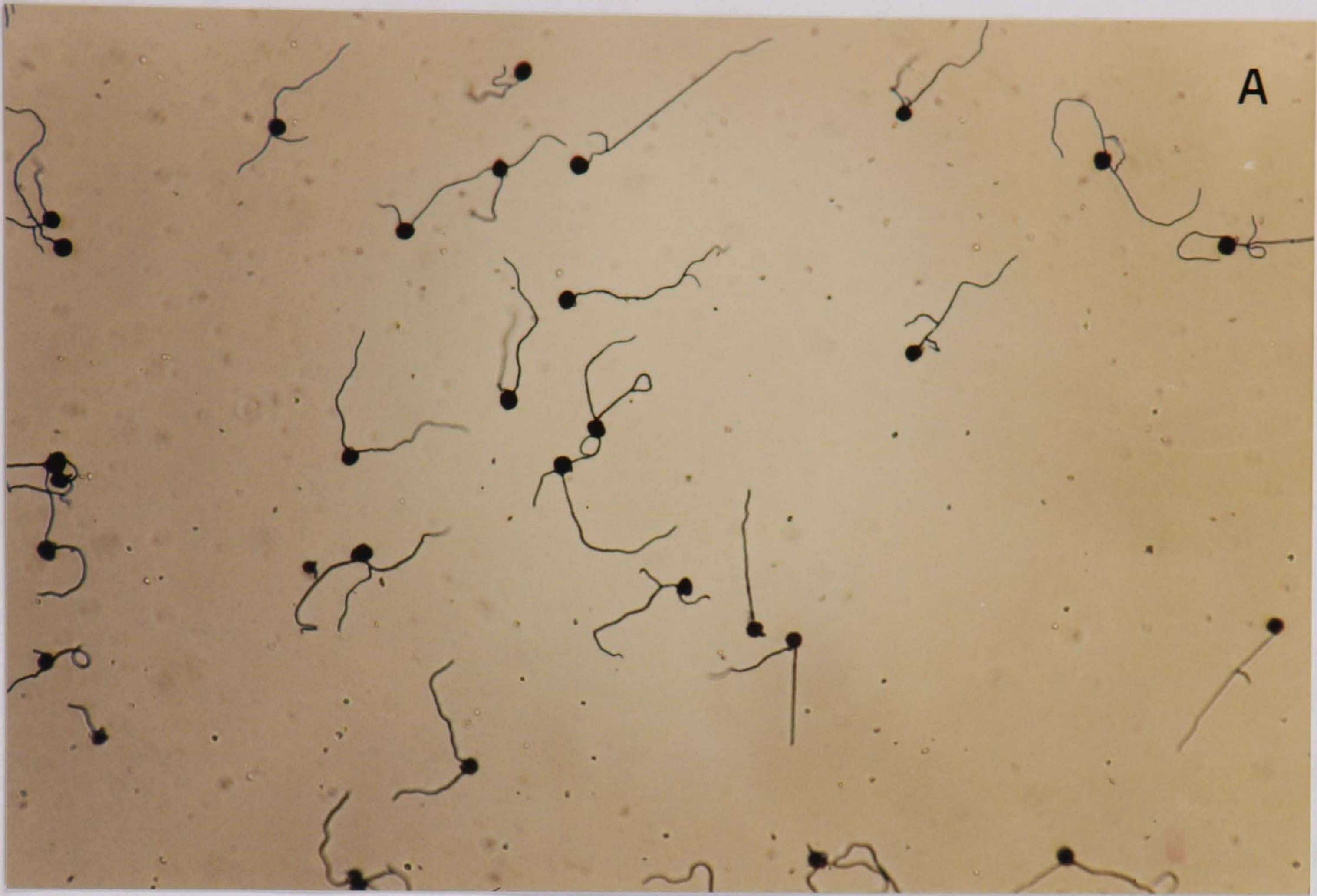
(A)

Type of inoculum	Incubation time (h)	
	4	6
*E.nigrum*996	14.400	32.017
*E.nigrum*98	11.687	33.650
	LSD = 4.273	

(B)

Type of inoculum	Incubation time (h)		
	8	12	23
*E.nigrum*996	5.383	9.943	38.163
*E.nigrum*98	5.470	12.023	77.997
	LSD = 1.688		

[a] Data are means of three replicates



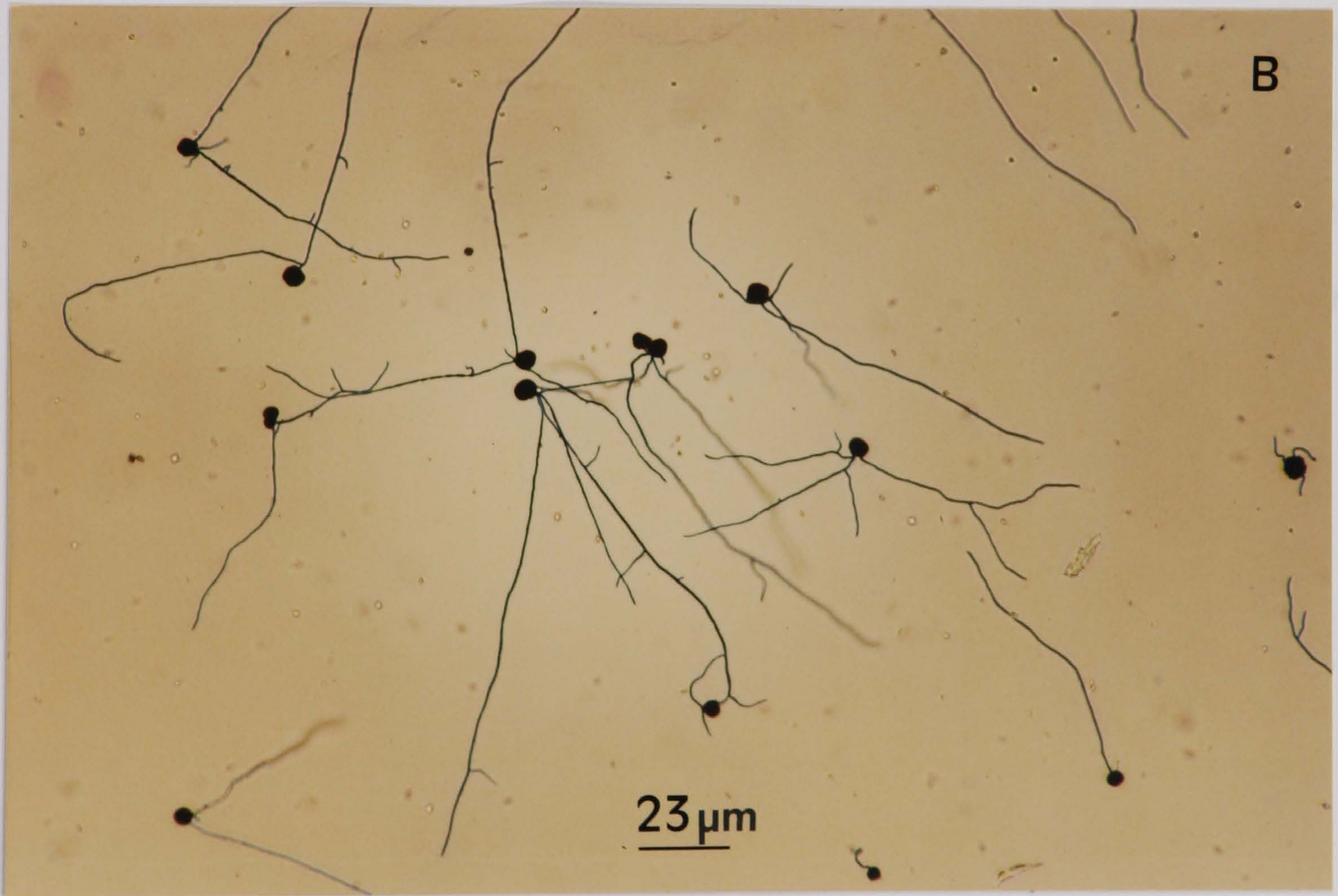


Plate 3.2. Germination of spores of *E.nigrum* on water agar at 0.935 a_w after 23 h incubation A: *E.nigrum*996. B: *E.nigrum*98.

3.5.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w

Growth of *E.nigrum* spores produced on media at 0.996 a_w (-0.7 MPa) and at 0.98 a_w (-3.0 MPa) were examined at 10, 17 and 25°C (Fig. 3.19, 3.20, 3.21). Maximum growth rates were obtained at 17°C and 0.996 a_w for both types of inoculum on PDA, 1/10 strength PDA and on a minimal salts medium. Minimum growth rates were recorded at 0.95 a_w for both culture types; at this water stress level no growth occurred in many cases. Growth was negligible at 35°C for all treatments.

Interestingly, on PDA (Fig. 3.19) *E.nigrum*98 had significantly higher growth rates than *E.nigrum*996 at 0.996 a_w when plates were incubated at 10 or 17°C, and at 0.99 a_w when the incubation temperature was 10 or 25°C (Plate 3.3 a, b). At 0.984 a_w there was no significant difference between the growth rates of both inoculum types. *E.nigrum*996 did not have higher growth rate than *E.nigrum*98 on PDA at any a_w or temperature tested, with the exception of 0.95 a_w and 25°C, where *E.nigrum*996 was able to grow, while *E.nigrum*98 did not grow over the experimental time period. The optimum temperature for growth was 17°C at 0.996, 0.990 and 0.984 a_w, with the exception of *E.nigrum*98 at 0.99 a_w, where the maximum growth rate was at 25°C.

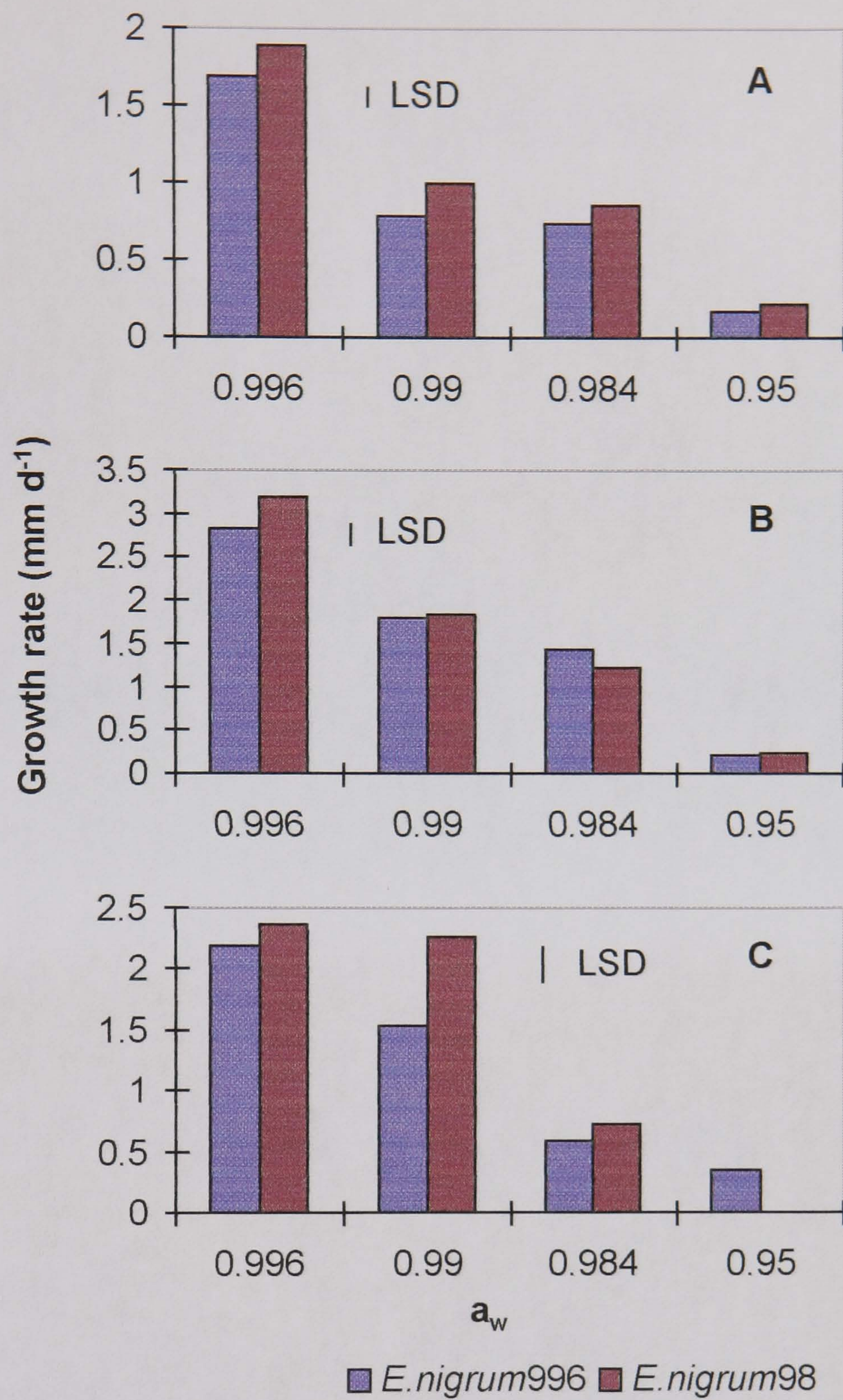
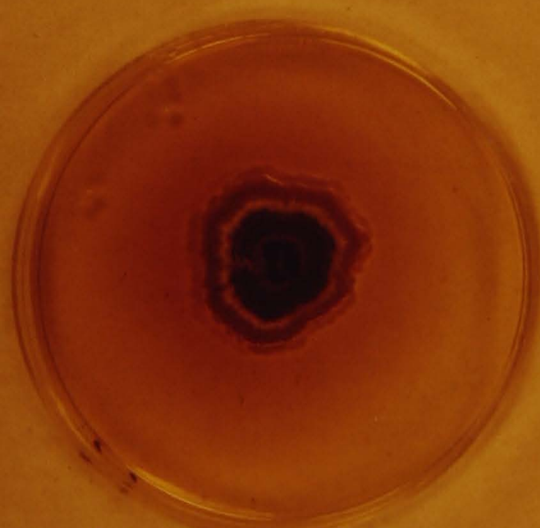


Figure 3.19. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **PDA** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates.



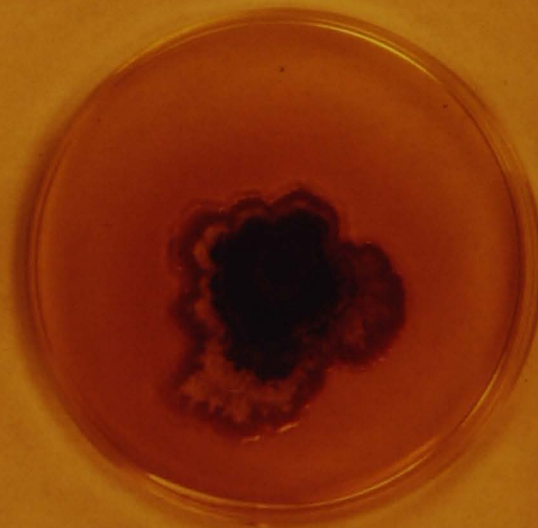


Plate 3.3. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on PDA.

a) at 0.990 a_w and 10°C, b) at 0.990 a_w and 25°C.

On 1/10 PDA and 0.996 a_w (Fig 3.20) *E.nigrum*98 grew faster than *E.nigrum*996 at 10°C, while at 17 or 25°C *E.nigrum*996 grew faster. Both inoculum types had similar growth rates at 0.990 a_w. At 0.984 a_w *E.nigrum*98 grew significantly faster than *E.nigrum*996 at 10 and 17°C, with no difference detected at 25°C. At 0.95 a_w growth was negligible at all temperatures tested. However, *E.nigrum*98 was able to grow at 17°C. The optimum temperature for growth was 17°C and 0.996 a_w. At 0.990 and 0.984 a_w both types of inoculum had maximum growth rates at 25°C. It was notable that colonies of *E.nigrum*98 caused a more intense pigmentation of the culture medium at 10°C, at all a_w levels. In other cases sporulation of *E.nigrum*98 cultures was more profuse (Plate 3.4 a, b).

On MM (Fig. 3.21) *E.nigrum*996 had higher growth rates than *E.nigrum*98 at 0.996 a_w and all the temperatures tested. *E.nigrum*98 grew significantly faster than *E.nigrum*996 at 0.990 a_w and 17°C, and at 0.984 a_w and 25°C. At 0.95 a_w there were no differences between the two inoculum types with no growth occurring at 0.95 a_w and 25°C. Optimum temperature for growth was 17°C at 0.996 a_w. At 0.984 a_w *E.nigrum*996 had maximum growth rate at 17°C, while *E.nigrum*98 grew faster at 25°C. Again, colonies of *E.nigrum*98 had more intense pigmentation than *E.nigrum*996 colonies at: 10°C and 0.996 a_w, 10°C and 0.984 a_w (Plate 3.5 a), 10°C and 0.950 a_w, 17°C and 0.990 a_w (Plate 3.5 b) and 25°C and 0.990 a_w (Plate 3.5 c). Sporulation by *E.nigrum*98 was more profuse at 17°C and 0.990 a_w (Plate 3.5 b).

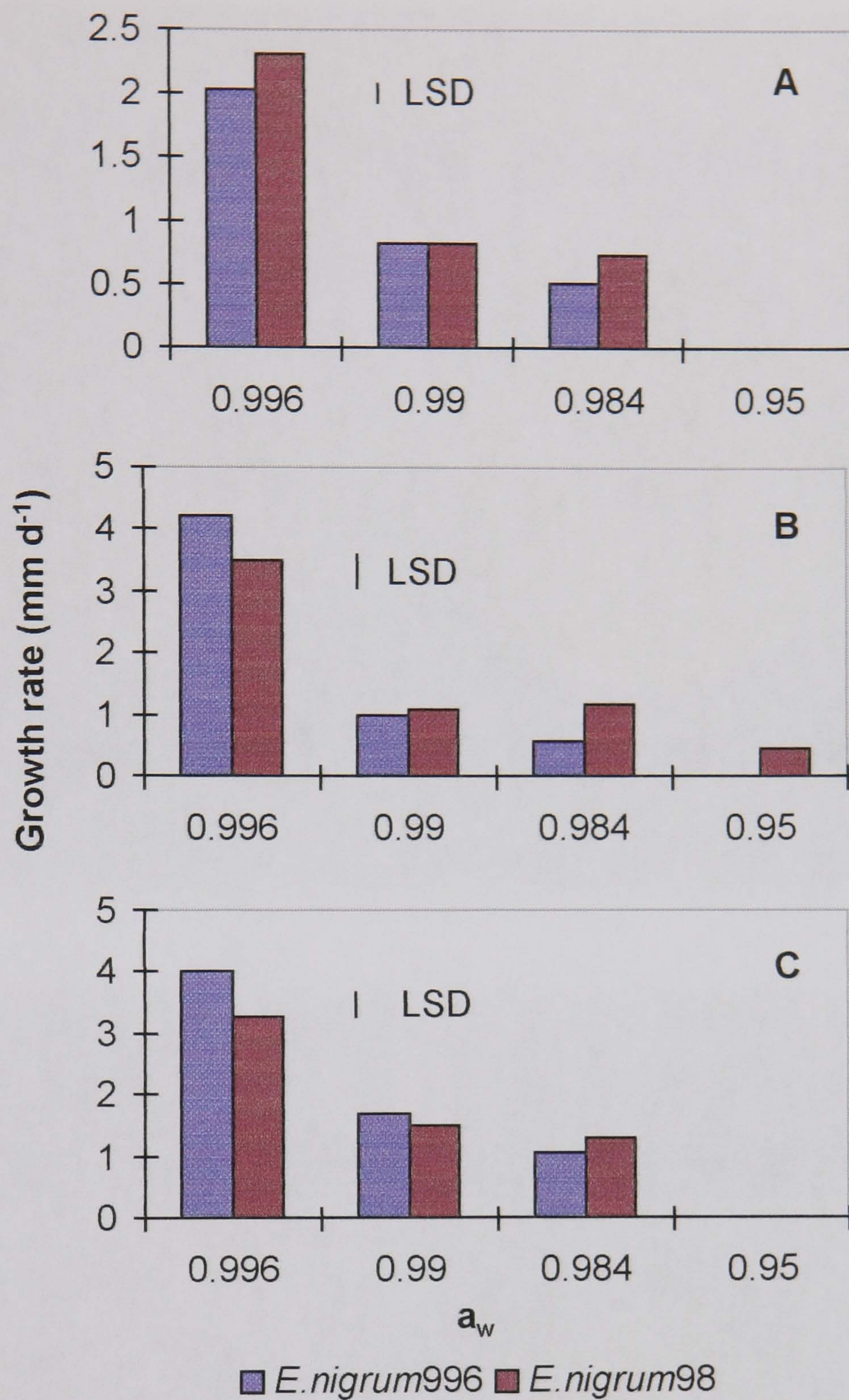
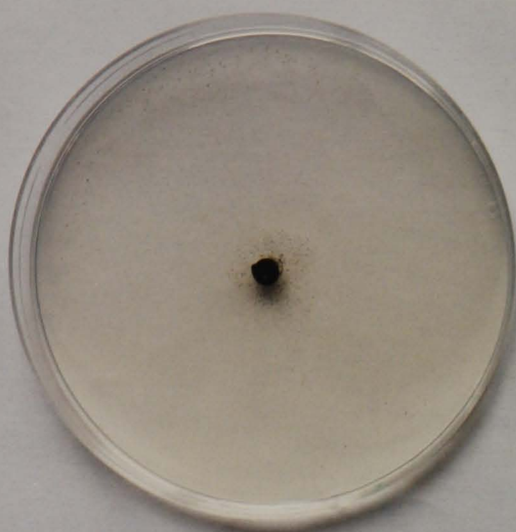
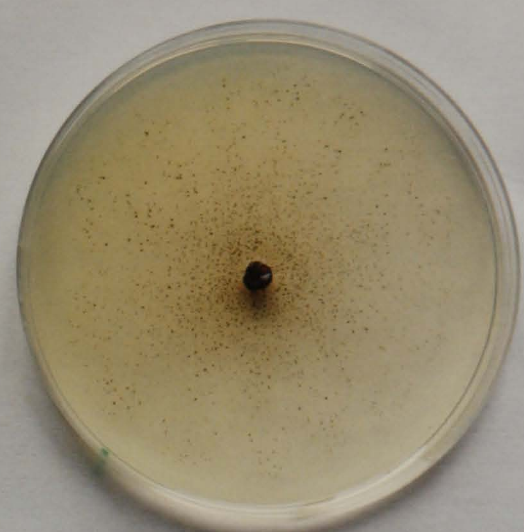


Figure 3.20. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **1/10PDA** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates.

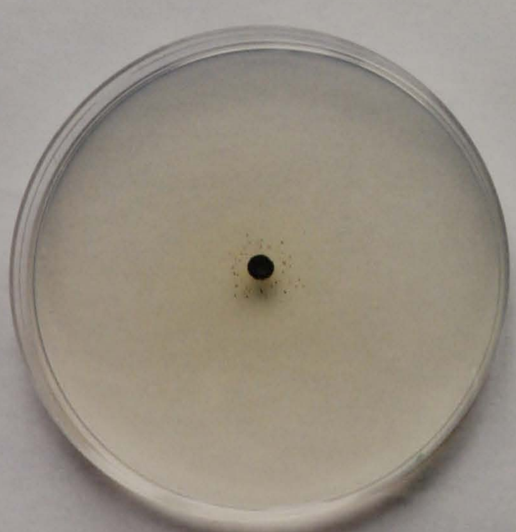
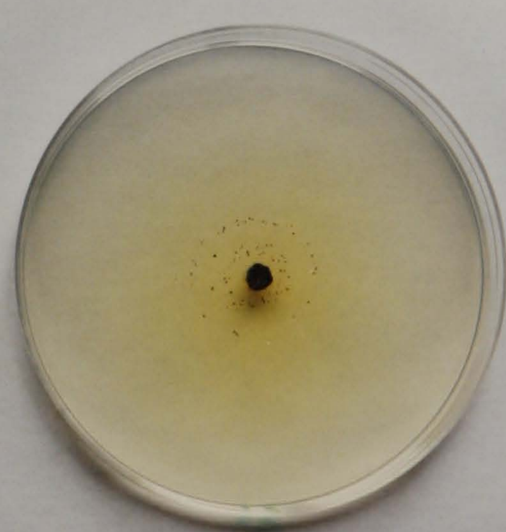



Plate 3.4. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on 1/10PDA a) at 0.996 a_w and 17°C, b) at 0.990 a_w and 25°C.

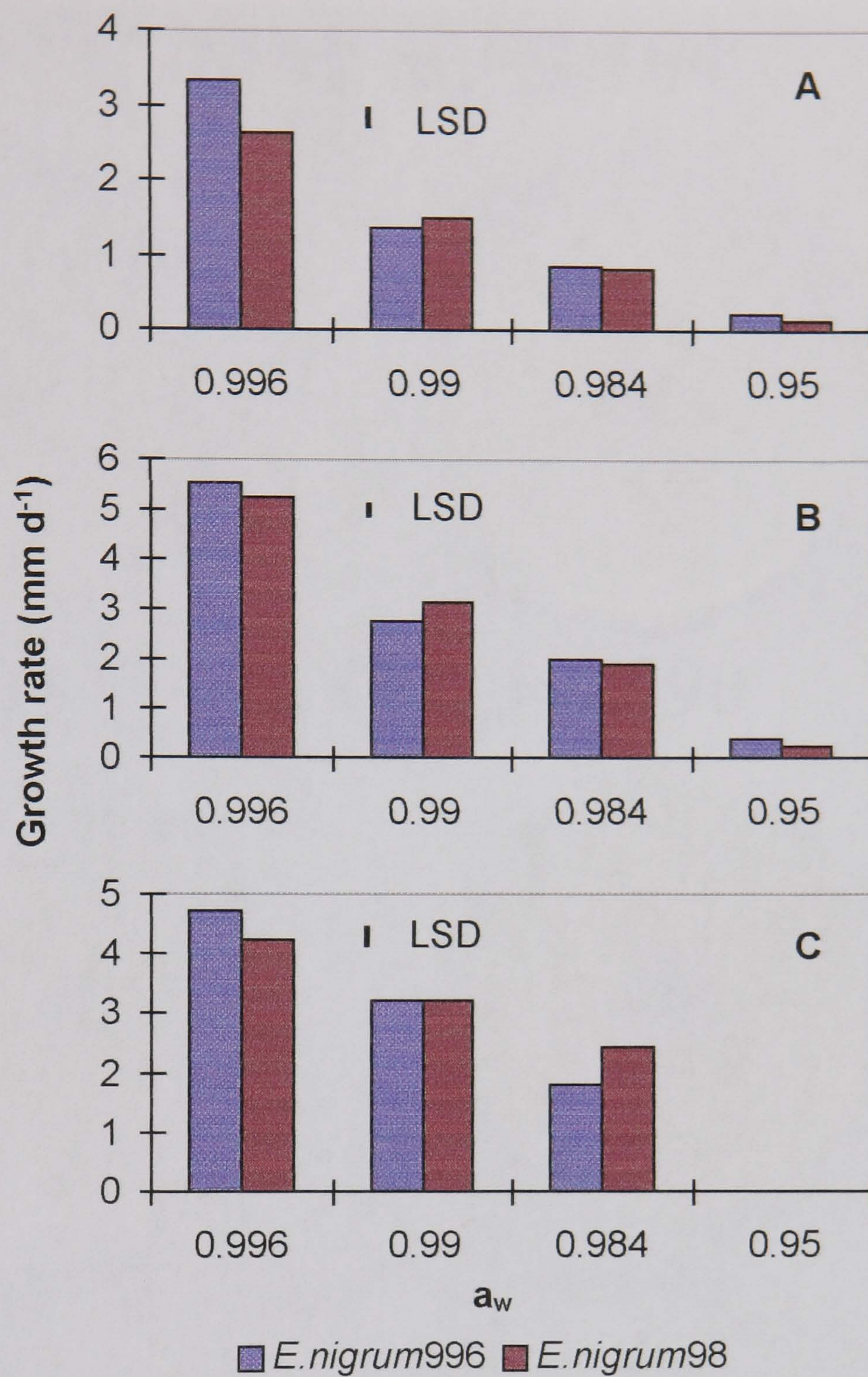
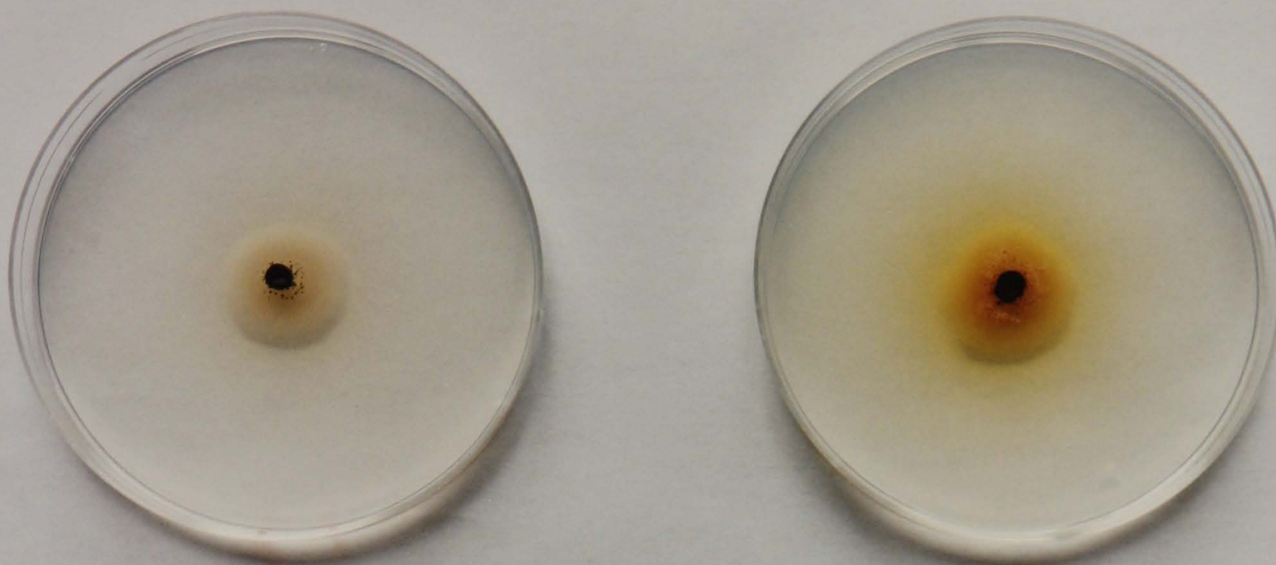
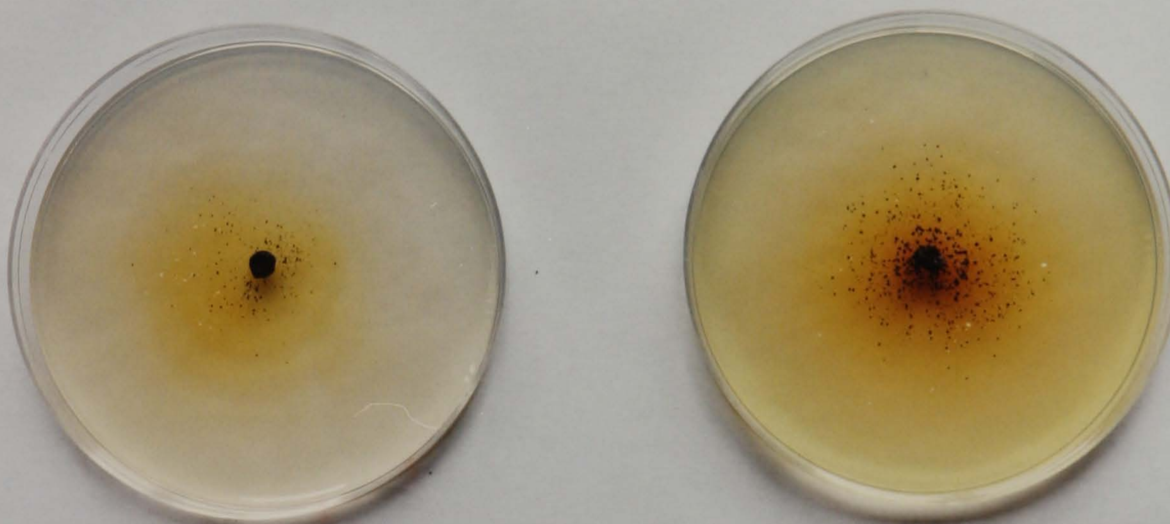
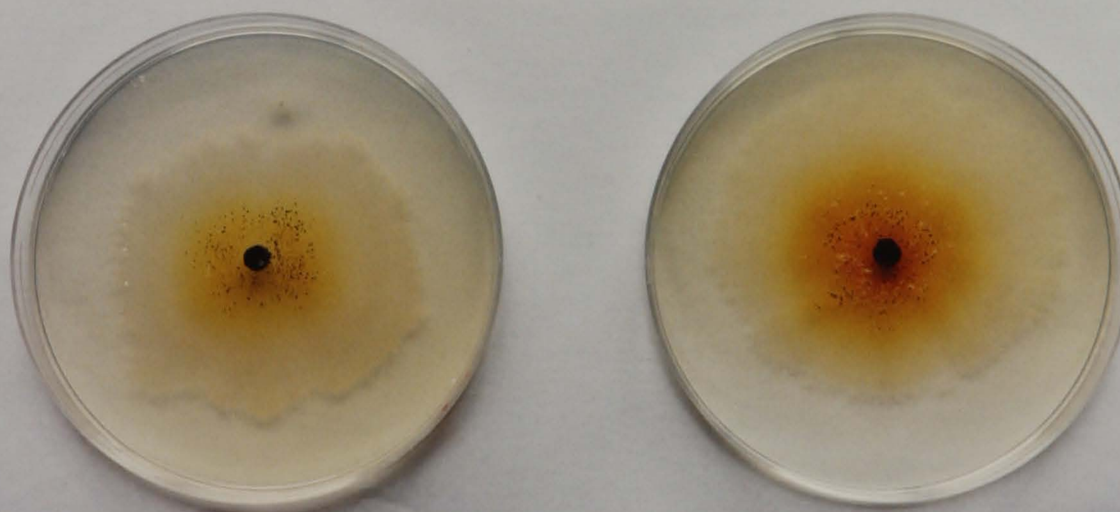


Figure 3.21. Growth rates of *E.nigrum*996 and *E.nigrum*98 on **MM** at different a_w levels and different temperatures: 10°C (A), 17°C (B) and 25°C (C). Data are means of five replicates.







*E.nigrum*996 *E.nigrum*98

Plate 3.5. Comparison of 20-d-old colonies of *E.nigrum*996 and *E.nigrum*98 on MM a) at 0.984 a_w and 10°C, b) at 0.990 a_w and 17°C, c) at 0.990 a_w and 25°C.

3.5.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans*

(a) Calibration curves

Calibration curves are shown in Fig. 3.22 for trehalose, glucose, glycerol, erythritol, arabitol and mannitol. The r^2 were 0.992, 0.994, 0.999, 0.999, 0.997 and 0.989 respectively.

(b) Solutes accumulated by *E.nigrum*

When mycelium and spores were pooled and homogenised (Table 3.19) a significant difference was observed in the amounts of glycerol, arabitol and mannitol in biomass produced in media at 0.996 a_w and at 0.98 a_w. The amounts of these three sugar alcohols (polyols) were higher in biomass grown on media at 0.98 a_w. No significant differences between the treatments were observed in the amounts of trehalose and glucose accumulated. No erythritol was detected in either of the treatments. When mycelium and spores were analysed separately and without homogenisation, a number of differences were observed (Tables 3.20 and 3.21): In mycelium produced at 0.98 a_w the amount of glycerol was significantly higher than in that produced at 0.996 a_w. No significant differences were observed in the amounts of the other solutes analysed. In spores produced at 0.98 a_w the amounts of glycerol and arabitol were significantly higher than in spores produced at 0.996 a_w. In the 0.996 a_w treatment, the highest polyol accumulated was mannitol, while in the 0.98 a_w treatment it was glycerol. No significant differences were observed in the amounts of the other solutes analysed. The amounts of all the solutes were, in general, lower when the biomass was homogenised prior to analysis.

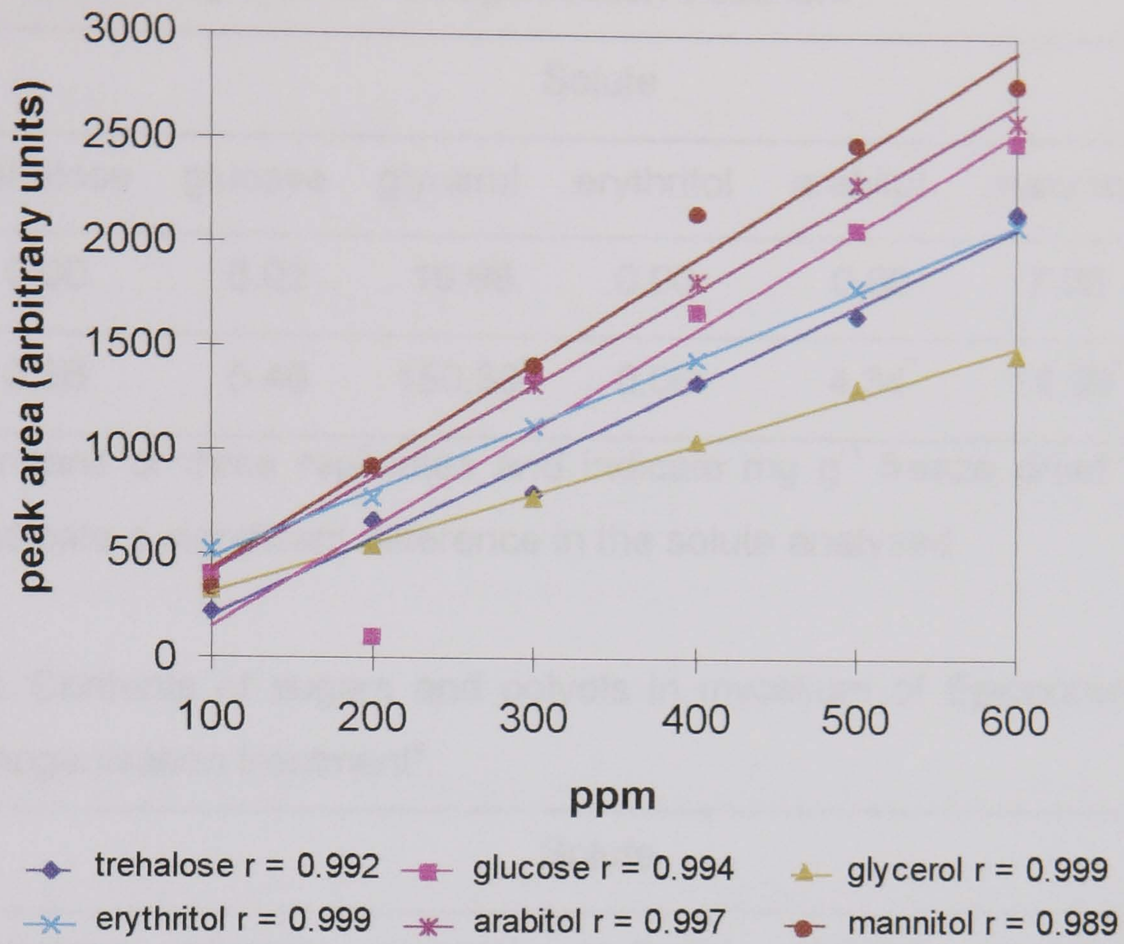


Figure 3.22. Calibration curves for the solutes analysed

Table 3.19. Contents of sugars and polyols in 10-d-old mycelium and spores of *Epicoccum nigrum* subjected to homogenisation treatment[a].

	Solute						
a_w	trehalose	glucose	glycerol	erythritol	arabitol	mannitol	Total
0.996	0.00	6.02	16.68	0.00	0.00	7.93	30.63
0.98	0.56	5.46	150.30*	0.00	4.34*	14.93*	175.59

[a]Data are means of three replicates and indicate mg g^{-1} freeze dried biomass. Asterisks indicate a significant difference in the solute analysed.

Table 3.20. Contents of sugars and polyols in mycelium of *Epicoccum nigrum* without homogenisation treatment[a].

	Solute						
a_w	trehalose	glucose	glycerol	erythritol	arabitol	mannitol	Total
0.996	56.21	74.85	2.38	0.20	22.68	123.89	280.21
0.98	21.69	87.04	382.29*	0.00	25.41	82.04	598.47

[a]Data are means of three replicates and indicate mg g^{-1} freeze dried biomass. Asterisks indicate a significant difference in the solute analysed.

Table 3.21. Contents of sugars and polyols in spores of *Epicoccum nigrum* without homogenisation treatment[a].

	Solute						
a_w	trehalose	glucose	glycerol	erythritol	arabitol	mannitol	Total
0.996	15.91	75.85	15.84	1.74	4.45	99.93	213.72
0.98	22.88	78.91	149.31*	0.00	16.37*	66.79	334.26

[a]Data are means of three replicates and indicate mg g^{-1} freeze dried biomass. Asterisks indicate a significant difference in the solute analysed.

(c) Temporal changes in accumulation of polyols and sugars in mycelium and spores of *E.nigrum*

Mycelium: Results of the temporal accumulation of solutes in mycelium of *E.nigrum* are shown in Fig. 3.23 and 3.24. The content of trehalose increased with time in mycelium produced at both 0.996 a_w and 0.98 a_w. However, for all the sampling times trehalose content was higher in mycelium produced at 0.996 a_w, and significantly so after 20 days. The content of glucose decreased with time in mycelium produced at 0.996 a_w but there was no clear trend of accumulation in mycelium produced at 0.98 a_w, but in the latter treatment concentrations were always smaller. The content of glycerol was always much higher in mycelium produced at 0.98 a_w, with a maximum concentration in 5-day-old mycelium. Temporal accumulation patterns of arabitol were similar in both mycelial treatments with no clear trend with time. For all the dates tested arabitol content was higher in mycelium produced at 0.98 a_w, the difference being significant after 15 and 20 d and maximum in 20 d mycelium. The content of mannitol increased with time until the 10th-20th day and then decreased in both types of mycelium. Mycelium produced at 0.996 a_w had a higher mannitol content at the 10 and 20 d sample time. No erythritol was detected in either type of mycelium.

Spores: In spores of *E.nigrum* (Fig. 3.25, 3.26) the content of trehalose decreased with time until the 15th day and then increased only in spores produced at 0.98 a_w. The content of glucose increased with time until the 10th-15th day and then decreased in both types of spores. Content of glycerol was always much higher in spores produced at 0.98 a_w but did not show marked changes with time.

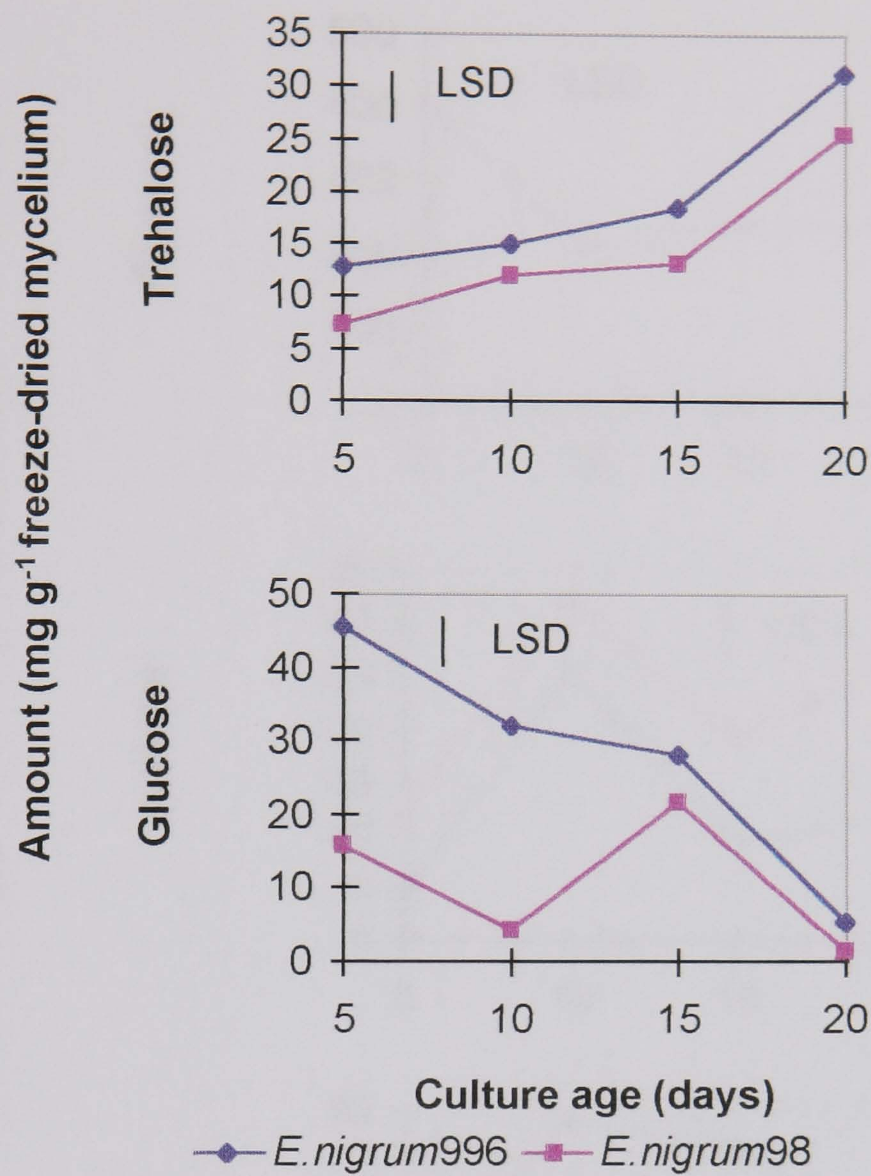


Figure 3.23. Accumulation of trehalose and glucose in mycelium of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

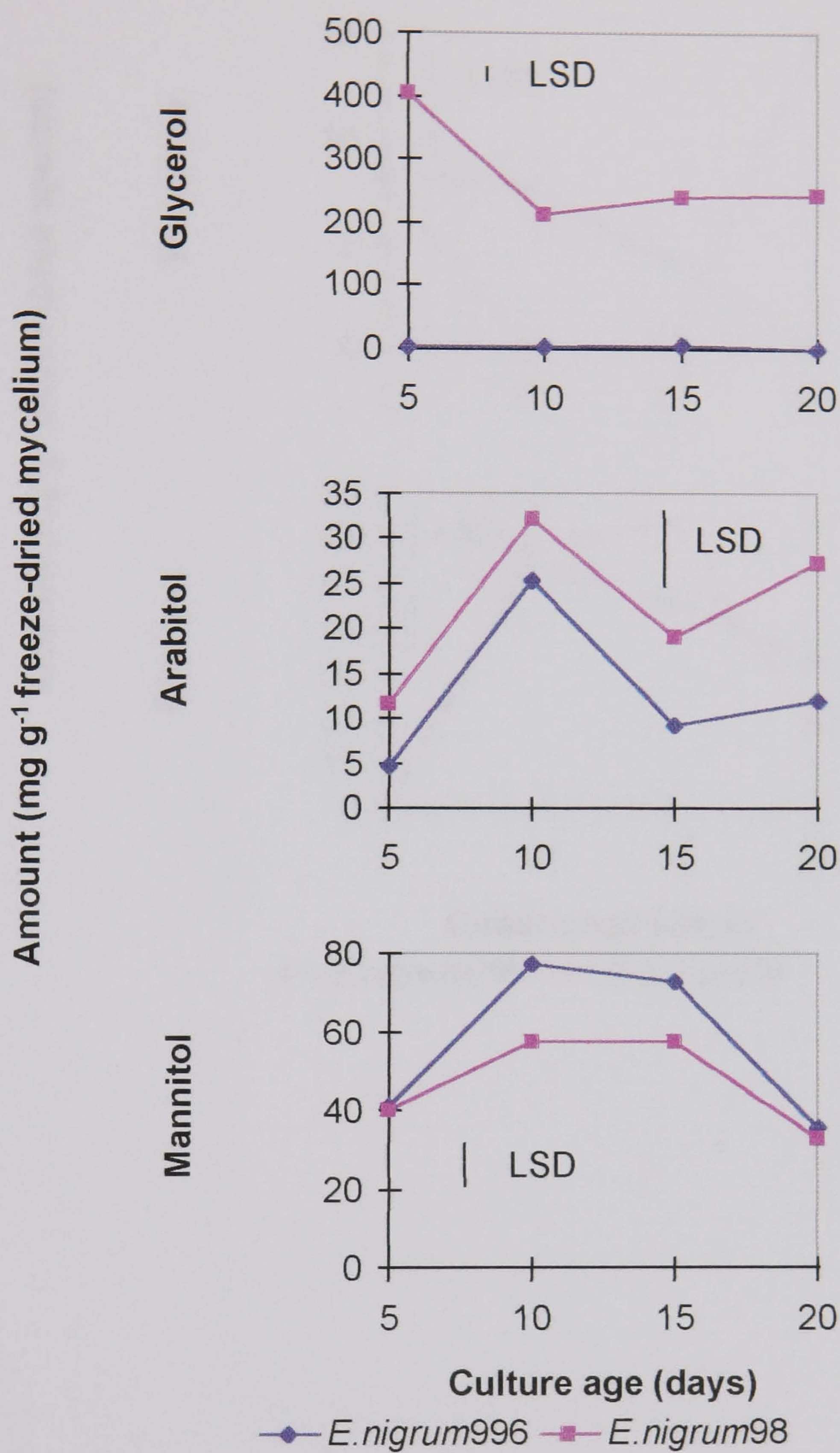


Figure 3.24. Accumulation of glycerol, arabitol and mannitol in mycelium of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

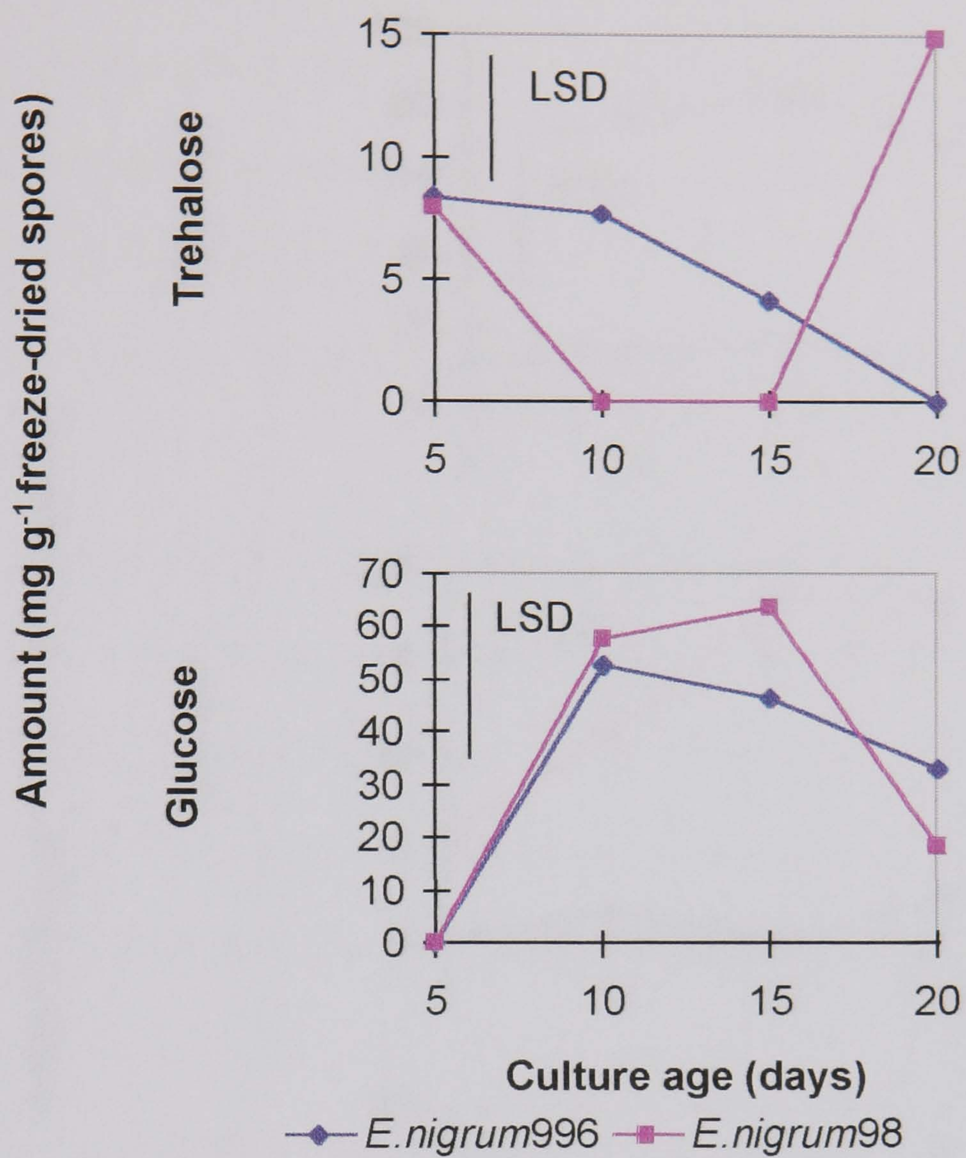


Figure 3.25. Accumulation of in trehalose and glucose in spores of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

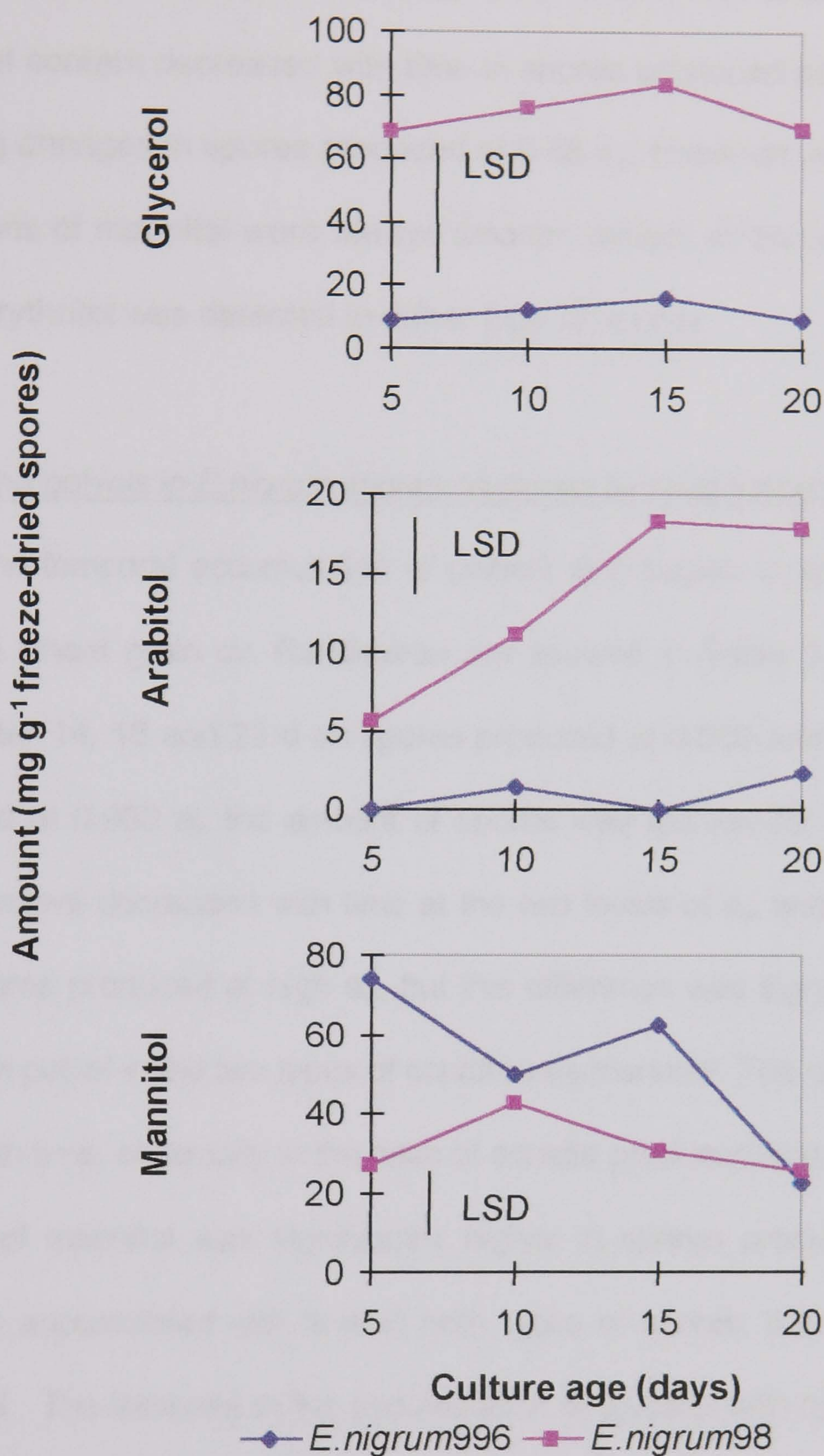


Figure 3.26. Accumulation of glycerol, arabitol and mannitol in spores of *E.nigrum*996 and *E.nigrum*98. Data are means of three replicates

The content of arabitol increased with time in spores produced at 0.98 a_w but did not do so in spores produced at 0.996 a_w, which always had lower concentrations. The mannitol content decreased with time in spores produced at 0.996 a_w and did not show big changes in spores produced at 0.98 a_w. However, at the lower a_w the concentrations of mannitol were always smaller, except at the last sampling time (20 d). No erythritol was detected in either type of spores.

(d) Sugars and polyols in *E.nigrum* spores produced by solid fermentation

Results of the temporal accumulation of polyols and sugars in spores of *E.nigrum* produced on wheat grain cv. Rendeveau are showed in Table 3.22. Analysis was done only after 14, 18 and 23 d on spores produced at 0.980 and 0.996 a_w. Earlier, after 7 d and at 0.960 a_w the amount of spores was too low for detailed analysis. Trehalose content decreased with time at the two levels of a_w tested. It was always higher in spores produced at high a_w, but this difference was significant only at day 23. The main polyol in the two types of conidia was mannitol. The content of mannitol increased with time, especially in the case of conidia produced at 0.996 a_w. At day 23 the amount of mannitol was significantly higher in spores produced at 0.996 a_w. Glycerol also accumulated with time in both types of spores, but in lower amounts than mannitol. The increase in the accumulation of glycerol with time was higher for spores produced at 0.980 a_w. By day 23 the amount of glycerol was significantly higher in spores produced at 0.980 a_w. Arabitol was found in lower amounts than glycerol in both types of spores, but this polyol accumulated specifically in spores produced at the lower a_w. At days 18 and 23 the amount of arabitol was significantly higher in spores produced at 0.980 a_w. Glucose content increased with time both in spores produced at 0.996 and 0.980 a_w. At day 14 the content of glucose was

significantly higher in spores produced at 0.980 a_w. No accumulation of erythritol was detected in any of the samples.

Table 3.22. Accumulation of solutes in spores of *E.nigrum* produced on wheat grain cv. Rendeveau at two levels of water activity (a_w). Data indicate mg g^{-1} freeze dried spores[a]

	Days after inoculation						
	14		18		23		
Solute	0.996 a_w	0.980 a_w	0.996 a_w	0.980 a_w	0.996 a_w	0.980 a_w	LSD
Trehalose	14.61	10.96	7.41	3.41	6.77	1.89	4.78
Glucose	4.09	13.15	32.01	29.45	34.79	38.17	5.11
Glycerol	0.28	0.92	6.67	6.33	7.79	10.03	2.14
Arabitol	0.00	0.36	0.17	2.27	0.42	3.59	1.47
Mannitol	4.06	6.08	29.84	23.5	37.91	24.97	7.37

[a] Data are means of three replicates

Results of the temporal accumulation of solutes in spores produced on wheat grains cv. Brigadier modified with water/glycerol solutions are shown in Table 3.23. In this case trehalose was only detected in small amounts at day 21, when the trehalose content was significantly higher in spores produced at 0.996 a_w. The main polyol was again mannitol in the three types of spores analysed, with the highest amount being detected at day 21 in spores produced at 0.996 a_w. Glycerol also accumulated with time, and the increase was higher in spores produced at 0.98 a_w. Arabitol accumulated with time, but in contrast to that observed with the first wheat cultivar, accumulation was higher in spores produced at high a_w. Glucose was accumulated in larger quantities in spores produced at 0.98 a_w, when the a_w of the substrate was adjusted with a solution of glycerol/water. No accumulation of erythritol was detected in any of the samples.

Table 3.23. Accumulation of solutes in spores of *E.nigrum* produced on wheat grain cv. Brigadier at two levels of water activity (a_w). Data indicate mg g^{-1} freeze dried spores[a]

Solute	a_w	Days after inoculation			LSD
		7	14	21	
Trehalose	0.996	0.00	0.00	1.78	
	0.98	0.00	0.00	0.56	
	0.98[g]	0.00	0.00	0.00	0.61
Glucose	0.996	0.00	5.12	9.33	
	0.98	0.00	0.56	15.84	
	0.98[g]	0.00	1.47	40.78	11.91
Glycerol	0.996	0.00	2.36	3.64	
	0.98	0.00	5.01	17.23	
	0.98[g]	0.00	2.55	13.76	6.19
Arabitol	0.996	1.61 (0.11)	2.72 (0.16)	17.76 (0.43)	
	0.98	1.78 (0.13)	2.25 (0.15)	8.96 (0.30)	
	0.98[g]	3.55 (0.19)	3.39 (0.18)	4.28 (0.21)	(0.08)
Mannitol	0.996	16.49 (0.42)	16.52 (0.42)	34.63 (0.63)	
	0.98	0.50 (0.04)	0.81 (0.07)	19.03 (0.45)	
	0.98[g]	18.10 (0.44)	9.26 (0.31)	16.97 (0.42)	(0.07)

[a] Data are means of three replicates. Data in brackets are transformed by asin (sqrt(x/100)). [g] a_w was adjusted with a solution of glycerol/water

(e) Sugars and polyols in the spore washings from solid substrate fermentation

Results from cv. Rendeveau are shown in Table 3.24. Trehalose was higher in the case of washings from spores produced at 0.996 a_w, as it happened with the spores (content of trehalose was higher in spores produced at 0.996 a_w). The main polyol present in spore washings was mannitol. The amounts of mannitol increased with time in the extraction solution of both spore types. However, mannitol was present in bigger amounts in the case of 0.996 a_w treatment. The second highest quantity of

polyol was glycerol in the case of 0.996 a_w, and arabitol in the case of 0.98 a_w. No difference was detected in the amount of glycerol in the extraction liquid of both spore types. In both cases the amount of glycerol increased with time, but unlike the content in spores, this increase was not higher for *E.nigrum*98. Arabitol quantities increased with time in both cases, unlike the content in spores, which increased only in *E.nigrum*98 spores and was always very low in *E.nigrum*996 spores. Arabitol amounts removed were higher in the case of *E.nigrum*98. Glucose was present in the washing liquid consistently in high amounts, in many cases out of the range of the calibration curves. The amount of glucose present increased with time for both spore types, as with the content in spores.

Table 3.24. Concentration (ppm) of solutes in the washings used to extract *E.nigrum* spores from wheat grain cv. Rendeveau at two levels of water activity (a_w)[a]

	Days after inoculation						
	14		18		23		
Solute	0.996 a_w	0.980 a_w	0.996 a_w	0.980 a_w	0.996 a_w	0.980 a_w	LSD
Trehalose	31.333	0.000	0.000	2.567	60.327	0.000	50.120
Glucose	521.07	449.83	>600.00	589.27	>600.00	>600.00	128.65
Glycerol	15.33	2.67	113.50	109.82	334.37	325.60	120.98
Arabitol	36.33	199.30	159.00	251.43	182.37	422.60	98.13
Mannitol	308.30	90.86	>600.00	281.43	>600.00	>600.00	120.76

[a] Data are means of three replicates

Results from cv. Brigadier are shown in Table 3.25. At day 7 amounts of solutes detected were negligible. The main polyol present in spore washings was mannitol. Mannitol was present in bigger amounts in the case of 0.996 a_w treatment, at day 14. The second highest quantity of polyol was arabitol, which was washed out in higher

quantities in the case of 0.98 a_w treatment (adjusted with water). The amount of glycerol washed out increased with time for the 0.98 a_w treatment, and with a_w adjusted with glycerol/water. The amount of glucose present increased with time for spores produced at 0.98 a_w (adjusted with water or glycerol/water).

Table 3.25. Concentration (ppm) of solutes in the washings used to extract *E.nigrum* spores from wheat grain cv. Brigadier at two levels of water activity (a_w)[a]

	Days after inoculation						
	14			21			
Solute	0.996 a_w	0.98 a_w	$0.98^g a_w$	0.996 a_w	0.98 a_w	$0.98^g a_w$	LSD
Trehalose	26.70	0.00	0.00	0.00	44.45	33.33	NS
Glucose	474.00	9.33	147.30	179.33	342.00	695.00	294.31
Glycerol	1.67	6.67	0.00	47.5	42.67	81.33	74.87
Arabitol	92.5	54.2	61.3	96.00	340.00	105.00	116.18
Mannitol	404.00	112.00	161.00	282.00	224.00	206.00	195.70

[a] Data are means of three replicates. NS: not significant

(f) Polyols and sugars accumulated by *P.frequentans*

Since *P.frequentans* is another potential BCA for control of twig blight in the phylloplane, studies on physiological accumulation of sugars/sugar alcohols were conducted to try and improve ecological competence and water stress tolerance. The fungus was grown on PDB at high (0.996) and reduced a_w (0.95).

When mycelium and spores of the two treatments were homogenised (Table 3.26) significant differences were observed in the amounts of trehalose, glycerol, arabitol and mannitol endogenously accumulated. For all of the sugars and

polyols the amounts were higher in biomass produced in medium at lowered a_w (0.95).

When mycelium and spores were not homogenised (Table 3.27) significant differences were observed in the amounts of trehalose, glucose, glycerol, arabitol and mannitol. The amount of glycerol was higher in biomass produced under water stress (0.95 a_w). The amounts of trehalose, glucose, arabitol and mannitol were higher in biomass produced at 0.996 a_w. Overall, the amounts of sugars and polyols were lower when the biomass was homogenised prior to analysis.

Table 3.26. Contents of sugars and polyols in mycelium and spores of *Penicillium frequentans* subjected to homogenisation treatment[a].

	Solute						
a_w	trehalose	glucose	glycerol	erythritol	arabitol	mannitol	Total
0.996	0.00	17.13	9.22	7.06	1.69	16.54	51.64
0.95	11.28*	6.17	189.30*	5.66	8.10*	24.11*	244.62

[a]Data are means of three replicates and indicate mg g^{-1} freeze dried biomass. Asterisks indicate a significant difference in the solute analysed.

Table 3.27. Contents of sugars and polyols in mycelium and spores of *Penicillium frequentans* without homogenisation treatment[a].

	Solute						
a_w	trehalose	glucose	glycerol	erythritol	arabitol	mannitol	Total
0.996	11.62*	75.97*	2.42	115.05	9.31*	128.99*	343.36
0.95	4.38	13.19	273.83*	0.00	2.85	7.00	301.25

[a]Data are means of three replicates and indicate mg g^{-1} freeze dried biomass. Asterisks indicate a significant difference in the solute analysed.

(g) Temporal changes in accumulation of sugars and polyols in mycelium and conidia of *P.frequentans*

Results of the time course of accumulation of sugars and polyols in biomass of *P.frequentans* are shown in Figs. 3.27 and 3.28. The trehalose content decreased from the 5th till the 10th day in biomass produced at both 0.996 a_w and 0.95 a_w. From the 10th until the 15th day the content of trehalose increased in biomass produced at 0.95 a_w. The content of glucose decreased with time in both types of biomass, and the concentration was always higher in biomass produced at 0.996 a_w. Glycerol content was always much higher in biomass produced at 0.95 a_w, the maximum amount being recorded at day 5. The erythritol content decreased with time in biomass produced when water was freely available (0.996 a_w) and increased in biomass produced under drier conditions (0.95 a_w), which always showed smaller endogenous amounts. The content of arabitol was always very low in both types of biomass. The content of mannitol was always higher in biomass produced at the higher water availability treatment (0.996 a_w).

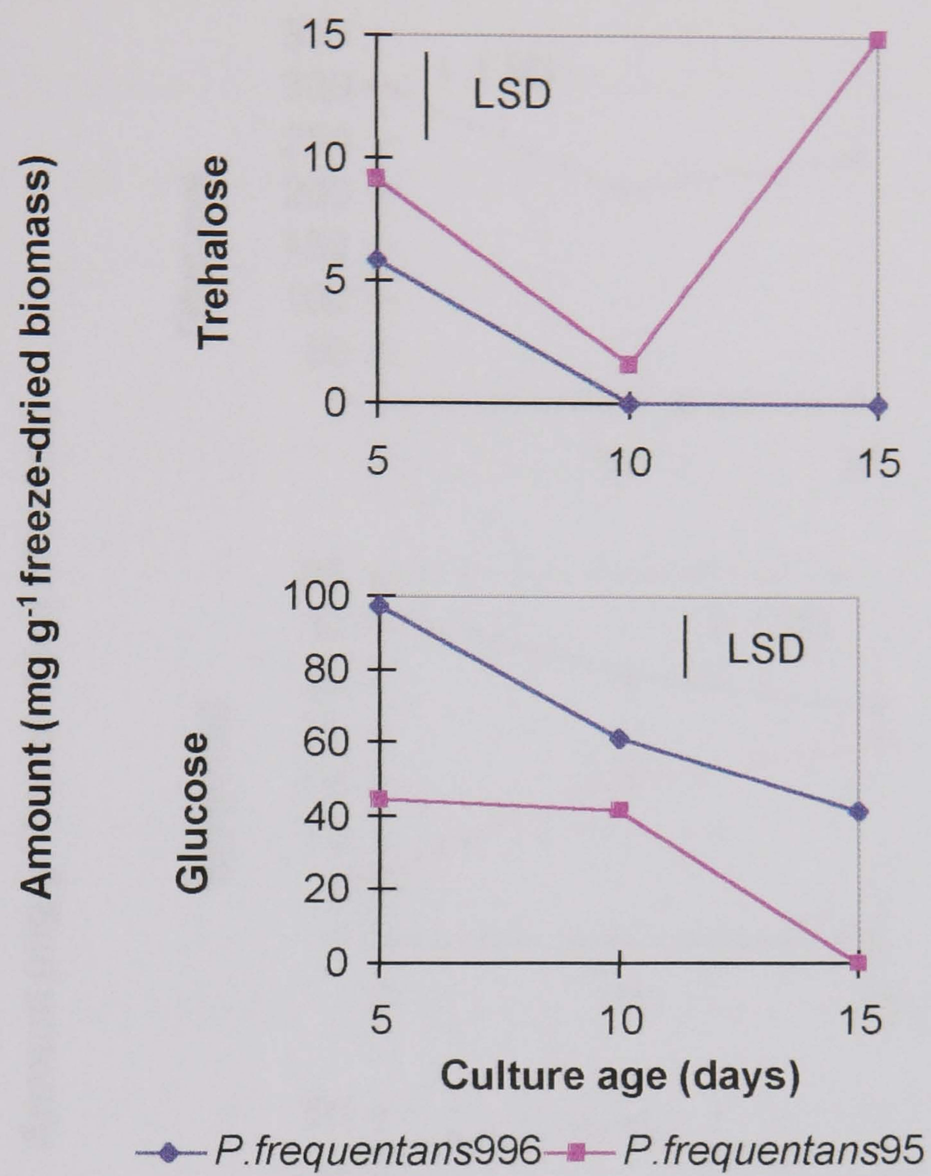


Figure 3.27. Accumulation of trehalose and glucose in biomass of *P.frequentans*996 and *P.frequentans*95. Data are means of three replicates

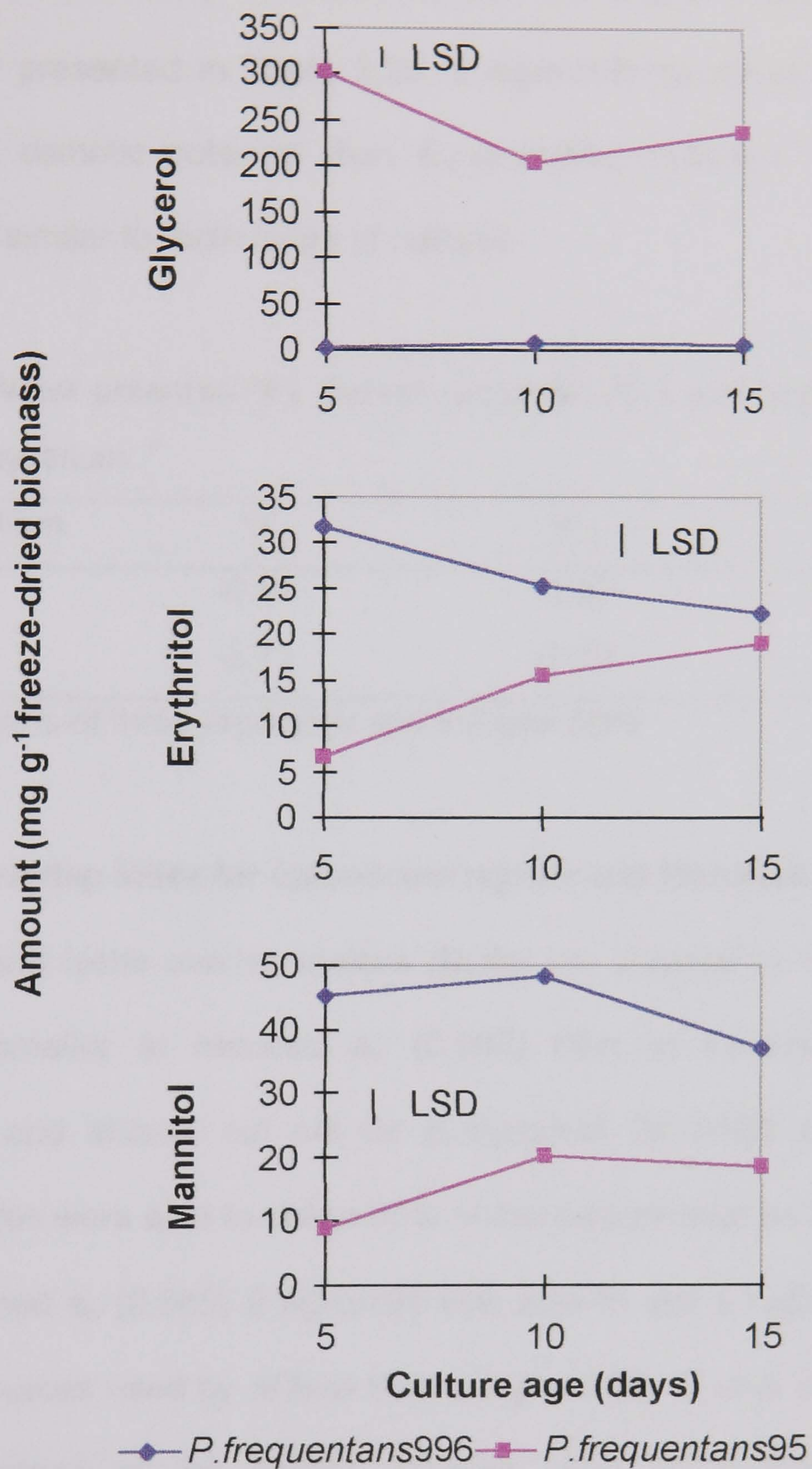


Figure 3.28. Accumulation of glycerol, erythritol and mannitol in biomass of *P.frequentans*996 and *P.frequentans*95. Data are means of three replicates

3.5.4. Turgor pressure in *Epicoccum nigrum* mycelium

Values of water potential, osmotic potential and turgor pressure for *E.nigrum* mycelium are presented in Table 3.28. *E.nigrum*98 mycelium had lower water potential and osmotic potential than *E.nigrum*996 mycelium. However, turgor pressure was similar for both types of cultures.

Table 3.28. Water potential (Ψ), osmotic potential (Ψ_{Π}) and turgor pressure (Ψ_P) of *E.nigrum* mycelium. [a]

Type of mycelium	Ψ	Ψ_{Π}	Ψ_P
*E.nigrum*996	-0.61	-1.07	0.46
*E.nigrum*98	-2.71	-3.19	0.48

[a] Data are means of three replicates and indicate MPa

3.5.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa*

Niche sizes and niche overlap indices (NOIs) are showed in Table 3.29. Niche sizes were smaller at reduced a_w (0.985) than at normal a_w (0.996) for *E.nigrum*996 and *M.laxa*, but not for *E.nigrum*98. At 0.996 a_w both types of *E.nigrum* spores were able to utilise 85% of the carbon sources that *M.laxa* used, while at reduced a_w (0.985) *E.nigrum*98 was able to use a higher percentage of the carbon sources used by *M.laxa* than *E.nigrum*996. *M.laxa* was able to use a higher percentage of the carbon sources used by *E.nigrum*996 than by *E.nigrum*98, especially at reduced a_w (0.985 a_w).

Table 3.29. NOIs for *E.nigrum* paired with *M.laxa*, derived from carbon source utilisation data, at two a_w levels

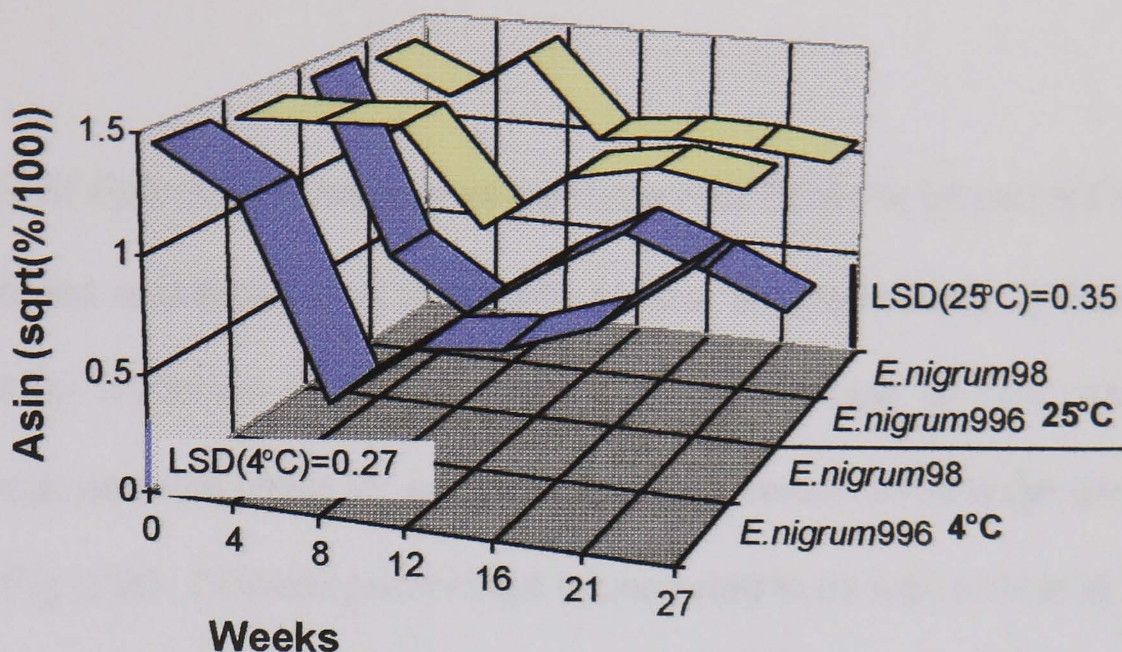
	a_w					
	0.996			0.985		
Spore type	Niche size[a]	$NOI_{M.laxa}$[b]	$NOI_{E.nigrum}$[c]	Niche size	$NOI_{M.laxa}$	$NOI_{E.nigrum}$
*E.nigrum*996	44	0.85	0.66	34	0.76	0.71
*E.nigrum*98	45	0.85	0.64	46	0.90	0.57

[a] Per 96 carbon sources. The niche size for *M.laxa* was 34 at 0.996 a_w and 29 at 0.985 a_w. [b] $NOI_{M.laxa}$ represents the proportion of the carbon compounds utilised by *M.laxa* that were also utilised by *E.nigrum*. [c] $NOI_{E.nigrum}$ represents the proportion of the carbon compounds utilised by *E.nigrum* that were also utilised by *M.laxa*.

3.5.6. Viability of *Epicoccum nigrum* spores after storage

Viability of fresh *E.nigrum* spores was different for the two spore types studied. *E.nigrum*98 spores survived better than *E.nigrum*996 spores, at either 4 or 25°C (Fig. 3.29). *E.nigrum*996 spores lost viability very quickly: after 8 weeks at 4°C and only after 4 weeks at 25°C, there were significant decreases in survival. However, after this first decrease, viability was maintained, and even in some cases data indicated abnormal increases. Viability of *E.nigrum*98 spores generally remained stable until the end of the experiment (27 weeks storage).

Freeze-drying affected the viability of both spore types studied: it was reduced from 98 to 67% for *E.nigrum*996 spores and from 96.67 to 50.67% for *E.nigrum*98 spores. Freeze-drying also affected severely the ability of spores to survive during storage: viability decreased very quickly for both spore types and at the two temperatures tested, and it was zero in all cases after 12 weeks storage (Fig. 3.29).



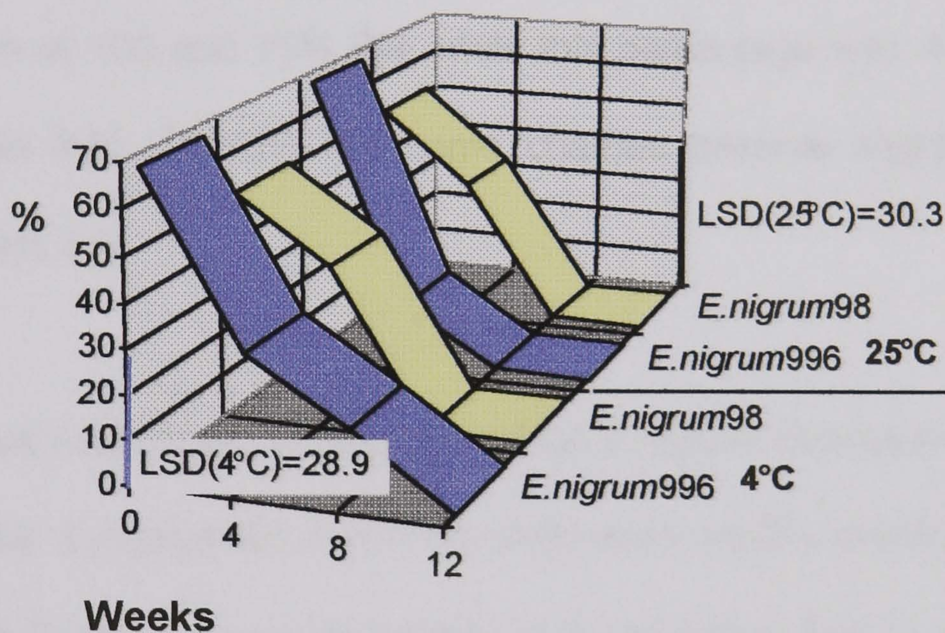


Figure 3.29 . Viability of fresh and freeze-dried *E.nigrum* spores stored at 4 or 25°C. Data are means of three replicates and indicate percentage germination on Potato Dextrose Agar (PDA). Incubation times were 8 and 20 h for fresh and freeze-dried spores, respectively.

3.6. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans*

3.6.1. Efficacy of *Epicoccum nigrum* as post-harvest BCA for brown rot of fruit

Untreated cherries and those inoculated with *M.laxa*, showed rotting symptoms and were covered by mycelium of the pathogen. The percentage of fruit covered by *M.laxa* mycelium was estimated for each fruit as the disease percentage and results are shown in Fig. 3.30. Disease percentage in untreated fruits was similar at 100 and 75% relative humidities (RH), and significantly lower at 33% RH. At the end of the experiment (10 days after inoculation) nearly all fruits were completely covered by *M.laxa* mycelium at 100 and 75% RH, while this percentage was 47% at 33% RH (Fig. 3.30, Plate 3.6). At 33% RH some cherries became slightly shrunken in appearance (Plate 3.6).

The cherries were treated with spores of the BCA *E.nigrum* obtained from unmodified and low a_w media. *E.nigrum*996 and *E.nigrum*98 were equally effective in controlling fruit rot. At days 6 and 7 nearly all treated fruits appeared free of mycelium of the pathogen. At day 10 maximum disease percentages on treated fruits were registered at 100% RH, and minimum at 33% RH, but all of them were significantly lower than the untreated controls (Fig. 3.30, Plate 3.6). In a few cases growth of *E.nigrum* was observed on cherries.

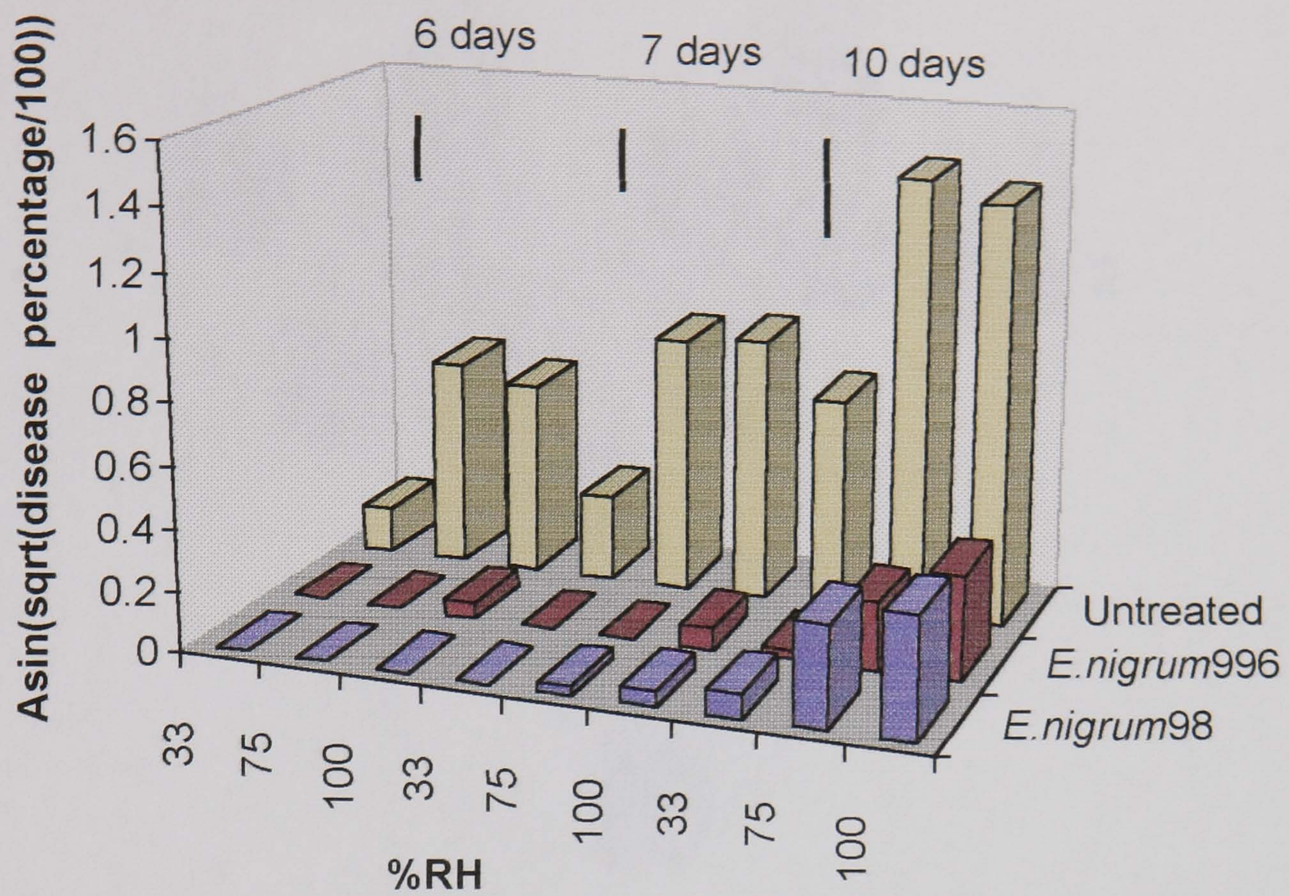


Figure 3.30. Control of brown rot of cherries by *E.nigrum*996 and *E.nigrum*98, at different relative humidities (%RH), assessed 6, 7 and 10 days after inoculation with *M.laxa*. Data are means of five replicates. Bars indicate LSD for each evaluation date.

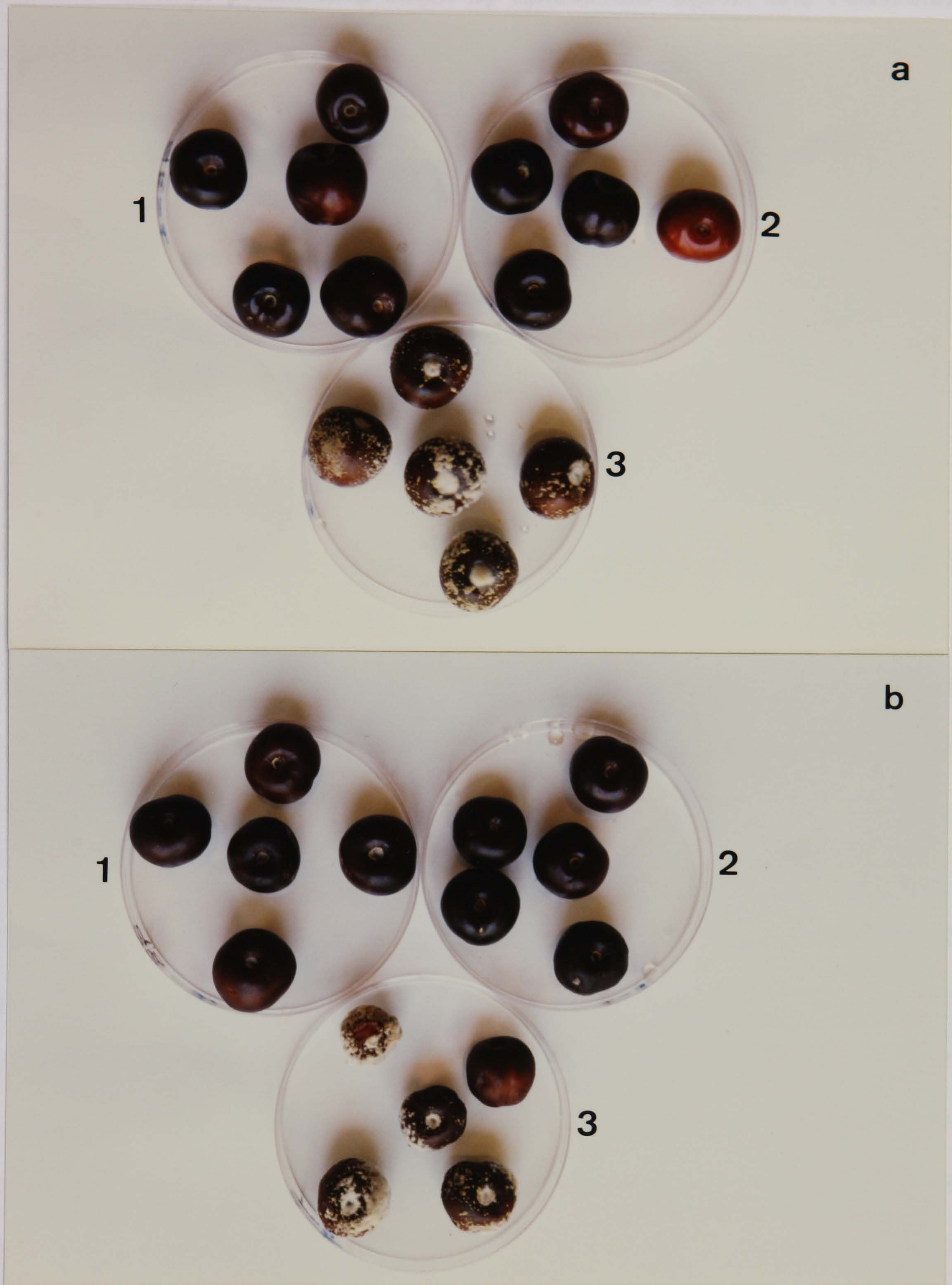


Plate 3.6. Disease control of rot of cherries (*M.laxa*) by *E.nigrum* at 75% (a) and 33% (b) relative humidity, ten days after inoculation. 1: fruits treated with *E.nigrum*98, 2: fruits treated with *E.nigrum*996, 3: untreated fruits and inoculated with the pathogen.

3.6.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans*

(a) Disease development

Inoculation of shoots with *M.laxa* caused blight symptoms similar to natural infections and to those described in previous experiments (Melgarejo *et al.*, 1986; De Cal *et al*, 1990). A progressive wilting advanced along the twig from the inoculation site, and secretion of gums was observed (Plate 3.7).

(b) Disease control

Different preparations of the BCAs *E.nigrum* and *P.frequentans* were applied, produced under water stress conditions or unstressed, and applied with or without nutrients. Shoots treated with the fungicide captan or untreated were used as controls. Treatments were applied four times and disease control was monitored for 44 days.

The development of lesions is shown in Fig. 3.31. On the last two dates of evaluation significant differences were found in the length of lesions of twigs from different treatments. Table 3.30 shows the contrast analysis of different treatments applied. Biological treatments reduced lesion length compared to the untreated control to a similar extent to the chemical treatment with captan. When the antagonists were produced in a medium at reduced a_w (0.98-0.95), lesion length was smaller than when antagonists were produced at 0.996 a_w. This difference was also observed when comparing *E.nigrum* treatments only, but not in the case of *P.frequentans*.

Plate 3.7. Symptoms of disease induced by *Monilinia laxa* on peach twigs. Rating as described in section 2.9.2.

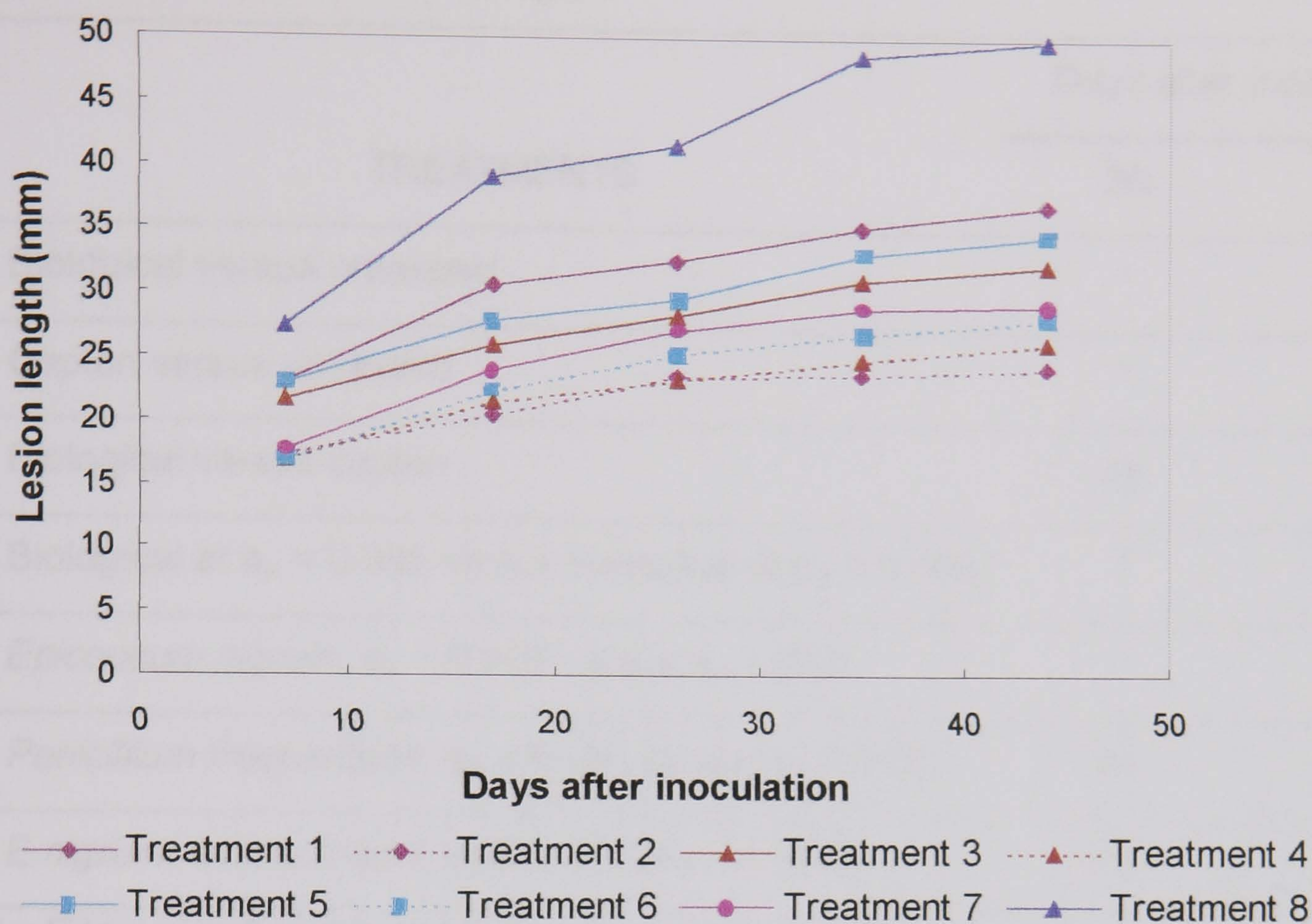


Figure 3.31. Evolution of lesions induced by *M.laxa* on peach twigs treated with different preparations of the antagonists *E.nigrum* and *P.frequentans*.

Treatment 1: *E.nigrum*; conidia + mycelium + nutrients 1; $a_w = 0.996$

Treatment 2: *E.nigrum*; conidia + mycelium + nutrients 1; $a_w = 0.98$

Treatment 3: *E.nigrum*; conidia + mycelium; $a_w = 0.996$

Treatment 4: *E.nigrum*; conidia + mycelium; $a_w = 0.98$

Treatment 5: *P.frequentans*; conidia + mycelium + nutrients 2; $a_w = 0.996$

Treatment 6: *P.frequentans*; conidia + mycelium + nutrients 2; $a_w = 0.95$

Treatment 7: captan

Treatment 8: untreated control

Table 3.30. Contrast analysis of different treatments applied to control *Monilinia laxa*. Comparison of lesion lengths

TREATMENTS	Days after inoculation	
	35	46
Biological versus untreated	*	*
Captan versus untreated	*	*
Biological versus captan	ns	ns
Biological at $a_w = 0.996$ versus biological at $a_w < 0.996$	*	*
Epicoccum nigrum: $a_w = 0.996$ versus $a_w = 0.98$	*	*
Penicillium frequentans: $a_w = 0.996$ versus $a_w = 0.95$	ns	ns
E.nigrum: with nutrients versus without nutrients	ns	ns

* = F test significant at $p = 0.05$; ns = not significant

Fig. 3.32 shows the evolution of the disease index, rating the appearance of the twig. Significant differences were observed from the 3rd sampling date. Table 3.31 shows the contrasts analysis of the different treatments applied.

The extent of colonisation of twigs by *M.laxa* in the different treatments are compared in Table 3.32. No significant differences were observed when analysing data of the extent of pathogen colonisation.

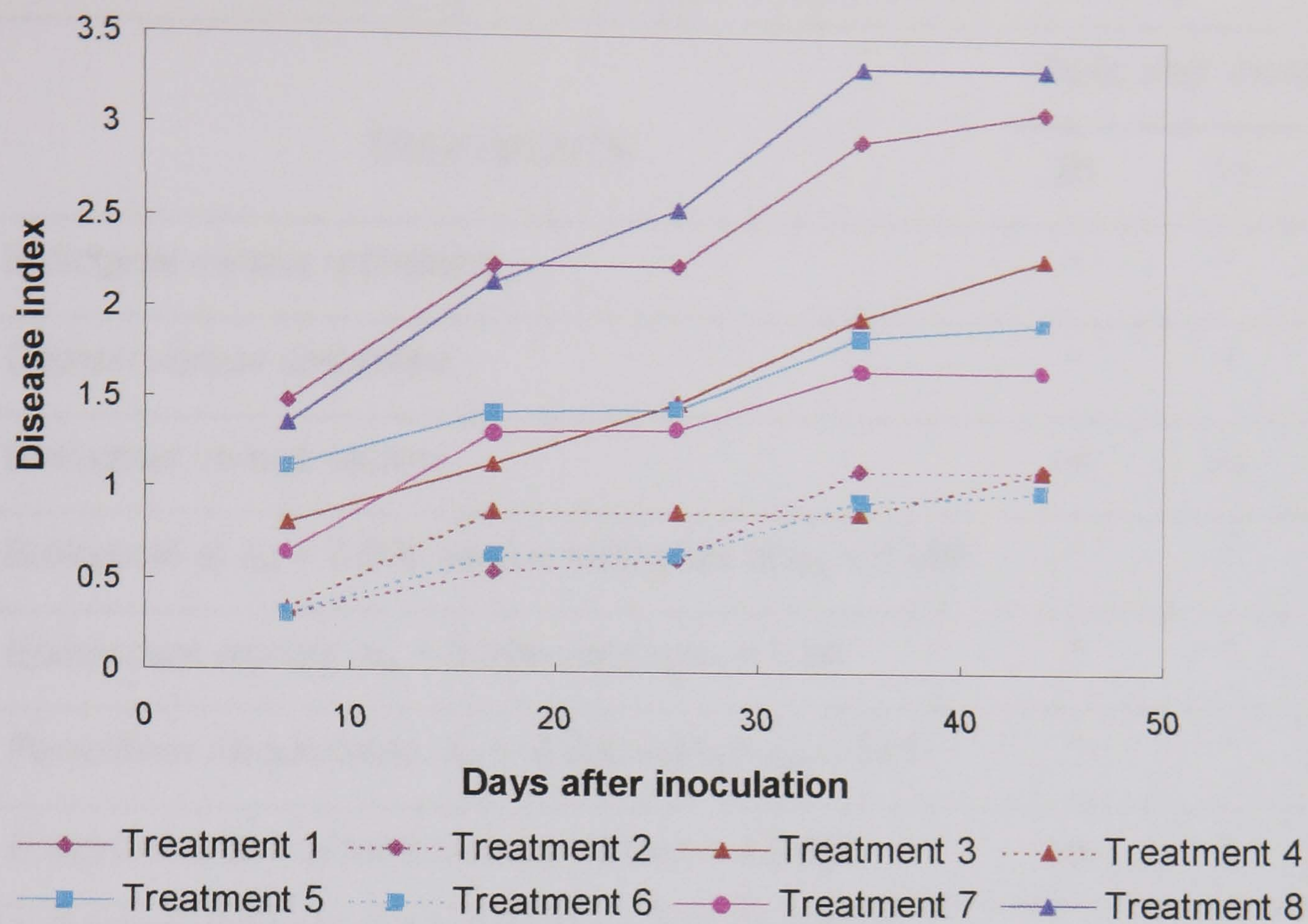


Figure 3.32. Evolution of disease index of peach twigs inoculated with *M.laxa* and treated with different preparations of the antagonists *E.nigrum* and *P.frequentans*.

Treatment 1: *E.nigrum*; conidia + mycelium + nutrients 1; $a_w = 0.996$

Treatment 2: *E.nigrum*; conidia + mycelium + nutrients 1; $a_w = 0.98$

Treatment 3: *E.nigrum*; conidia + mycelium; $a_w = 0.996$

Treatment 4: *E.nigrum*; conidia + mycelium; $a_w = 0.98$

Treatment 5: *P.frequentans*; conidia + mycelium + nutrients 2; $a_w = 0.996$

Treatment 6: *P.frequentans*; conidia + mycelium + nutrients 2; $a_w = 0.95$

Treatment 7: captan

Treatment 8: untreated control

Table 3.31. Contrast analysis of different treatments applied to control *Monilinia laxa*. Comparison of disease index rating the appearance of the twig

TREATMENTS	Days after inoculation		
	26	35	46
Biological versus untreated	*	*	*
Captan versus untreated	*	*	*
Biological versus captan	ns	ns	ns
Biological at a_w = 0.996 versus biological at a_w < 0.996	*	*	*
Epicoccum nigrum: a_w = 0.996 versus a_w = 0.98	*	*	*
Penicillium frequentans: a_w = 0.996 versus a_w = 0.95	*	*	*
E.nigrum: with nutrients versus without nutrients	ns	*	ns

* = F test significant at p = 0.05; ns = not significant.

Table 3.32. Extent of *M.laxa* colonisation on twigs evaluated 46 days after inoculation.

TREATMENT	Colonisation (mm)
1: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.996	39.3
2: *E.nigrum*; conidia + mycelium + nutrients 1; a_w = 0.98	26.2
3: *E.nigrum*; conidia + mycelium; a_w = 0.996	41.3
4: *E.nigrum*; conidia + mycelium; a_w = 0.98	39.3
5: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.996	51.2
6: *P.frequentans*; conidia + mycelium + nutrients 2; a_w = 0.95	39.3
7: captan	37.7
8: untreated	56.0

CHAPTER 4

DISCUSSION

I: STUDIES ON *Penicillium oxalicum* FOR BIOCONTROL OF *Fusarium* WILT OF TOMATO IN THE RHIZOSPHERE

4.1. PRODUCTION OF *Penicillium oxalicum* INOCULUM: INDUCTION OF SUBMERGED CONIDIATION

The major factors responsible for induction of sporulation of *P.oxalicum* in submerged culture have been determined for the first time, as part of the development process of this fungus as a BCA. Sporulation of *P.oxalicum* in solid substrates and submerged culture may be triggered by different mechanisms. Submerged sporulation did not occur using media which supported sporulation in Petri dishes (such as Czapek Dox or Maxwell and Lumsden medium). Mannose used as a carbon source supports higher sporulation than other carbon sources in solid media (Pascual *et al.*, 1997), but it failed to induce sporulation of *P.oxalicum* in submerged culture. This agrees with the behaviour observed for most fungi, which will remain entirely vegetative in submerged culture (Smith, 1978). Dryness and oxygen supply are major factors inducing sporulation in aerated solid culture. In submerged culture the (partial) absence of these major factors makes other factors play a more important role in inducing sporulation. The equilibrium between the vegetative and sporulative phase is much finer and factors needed for sporulation are much more specific than in surface culture: nutritional parameters probably need to have determined values to support sporulation, and this is not fulfilled by complex media such as malt extract agar, molasses based medium, corn steep medium or brewers yeast medium.

Only one of the methods described by Morton *et al.* (1958) was successful in stimulating sporulation in pellets in submerged culture. When glucose concentration was increased after 24 h culture (C:N=187.5) no sporulation was observed. By contrast when mycelial pellets were transferred from medium A to a nitrogen-free medium (medium B), conidiophores and conidia could be observed 24 h after transfer. This seems to indicate that nitrogen depletion rather than the C:N ratio itself is the stimulus that triggers sporulation in *P.oxalicum*. C:N ratio has also previously been shown to be the stimulus triggering sporulation in other fungi (Jackson and Bothast, 1990).

Other stimuli can act synergistically after transfer to a medium without nitrogen. We observed an increase in sporulation by the addition of calcium or PEG 6000 at a rate of 400 g l^{-1}. PEG 6000 at a rate of 200 g l^{-1} did not have any effect. PEG 6000 reduces the matric potential in the culture medium, and water stress is a factor that induces and influences sporulation in other fungi (Inch and Trinci, 1987; Humphreys *et al.*, 1989; Jin *et al.*, 1991). When *P.oxalicum* was grown in aerated cultures sporulation was reduced at decreasing matric potentials (Pascual *et al.*, 1997). The mechanism by which water potential affects sporulation is however not known. Morton (1961) showed that the aerial stimulus to sporulation does not depend on water loss from the mycelium, but to the formation of surface-active material when the mycelium becomes aerial. This surface active material was suggested to be a protein, and is probably hydrophobin. If water loss is also not responsible for sporulation in submerged culture, the role of decreased water potential in sporulation may be related to the production of this surface-active material, or PEG 6000 could be acting directly as the surface activator.

Medium F amended with calcium is a culture medium with a C:N=8.5, known to induce sporulation in submerged cultures of other species of *Penicillium* (Foster *et al.*, 1945; Pitt and Poole, 1981; Ugalde and Pitt, 1983a). Calcium associates with the plasma membrane, the tonoplast and the mitochondria (Ugalde and Pitt, 1984), and the mechanism whereby the conidiation stimulus from bound Ca^{2+} is transduced could be related to its effect on the transmembrane electric potential (Ugalde and Pitt, 1986). Other divalent cations can also induce conidiation, but they are less effective (Hadley and Harrold, 1958). It has been proposed that they interfere with a plasma membrane environmental sensor responsible for triggering conidiation (Ugalde and Roncal, 1995). In the present study no sporulation occurred when *P.oxalicum* was grown on medium F modified with different amounts of calcium. On the other hand *P.oxalicum* produced spores when calcium was added to medium A and the induction of sporulation was maximum when C:N ratio was 93 (representing a reduction in the amount of nitrogen to 1.5 g l^{-1} KNO_3). Therefore it seems that when no calcium is present in the culture medium only the total depletion of nitrogen is able to stimulate sporulation. On the other hand when calcium is present it is still possible to obtain sporulation but only at determined values of the carbon:nitrogen ratio in the medium. Hadley and Harrold (1958) studying sporulation of *Penicillium notatum* found that the calcium requirement for sporulation diminished as the total nutrient concentration was lowered, and was similarly reduced if the concentration of the nitrogen source alone was lowered. However, reductions in the levels of other single nutrients caused relatively small alterations in the calcium requirement. This confirms that calcium and nitrogen concentration are major stimuli triggering sporulation in *Penicillium* spp., although the response varies slightly amongst the different species.

Apart from its direct effect on sporulation, calcium has also been shown to diminish the water stress produced by non-permeating solutes (PEG 4000) in other fungi (Busse and Bottomley, 1989). It was shown that PEG 6000 *per se* increased sporulation of *P.oxalicum*, but the use of both calcium and PEG could result in higher spore yields because of this synergistic effect.

From a practical point of view it is very important that the method developed in this work to induce sporulation of *P.oxalicum* avoids the need for transferring the mycelium after 24 h growth, as this would be a very difficult approach to produce inoculum on an industrial scale using fermenters. Furthermore, the amount of spores obtained needs to be economically acceptable for industrial production of the fungus. Sporulation was also obtained using PDB as culture medium with modified C:N ratio and added calcium. However, levels of sporulation were lower than those obtained with Morton's method. This means that other components present in Morton's medium can also play a role in the amount of spores produced once the sporulation process has been triggered. But it is important to point out that other complex media could be used once they are modified to have the major properties responsible for sporulation (e.g. C:N ratio and calcium). This opens the possibility of using cheap culture media based on by-products, which is an important consideration in the economic industrial production of the fungus. It is also important to notice that the most efficient way to produce spores in PDB was by modifying it to C:N=93 and diluting it by 50%, because in this way the use of the extra carbon source is minimised, contributing to a more economic process. The lower sporulation indices observed for undiluted medium indicated that the fungus is able to grow as vegetative mycelium for longer before

sporulation starts, and this would be a disadvantage when separating the spores from the rest of the culture.

4.2. CHARACTERISATION OF *Penicillium oxalicum* SPORES

4.2.1. Study on growth of *Penicillium oxalicum* in sterile soil

Germination of *P.oxalicum* in soil was maximum at the lowest water potential values tested (-7.0 and -4.0 MPa). In studies *in vitro* (Pascual *et al.*, 1997) germination of *P.oxalicum* was not affected over a wide range of water potential. It was only reduced at water potential values lower than -4.4 and -7.4 MPa, matric and osmotic potential, respectively. This behaviour is different to that observed in this study in soil, where matric forces are of primary importance. In the wettest soil, water availability for the fungus was higher but aeration of soil was reduced, resulting in a shortage of oxygen, which is an essential factor for spore germination (Griffin, D.H., 1981). In contrast, germ tube extension was not affected by this shortage of oxygen at least 24 h after inoculation. The endogenous reserves within the conidia may have enabled initial germ tube extension to occur.

Differences in germination after 24 h incubation did not result in differences in the cfu values. This indicates that ungerminated spores were viable. Growth of germinated spores was not extensive enough to produce an increase in the cfu value. After five days incubation differences in germination were maintained, and there was an increase in cfu numbers in dry treatments. However, no differences in germ tube length were observed between treatments. A likely explanation is that more growth was actually produced in dry treatments but the processing of samples resulted in

breakdown of long germ tubes, producing both an increase in cfu number and an underestimation of germ tube length.

The relatively xerotolerant character of *P.oxalicum* was shown in this experiment. Populations in soil showed a decreasing trend when water potential was -1.0 MPa. It increased slightly with time when soil water potential was -1.5 MPa, the permanent wilting point of plants (Griffin, D.H., 1981). The behaviour of *P.oxalicum* at the lowest water potential tested (-7.0 MPa) indicated that growth was favoured in these conditions until the 30th day. The subsequent decreasing trend in the population could indicate some autolysis of the fungus was taking place, perhaps due to lack of a readily available carbon source. In the rest of the treatments without nutrients, a maximum cfu value was not reached during the period of time studied and data showed a more linear trend. The xerotolerant nature is important for effective introduction of antagonists into soil, as soil microbial populations are controlled more by water availability than temperature (Acea and Carballas, 1990). Different species of *Penicillium* have been described as xerotolerant, such as *P.hordei* and *P.janthinellum* (Magan and Lynch, 1986) or as xerophiles, including *P.implicatum*, *P.phoeniceum*, *P.janczewskii*, *P.corulophilum*, *P.baarnense*, *P.restrictum* and *P.purpurogenum* (Hocking and Pitt, 1979).

Differences in cfu values could have been due to the soil washing method used. This favours heavily sporulating species, but not those predominantly present as mycelium (De Cal and Melgarejo, 1992). The method may have therefore underestimated mycelial growth of the fungus. But it is reasonable to consider that the germination differences observed in the microscope study were causing the differences in cfu

values, because in all the treatments mycelial growth was observed from germinated spores.

In a previous study (Pascual *et al.*, 1997) the effect of different sources of carbon and nitrogen on growth and sporulation of *P.oxalicum* was tested *in vitro*. It was shown that *P. oxalicum* has a preference for organic nitrogen sources, such as peptone or amino acids. Several fungi can not use inorganic nitrogen (Gleason 1976), requiring glutamate, asparagine or other amino acids. Fructose, xylose, mannose, maltose and sucrose were the carbon sources that stimulated growth of *P. oxalicum* most. With the exception of mannose, they were all stimulatory for growth of *Penicillium frequentans* and *Penicillium purpurogenum* (De Cal *et al*, 1993). Maltose was also found to stimulate *Pythium* growth (McQuilken *et al*, 1992). In this study *P. oxalicum* populations increased in sterile soil where nutrients favouring growth *in vitro* (0.8 % mannose, 0.08 % arginine) were added. Nutrients were not able to counteract the negative effect of excess water in soil at the initial growth stages. However, in later stages of mycelial growth, nutrient supply is essential. Thus, in dry soil treatments growth stops after 20-30 days. The effect of water potential is therefore reduced and nutrients start having a significant effect on populations of *P.oxalicum*. Other types of nutrients have been applied with *P.oxalicum* in biocontrol experiments: a solution of malt and yeast extracts did not produce an increase in population of *P.oxalicum* in sterile soil (De Cal *et al.*, 1995). Nutrient addition can play an important role in the formulation of the fungus; it has been seen that certain types of nutrients produced a better level of soil colonisation, and this improved colonisation can result in more efficient biocontrol.

4.2.2. Surface properties and adhesion of aerial and submerged spores

From the obtained results it can be said that surface hydrophobicity is probably similar for aerial and submerged spores, and that the differences observed depended on the method used.

Surface hydrophobicity assessed using the adhesion test indicated that submerged spores of *P.oxalicum* were more hydrophobic than aerial spores. This is opposite of what would be expected if hydrophobins were only responsible for the adhesion, and if they were deposited in the walls of aerial structures, but not in submerged ones, as has been shown for other fungi, such as *Schizophyllum commune* (Wessels, 1992). The method for measuring hydrophobicity by an adhesion test is based on the assumption that there is a correlation between the hydrophobicity of cells and their adherence to polystyrene (Mozes and Rouxhet, 1987). However, surface properties other than hydrophobicity have the potential to influence binding (Ternure and Hoch, 1993). The effect of hydrophobic interactions can be modified by other factors such as electrostatic forces, which can be occurring between cell/cell and cell/support in the case of polystyrene plates, because the surface of plates of polystyrene has been shown to be negatively charged (Mozes and Rouxhet, 1987). Spores of many fungi are also negatively charged (Kuo and Hoch, 1996). Attachment of pycnidiospores of *Phyllosticta ampelicita* is related to hydrophobic and ionic interactions: pycnidiospores attach first by electrostatic and van der Waals forces and then the attachment is firm if the substrate is hydrophobic (Kuo and Hoch, 1996). However, the presence of electrostatic charges on the conidial surface does not mean necessarily that they have a role in adhesion, as has been shown for some entomopathogenic fungi (Boucias *et al.*, 1988). The results obtained in the present study could be explained in

two ways: a) hydrophobins assemble on the surface of submerged conidia, but they do so to a higher extent than on aerial conidia, and b) other forces, such as electrostatic interactions are interfering and modifying the effect of hydrophobic interactions. If, in the case of *P.oxalicum*, adhesion to hydrophobic and hydrophilic surfaces is not only governed by hydrophobic interactions, then you can get equal or higher adhesion to hydrophilic than to hydrophobic surfaces, as observed with the aerial spores. Conidia of *Nectria haematococca* showed equal attachment to hydrophobic and hydrophilic artificial surfaces (Braun and Howard, 1994). However, in many cases it has been observed that adhesion of fungal spores is higher to hydrophobic than to hydrophilic surfaces. Examples include *Uromyces* urediospores and germlings (Terhune and Hoch, 1993), *Magnaporte grisea* (Braun and Howard, 1994), *Colletotrichum musae* (Sela-Buurlage *et al.*, 1991) and *Phyllosticta ampelicida* (Kuo and Hoch, 1996).

It was also observed by SEM that submerged spores appeared covered by an external material that could have a role in adherence if it was not completely washed off before the adhesion test. This has also been observed for other fungi: adhesion of *Cochiobolus heterostrophus* to host plant surfaces is produced by the extracellular matrix materials (ECM); hydrophobic interactions and ECM are also responsible for adhesion of *Uromyces appendiculatus* (Braun and Howard, 1994). In other cases glycoproteins are suggested to have a role in adhesion to polystyrene or plant surfaces, which is mediated by ions. The yeast *Rhodosporidium toruloides* adhered to a higher extent in the presence of Ca^{2+} and Mg^{2+} than in distilled water (Buck and Andrews, 1995). In the present study, submerged *P.oxalicum* spores were produced in a medium containing Ca^{2+}. Any remaining Ca^{2+} on the surface of spores could then

play a role in adhesion if glycoproteins are involved. This would explain the higher adhesion of submerged spores to polystyrene plates. Ca^{2+} could act by binding negative charges on spore surfaces and polystyrene plates.

Conflicting results were obtained from the two methods used to measure hydrophobicity. The second method assessed the distribution ratio of cells between water and an organic phase (Mozes and Rouxhet, 1987). Results indicated that aerial spores of *P.oxalicum* were slightly more hydrophobic than submerged spores. Thus, these results were more in accordance with the idea that hydrophobins are excreted into the culture medium in submerged culture, while they are deposited in the walls of aerial structures (Wessels, 1992). Mozes and Rouxet (1987) using five different methods for measuring hydrophobicity found that there was a good agreement among the five methods for the very hydrophobic and the very hydrophilic microorganisms. The hydrophobic microorganism used was *Moniliella pollinis*, which gave hydrophobicity index of 99 in the phase distribution test at pH=3. At this same pH aerial *P.oxalicum* spores gave a hydrophobicity index of 96.56 while the value for submerged spores was 91.70. Thus, *P.oxalicum* spores can be considered as hydrophobic using this method. This method has also been used to compare hydrophobicity of aerial and submerged spores of the entomopathogenic fungus *Beauveria bassiana*, and it was found that hydrophobicities of the two types of conidia were quite similar and higher than that of blastospores. As in our case, the hydrophobicity index decreased with increasing pH values (Hegedus *et al.*, 1992). However, aerial spores of other fungi behaved differently: hydrophobicity indices were 100 for pH 3, 6 or 9 in the case of *Metarhizium anisopliae*, and it was maximum at pH 6 for *Nomuraea rileyi* (Boucias *et al.*, 1988). Comparison of hydrophobicity of

aerial and submerged spores has also been done for *Trichoderma harzianum* using another method (Muñoz *et al.*, 1995). Results showed that submerged spores were hydrophilic, while aerial ones were highly hydrophobic. Observation of these spores by SEM showed that aerial spores appeared covered by an extracellular mucilage and submerged spores were mostly collapsed, not clustered and larger than aerial spores. In my study, submerged spores and not aerial spores were covered by a matrix. Submerged spores did not collapse, and were similar in size and arrangement to aerial spores. Foster *et al.* (1945) showed that conidia of *P.notatum* formed in submerged liquid culture were morphologically similar to those of surface culture. This indicates that surface properties of submerged spores/aerial spores can be very different depending on the species studied and shows the difficulty of generalising about the processes occurring during submerged cultivation.

Adhesion of germ tubes of *P.oxalicum* to hydrophobic and hydrophilic surfaces was similar for both germ tubes from aerial or submerged spores. Both types of germ tubes were produced by submerged cultivation by incubating the spores (aerial or submerged) in PDB shake flasks. According to Wessels (1992), rodlet layers conferring hydrophobicity should not be formed on their surface. However, adhesion of both types of germ tubes was significantly higher to the hydrophobic than to the hydrophilic surface. This may suggest again that hydrophobins are actually assembling on the surface of submerged structures. Alternatively it is possible that the higher surface area of a germ tube compared to the surface area of an aerial spore is responsible for the higher attachment of the germ tube to the hydrophobic surface, even if the surface of the germ tubes contains less hydrophobins.

Aerial and submerged *P.oxalicum* spores adhered slightly to tomato roots only after 17 h incubation. In contrast adhesion of spores to the hydrophobic or hydrophilic surfaces was more rapid, and generally speaking, when it occurred, it was within 1 h. Adhesion to plant surfaces is similar to adhesion to hydrophobic surfaces for several air-borne fungi. Buck and Andrews (1995) showed that adhesion of the yeast *Rhodosporidium toruloides* to barley leaves and polystyrene showed similar kinetics: maximum adhesion occurred within 30 to 45 minutes. Conidia of *Colletotrichum musae* adhered to polystyrene to the same extent as to the host surface (Sela-Buurlage *et al.*, 1991). Conidia of *Botrytis cinerea* were found to adhere to the tomato cuticle immediately upon hydration, and this immediate adhesion depended at least in part on hydrophobic interactions (Doss *et al.*, 1993). This rapid adhesion indicated a passive, non-specific mechanism. Also, in the rhizosphere the rapid adherence of bacteria to radish roots probably does not involve specific recognition events, but in this case no correlation between bacterial adherence properties and bacterial surface hydrophobicity was found (James *et al.*, 1985). The slower adhesion to roots of *P.oxalicum* spores indicates that the process differs from that observed on artificial surfaces. It is possible that after 17 h incubation the spores had germinated (the spore suspension was made in water but the spores could use root exudates to germinate). It has been shown that germ tubes from both aerial and submerged spores adhere more to the hydrophobic surface. Therefore hydrophobic interactions could play a role after the spores had germinated. In a study on superficial fungal growth on tomato roots, Beswetherick and Bishop (1993) found that the saprophyte *Cunninghamella elegans* did not adhere to plant cells after 1 day, while adhesion was observed for pathogenic and non-pathogenic necrotrophic fungi. *P.oxalicum* has also been cited occasionally as a weak pathogen on other hosts (Ride and Barber, 1987;

Jarvis *et al.*, 1990; Halfonmeiri and Solel, 1990). It is possible that this is related to its adhesion ability, and that this interaction could be involved in the triggering of defence mechanisms on the tomato plant.

4.2.3. Viability of *Penicillium oxalicum* spores after storage

P.oxalicum spores stored fresh survived well for 27 weeks (80-97 % survival), but aerial spores survived slightly better than submerged spores. Previous studies on the viability of submerged and aerial conidia of *Trichoderma harzianum* after 45 days storage at 25°C and 75 % relative humidity was 100 % for aerial spores and 15 % for submerged spores. It was suggested that this was at least partially due to the higher hydrophobicity of aerial spores, contributing to the maintenance of the dormant state (Muñoz *et al.*, 1995). With *P.oxalicum* the difference between the two spore types was much smaller, being 97 % survival for aerial spores, and 80 % for submerged spores, at 25°C. As discussed earlier, aerial spores were slightly more hydrophobic than submerged ones (using the phase exclusion assay), and thus our data agrees with the theory that hydrophobicity has a role in spore survival. It is possible that metabolic activity of submerged spores is higher than that of aerial ones because they are fully hydrated, as has been found for *Beauveria bassiana* (Thomas *et al.*, 1987). Other properties of the cell wall can probably have a role in the difference of survival observed for the two types of *P.oxalicum* spores. Cell walls of aerial conidia of *Trichoderma harzianum* were thicker than that of submerged conidia (Muñoz *et al.*, 1995), and rougher in the case of *Beauveria bassiana* (Thomas *et al.*, 1987). These properties make aerial conidia less vulnerable to adverse environmental conditions, as they remain in a dormant state.

Freeze-drying significantly reduced viability of the two spore types, but the reduction was much higher in the case of submerged spores. Drying is a crucial step that affects the general survival of fungi. The formation of intra- or intermolecular H bonds under conditions of excessive dehydration causes irreversible changes in some proteins which lead to enzyme inactivation (Brown, 1976). Air drying has been shown to affect the viability of the biocontrol fungus *Gliocladium roseum* (Jensen *et al.*, 1996). Some fungal species do not survive freeze-drying, and of those which survive, quantitative viability rates can be very low (Smith and Onions, 1983). Survival of submerged spores following freeze-drying could be improved by growing the fungus at reduced water potential. This has been shown for submerged spores of *Trichoderma harzianum*: survival following drying was improved by adding PEG 200 to the culture medium to reduce water potential (Jin *et al.*, 1991). Production at reduced water potential also has advantages of increased sporulation, as discussed earlier. The increased desiccation tolerance of *T.harzianum* spores was shown to be related to a higher content of trehalose in the spores (Jin *et al.*, 1991). McBride and Ensign (1987) reported that growth of *Streptomyces griseus* on media containing excess glucose yielded spores containing up to 25% of their dry weight as trehalose and with increased heat and desiccation resistance. In our case the culture medium used for the production of *P.oxalicum* contained excess glucose, but this was not enough for submerged conidia to survive drying as well as aerial conidia. Muñoz *et al.* (1995) when comparing trehalose content in aerial and submerged spores of *T.harzianum*, found that the content of trehalose was higher in submerged spores, which survived less than aerial ones during storage. This indicates that trehalose enhances survival to drying but does not contribute to survival during storage. Trehalose is known to function as an intracellular protectant during dehydration (Van

Laere, 1989) because of its unique ability to reversibly replace water upon dehydration of phospholipid bilayers (Crowe *et al.*, 1984). Externally supplied trehalose during freeze-drying could increase survival, as has been shown for *Saccharomyces cerevisiae* (Gadd *et al.*, 1987). Trehalose is one of the components recommended by Berny and Hennebert (1991) as an appropriate protecting medium for fungi.

Storage temperature did not have a remarkable effect on survival of fresh spores of *P.oxalicum* over the time periods used (27 weeks). Freeze-dried spores lost viability with time slightly faster at 25 than at 4°C. Temperature generally affects survival of fungi: low temperatures contribute to the dormant state of spores as metabolic activity is related to temperature, but the sensitivity to temperature varies with the species. *Gliocladium roseum* was stable at 4°C for 23 weeks, while viability decreased after 4-8 weeks at 15-20°C (Jensen *et al.*, 1996). Conidia of *Beauveria bassiana* survived better at 4 than at 25°C: after 16 weeks storage at 4°C germination of conidia was 50 %, while the percentage at 25°C was less than 20 % (Lane *et al.*, 1991). Microconidia of *Fusarium moniliforme* survived at 5°C for at least 15 weeks and for less than 10 weeks at 25 and 30°C (Liddell and Burgess, 1985). Solid substrate inocula of *Coniothyrium minitans* survived better at 5 and 15°C than at 30°C for at least four weeks (McQuilken and Whipps, 1995).

4.3. ASSESSMENT OF THE EFFICACY OF *Penicillium oxalicum*

P.oxalicum applied as aerial spores in water at $6x10^6$ spores ml^{-1} substratum was able to reduce *Fusarium* wilt of tomato both in the greenhouse and in the growth chamber experiments. Disease control was variable, with values ranging from 22 to 41% in

many cases. In other experiments the efficacy of the antagonist in greenhouse experiments was higher, giving 50 % disease control (De Cal *et al.*, 1995). *P.oxalicum* induces resistance in tomato plants (De Cal *et al.*, 1997a), and the process of induction may vary depending on the developmental stage of both plant and antagonist, this being responsible for the different degrees of control observed.

The present work has attempted to analyse different aspects of the biocontrol system studied, aiming to obtain the information needed for its possible improvement. The effect of spore concentration of *P.oxalicum* on disease control was studied in a growth chamber experiment. This will be discussed in the later Section (4.3.1.) together with data from other experiments about populations of *P.oxalicum* and *F.oxysporum* f.sp. *lycopersici* in the rhizosphere of tomato plants. Then the effect of *P.oxalicum* produced by different methods will be discussed (Section 4.3.2.), followed by the analysis of the effect of *P.oxalicum* as a growth promoting agent (Section 4.3.3.). Finally some comments will be made about growth promotion and induction of resistance by *P.oxalicum* (Section 4.3.4.).

4.3.1. Concentration of the *Penicillium oxalicum* treatment and populations of *Penicillium oxalicum* and *Fusarium oxysporum* f.sp. *lycopersici* in the rhizosphere

In many biocontrol systems, lack of consistency has been associated with low populations of the antagonist. This has been shown in the phyllosphere (Fokkema *et al.*, 1979; De Cal *et al.*, 1990) and in the rhizosphere: Raaijmakers *et al.* (1995) showed that the rhizosphere population densities of two *Pseudomonas* strains was an important determinant of their efficacy to suppress Fusarium wilt of radish. Hadar

et al. (1979) found a decreasing incidence of damping-off caused by *Rhizoctonia solani* with increasing concentrations of *Trichoderma harzianum* in soil. A dose-response relationship has been established for different biocontrol systems, and models have been fitted. Johnson (1994) proposed a model that was validated with data of antagonists working by different modes of action: competition and induction of resistance (data taken from Mandeel and Baker (1991). These same data and other on biocontrol by direct interaction of bacteria with pathogenic conidia were used by Montesinos and Bonaterra (1996) to develop two models that relate the densities of the BCA and the pathogen with disease response. In this last case differences in the values of key parameters are explained by the different mode of action of the two BCAs studied.

In the case of *P.oxalicum* it was observed that efficacy to control Fusarium wilt of tomato was not improved by applying the antagonist at $1x10^8$ spores ml^{-1} ($6x10^6$ spores ml^{-1} substratum) compared to $1x10^6$ ($6x10^4$ spores ml^{-1} substratum), but lower concentrations were ineffective. A similar behaviour was observed for *Pseudomonas* spp. working by competition for iron and induction of systemic resistance in the control of Fusarium wilt of radish (Raaijmakers *et al.*, 1995). However, our system seems to respond more to a yes/no pattern, i.e. reduction of disease index was achieved when the antagonist was applied at $6x10^6$, $6x10^5$ and $6x10^4$ spores ml^{-1} substratum, and no reduction was achieved for lower concentrations. Moreover, treatment with $6x10^4$ spores ml^{-1} substratum seemed slightly more effective than the others in reducing disease index, and treatment with $6x10^5$ spores ml^{-1} substratum was the only treatment able to increase the consumption of nutrient solution by inoculated plants. *P.oxalicum* induces resistance in tomato plants (De Cal *et al.*, 1997a). Therefore it seems that induction of resistance can be a yes/no or a

qualitative phenomenon. Results from the greenhouse experiment also showed no relationship between populations of *P.oxalicum* in the rhizosphere and disease reduction. At the end of the experiment, treatments with the smallest disease index were not those treatments with the highest rhizosphere populations. Therefore, establishment of a high population of the antagonist in the rhizosphere of tomato is not critical to get a high degree of disease reduction, indicating that competition is not essential for biocontrol. However, a minimum threshold concentration of the antagonist ($6x10^4$ spores ml^{-1} substratum) has to be used.

It is important to note that when *P.oxalicum* was applied in the growth chamber experiment at $6x10^6$ or $6x10^5$ spores ml^{-1} substratum, the resultant populations in the rhizosphere were similar. This may indicate the maximum threshold populations of the antagonist that roots can support had been reached. In the greenhouse experiment it was also observed that populations of *P.oxalicum* in the rhizosphere of tomato decreased with time especially in the treatment at $6x10^6$ spores ml^{-1} substratum. A decrease in *P.oxalicum* populations was also observed by De Cal *et al.* (1995). The important point is that an optimum concentration of the antagonist (between $6x10^4$-$6x10^5$ spores ml^{-1} substratum) has been established, because in both growth chamber and greenhouse experiments efficacy was not improved with treatments at higher concentration. This is important in order to calculate costs of production and indicates that in this case the amount of biomass to be applied will probably not be a limiting factor in the industrial production of *P.oxalicum*.

Populations of the pathogen in the rhizosphere of tomato plants were not reduced by *P.oxalicum*, in the growth chamber experiments, with the exception of the treatment with aerial spores in water at $6x10^4$ spores ml^{-1} substratum. Also, in the greenhouse

experiment, the observed reduction in wilting of tomato was not related to a decrease in population of *Fusarium oxysporum* f. sp. *lycopersici* in the rhizosphere, *per se*. It decreased with time but was not affected by the presence of the antagonist. This has also been observed by De Cal *et al.* (1995, 1997b). The isolate of *P.oxalicum* used by Windels (1981) did not affect rhizosphere populations of other microorganisms, except that a greater population of *Penicillium* spp. was observed. The isolate used in the present work produces lytic enzymes *in vitro* (Pérez-Leblic *et al.*, 1982) and it is able to reduce the population of microconidia of the pathogen in soil (De Cal *et al.*, 1995). However, in the rhizosphere, interactions between the pathogen and antagonist are modified by the presence of the plant, making the pathogen insensitive to the attack of the antagonist, or the antagonist unable to attack the pathogen. However, it is clear that disease reduction is unrelated to interactions between the pathogen and antagonist. This agrees with the fact that *P.oxalicum* works by inducing resistance in the host (De Cal *et al.*, 1997a).

4.3.2. Efficacy of different inoculum forms of *Penicillium oxalicum*

The efficacy of *P.oxalicum* varied depending on the type of inoculum used. Data from the growth chamber experiments indicate that generally aerial spores were more effective than submerged spores in reducing the disease. In the greenhouse experiment, *P.oxalicum* was able to reduce the disease index when produced by any of the methods used, 36 and 50 days after inoculation. However, at the end of the experiment (64 days after inoculation) disease index was only smaller in tomato plants treated with the antagonist produced on PDA or on solid substrate at 0.98 a_w. The different efficacy of inoculum types is a very important aspect to take into account if *P.oxalicum* is to be produced on a large scale. Although it has been seen that the

amount of inoculum of the antagonist needed will probably not be a limiting factor, it is required to evaluate those most effective and amenable to industrial production.

Different factors during the production of spores in aerial or submerged culture can affect the efficacy of the inoculum. Nutritional conditions have been shown to be very important for other BCAs. Lewis and Papavizas (1993) studied the efficacy of *Stilbella aciculosa* to control *Rhizoctonia solani* by growing the antagonist in solid fermentation on various substrates, with control being obtained with all substrates except perlite+corn cobs. Nutritional conditions during the production of fluorescent pseudomonads affected their ability to suppress growth of *Pythium ultimum in vitro*, with higher suppression obtained when using richer media for production (Pascual, 1992). Effectiveness of fungi used as BCAs against weeds also varied depending on nutritional conditions during production, with C:N ratio affecting the efficacy of *Colletotrichum truncatum* as a BCA (Schisler *et al.*, 1990). For the system described here it is especially important to note the effect of Ca^{2+} on secondary metabolism, which could have consequences for the biocontrol efficacy of *P.oxalicum*. Ibba *et al.* (1987) found that the production of the mycotoxin paxilline by *Penicillium paxilli* is inhibited by calcium-induced sporulation in submerged culture, and pointed out that calcium-induced conidiogenesis in submerged culture does seem to have consequences for the operation of secondary biosynthetic pathways, but it is not possible to recognise a consistent pattern of expression. Interestingly, they suggested some analogy between the system they studied with gibberellin fermentation. *P.oxalicum* promotes growth of tomato plants (Section 4.3.3.), and this could be by stimulation of hormone production by the plant or by the production of hormones by the fungus itself. If this is the case, it is possible that calcium-induced sporulation

affects the process. However, more research is needed to clarify this aspect of the work.

In growth chamber experiments the efficacy of mycelium produced by submerged cultivation was tested. For some BCAs, mycelium has been shown to be more effective than conidia (Lewis and Papavizas, 1985; Sharma and Singh, 1990). The culture medium used to produce mycelium was PDB, which is equivalent to PDA in its nutrient content, but the mycelium produced was not able to reduce the disease. This emphasises the necessity of finding methods for the production of spores of *P.oxalicum*, because this inoculum type is more effective.

In order to improve our understanding of the differences in efficacy between aerial and submerged spores, experiments were carried out using the liquid from submerged cultivation of *P.oxalicum*. This liquid had a variable effect on the disease, and it seems that the effect of this treatment was opposite to the effect obtained with submerged spores: usually when treatment with submerged spores reduced the disease the liquid from submerged cultivation was not able to do so. This suggests that there could be a factor(s) involved in the biocontrol which is released into the culture medium in different amounts from batch to batch, and that remains with the spores in inversely proportional amounts. More research is however needed to clarify this aspect.

Efficacy of aerial spores applied in water or with nutrients (Morton's medium) was slightly different. Both treatments reduced disease severity in some of the repetitions. However, increase in the consumption of nutrient solution was only produced by

aerial spores applied with nutrients. Growth of *P.oxalicum* in seedbeds is favoured by nutrients, as has been shown in Section 3.2.1. De Cal *et al.* (1995) also observed an increase in populations of *P.oxalicum* owing to the effect of nutrients (malt extract and yeast extract). It has been shown that the numbers of the antagonist in the rhizosphere is not a limiting factor in this biocontrol system. However, a change in the metabolic pattern of *P.oxalicum* in soil can occur by the effect of nutrients, and this can produce different effects on the tomato plant.

In the greenhouse experiment it was shown that the effectiveness of *P.oxalicum* was not dependent on the a_w of the medium. Modification of a_w of the medium is advantageous however, because yield of conidia was increased in both liquid medium (Section 3.1.2.) and in solid substrate (data not shown). An advantageous colonisation of soil by *P.oxalicum* produced at low a_w can not be expected, as soil supporting plant growth is not a dry environment.

4.3.3. Efficacy of aerial spore formulations for coating of tomato seeds for growth promotion

To test the growth promoting effect of *P.oxalicum* on tomato, an *in vitro* system was used. With this same method, growth promotion has been observed on seeds treated by immersion in a spore suspension of *P.oxalicum* (Garcia *et al.*, 1996). In the present study it was shown that the growth promotion effect of *P.oxalicum* was maintained, and in some cases improved when seeds were formulated with the antagonist. The application of antagonists by seed treatment is thought to be an economical alternative, since only a relatively small amount of inoculum is needed (Cliquet and Scheffer, 1996). Seed treatments have been widely used to apply biological control

agents. Methyl cellulose coating was used for example for *Pseudomonas aureofaciens* AB254 (Mathre *et al.*, 1994), and *Trichoderma* spp. (Cliquet and Scheffer, 1996). Alginate polymer was used for seed inoculation with *Pseudomonas fluorescens* (Russo *et al.*, 1996). A liquid coating formulation has been used for the application of biological seed treatments of *Trichoderma harzianum* (Taylor *et al*, 1991) and *P.oxalicum* has been used to coat pea seeds, although seeds were not formulated (Windels and Kommedahl, 1978).

The growth promotion effect observed varied depending on the medium in which the seeds were placed, and the tomato cultivar used. Seed germination was faster on water than on Hoagland agar. Hoagland solution is widely used as a nutrient solution for *in vitro* growth of plants. However, it is inhibitory to seed germination. *P.oxalicum* was able to activate seeds (cv. Precodor) and made them less sensitive to this inhibition. Formulations including nutrients (Morton´s medium) stimulated germination faster than those without nutrients. Nutrients probably stimulated growth of *P.oxalicum*, which activated seeds earlier. Standard treatment (seed bath) had the same effect as formulation with nutrients. In this case cells of *P.oxalicum* had not been dried and therefore were more active than dried cells included in the formulated seeds.

Once seeds had germinated growth was no longer inhibited on Hoagland agar and the growth promotion effect of *P.oxalicum* was also observed for tomato seeds cv. Lorena. Emergence of cotyledons was quicker for treated seeds on Hoagland agar and emergence of leaves was quicker for treated seeds on both Hoagland and water agar. This was also observed by Garcia *et al.* (1996). This growth promotion effect

was generally not affected by the type of formulation and the nutrients. However, in some cases a quicker response was observed for formulations containing nutrients. This was only observed for cv. Precodor, which always showed slower development than cv. Lorena. The quicker development of cv. Lorena could have masked the nutrient effect. At the end of the experiment the growth promotion effect of *P.oxalicum* resulted in a higher weight of seedlings. All treatments were able to do so for both varieties on Hoagland agar (except alginate formulation for cv. Precodor). However, on water agar only formulated seeds showed increased weight.

Populations of *P.oxalicum* on seeds were similar for all the formulation treatments, and higher in the seed bath treatment, but this difference did not correspond to a more intense growth promotion. The minimum populations of BCAs on seeds giving effective control will vary very much depending on the seed, the disease, the BCA and probably also the mode of action. Cliquet and Scheffer (1996) found that conidia of *Trichoderma* applied by an industrial film coating gave protection at 10^2 cfu/seed, but increasing numbers of healthy plants were found with increasing numbers of viable conidia on seeds. A minimum of log 7 to 7.5 cfu of *Pseudomonas aureofaciens* AB254/seed was required to protect sweet corn from *Pythium* seed rot (Callan *et al.*, 1997).

P.oxalicum formulated in tomato seeds survived air drying very well. The entrapment of living cells into a biopolymer such as alginate is known to protect cells against environmental stresses and to also improve their ability to compete (Mugnier and Jung, 1985). However, survival is different for different species. For example, the overall percentage survival of alginate-entrapped cells of *Azospirillum lipoferum* was

0.4 % after dehydration (Paul *et al.*, 1993). Mugnier and Jung (1985) showed that drying killed about 90 % of xanthan-entrapped cells of the bacterium *Rhizobium japonicum*. They also studied survival of *Penicillium candidum* during storage, and showed that it was not affected by a_w, but unfortunately did not show the percentage survival after drying.

4.3.4. Possible relationships to induced resistance

It is possible that the growth promotion effect of *P.oxalicum* on tomato seedlings is responsible for the induction of resistance to *Fusarium* wilt. Other possible mechanisms responsible for resistance induction could be the increased accumulation of metabolites such as phytoalexins or PR proteins. So far it has been demonstrated that resistance induction in tomato plants by *P.oxalicum* is not related to PR protein accumulation (Garcia *et al.*, 1997). Successful biocontrol of Fusarium wilts is difficult to achieve by reducing inoculum potential or protection of infection courts alone. Induction of resistance is a mechanism of action that can be specially suitable for biological control of diseases with mobile infection courts, such as wilts, because the BCA does not need to be present continuously at the infection court to protect the plant (Baker and Paulitz, 1993). Application of non-pathogens, such as *P.oxalicum*, that activate defence mechanisms may be a much better strategy, and may have more chance of success.

Different microorganisms have been reported to induce disease resistance against wilt diseases. Benhamou *et al.* (1996) reported induction of resistance by endophytic bacteria against *Fusarium oxysporum* f.sp. *pisi*, associated with ultrastructural modifications in pea root tissues. The endophyte *Acremonium kiliense* induced

resistance to wilt diseases of tomato, reducing the spreading of the pathogen within the plant, and similar to *P.oxalicum*, producing more efficient water transport (Bargman and Schonbeck, 1992). Plant growth stimulation and biocontrol has also been associated with Fusarium wilt control of tomato by *Serratia plymuthica* and *Pseudomonas* sp. (Frommel *et al.*, 1991), and with biocontrol of *Sclerotium rolfsii* and Fusarium wilt of cotton and tomato by other plant growth promoting pseudomonads (Gamliel and Katan, 1993). However, in the latter case a reduction of colonisation by the pathogens was registered, indicating a possible direct action of the antagonists on the pathogens.

Finally, as a summary of the results on the control of Fusarium wilt by *P.oxalicum* it can be seen that efficacy of the BCA is quite variable. If the efficacy depends on the interaction between the plant-BCA, it seems that the developmental/physiological stage of both plant and fungus are crucial factors in the biocontrol, with relatively narrow limits for the system to work. It is essential to improve our understanding of the mechanisms of this biocontrol system, to be able to improve the consistency of the results, and to enhance the disease control obtained with *P.oxalicum*.

II: STUDIES ON *Epicoccum nigrum* AND *Penicillium frequentans* FOR BIOCONTROL OF *Monilinia laxa* IN THE PHYLLOSPHERE

4.4. PRODUCTION OF *Epicoccum nigrum* BY SOLID SUBSTRATE FERMENTATION

Grain substrates are heterogeneous media commonly used for the production of spores of BCAs. Grains have been used for commercial production of spores of many entomopathogenic fungi, which cannot be effectively produced in liquid culture (Jenkins, 1996). *E.nigrum* is also a species which does not sporulate in liquid culture. While both starchy and oil based seeds have been examined, little attempt has been made to accurately manipulate the environmental factors in the solid substrate fermentation and study the impact on spore production and quality.

In the present study, a_w of the solid substrate was modified with water or a mixture of glycerol/water. No sporulation was obtained when maize grits was used as the solid substrate. Spore production on wheat grains was affected by the a_w of the substrate. Reduced a_w produces a reduction in growth rates of *E.nigrum* on culture media (Section 3.5.2). Reduction in spore yield is probably a consequence of reduction in growth. When the a_w of the solid substrate was adjusted with a mixture of glycerol/water spore production was increased compared to modification with water alone. Although a_w of the solid substrate was the same in both cases it was modified in a different way, resulting in a more efficient use of water by *E.nigrum* when a mixture of glycerol/water was used. Additionally,

glycerol can also function as a readily available carbon source favouring growth and subsequent sporulation. The important point is that an improved spore yield was obtained in a solid fermentation system at reduced a_w. Spores produced in this way may have the advantages described for *E.nigrum*98 spores compared to the ones produced at high a_w, but further research is needed in this area.

Previous studies have demonstrated that growth and sporulation of the isolate of *E.nigrum* used in this study were favoured on starch-based substrates, when compared to mono or disaccharides (De Cal *et al.*, 1993). Starch has also been shown to be the preferred carbon source for sporulation of a "mycelial" strain of *E.nigrum* (Bonnell and Levetin, 1982). This could be advantageous for production of spores by solid fermentation on heterogeneous solid substrates such as cereal grains, because starch is the main carbon source present in cereals. However, the lack of sporulation of the fungus on maize grits indicates that not only the main carbon source, but other nutritional characteristics are essential in determining the level of sporulation. Starch together with maltose has also been shown to be the most favourable carbohydrate for pigment development in *E.purpurascens* (Schol-Schwarz, 1959), and this is related to the production of antifungal compounds.

4.5. CHARACTERISATION OF *Epicoccum nigrum* AND *Penicillium frequentans*

4.5.1. Germination and germ tube extension of *Epicoccum nigrum* under freely available, and water stress conditions

*E.nigrum*996 spores germinated faster than *E.nigrum*98 spores when water was freely available (0.996 a_w) and under water stress (0.935 a_w). However, improved germ tube extension was observed for *E.nigrum*98 at reduced a_w (0.935). When grown at reduced a_w, osmotic adjustment in *E.nigrum* probably results in a reduction of cell water potential. When the fungus is placed onto media at reduced a_w the imbalance of water potential with the external environment is probably lower for *E.nigrum*98 than for *E.nigrum*996, and therefore the water uptake by *E.nigrum*98 should be more efficient. However, this did not result in improved germination, but it did produce improved germ tube extension. It seems that the early stages of germination in *E.nigrum* are less dependent on water availability than the later stages. Magan and Lacey (1984b) reported 0.88 a_w as the minimum for spore germination of *E.nigrum* isolates from the UK, and 0.89 for linear growth, at 25°C. Better germ tube extension may improve the capacity for rapid establishment and perhaps subsequent niche exclusion in the phyllosphere.

Improved germination and growth of propagules produced under stress has been reported for other fungi. Al-Hamdani and Cooke (1987) observed that sclerotia from growth-limited cultures, with enhanced polyol content, germinated more vigorously than other sclerotia. Hallsworth and Magan (1995) found that conidia of entomopathogenic fungi that had accumulated low molecular weight polyols

were associated with high germination rates and rapid germ tube growth relative to those from controls, at both high (0.989) and reduced a_w (0.887-0.951). They also found that the minimum a_w for germination was lower for modified conidia. Germination was generally much slower for the fungi studied by Hallsworth and Magan (1995) than for the *E.nigrum* studied here: at high a_w (0.989) it took 14 h for *Beauveria bassiana* spores to germinate (76.33% germination), and 35 h for *Paecilomyces farinosus* (47.33 % germination). In contrast, germination of *E.nigrum* was almost 100 % after only 6 h at 0.996 a_w, and after 23 h at 0.935 a_w. A higher germination rate may indicate that spores of *E.nigrum* maintain a higher metabolic activity, and perhaps the presence of a higher amount of available water and endogenous nutrients inside the spore.

Differences in metabolic rate/dormant state could also explain why no improved germination was obtained by spores of *E.nigrum* produced under water stress conditions. When measuring water potential (Ψ_w) of *E.nigrum*996 and *E.nigrum*98 mycelium, it was found that the former was higher than the latter (Section 3.5.4). It seems reasonable to consider that this difference is equivalent in spores, bearing in mind the enhanced accumulation of compatible solutes in *E.nigrum*98 spores (Section 3.5.3). Therefore water uptake may be more rapid by *E.nigrum*98 spores, or that the cell wall/membrane are modified allowing more rapid water ingress into the spores. A likely explanation is that not only the rate of water uptake is involved in the germination rate, but also the degree of dormancy of spores. *E.nigrum*996 spores are probably less dormant than *E.nigrum*98 spores, thus compensating for the slower rate of water uptake. An inhibitor could be involved in this process, but autoinhibition is most evident within very dense spore

populations and may be confined largely to these situations (Cooke and Whipps, 1993). The inhibition of the enzyme trehalase has been related to the maintenance of dormancy, trehalose being the main energy source for respiration during early germination (Thevelein *et al.*, 1984). No significant differences were found in the amount of trehalose in the two spore types of *E.nigrum* (Section 3.5.3), but the trehalase activity was not measured. From an ecological viewpoint spores produced under environmental stress (in this case low water availability) may be more primed as resting propagules than those produced under optimal environmental and nutritional conditions. This may also explain the higher survival of *E.nigrum*98 spores during storage (Section 3.5.6).

4.5.2. Growth rates of *Epicoccum nigrum* at standard and reduced a_w

In this study growth of *E.nigrum* was optimum at 17°C and 0.996 a_w. Growth rates were 2.8-3.2, 3.5-4.2, and 5.2-5.5 mm d^{-1} on PDA, 1/10PDA and minimal medium respectively. *E.nigrum* is a species that demonstrates a great deal of variability in general (Schol-Swarz, 1959). Mycelial growth rates of *E.nigrum* are markedly influenced by the isolate. Other isolates from peach twigs, with lower or no antagonistic ability against *M.laxa* showed higher growth rates than isolate 282. However, other isolates with antagonistic activity against plant pathogenic fungi, had similar growth rates to those reported here. Webber and Hedger (1986) reported growth rate of *E.nigrum* antagonistic to *C.ulmi* of 1.8 mm d^{-1} on Malt Extract Agar (MEA) at 22°C. This culture medium and temperature were not tested, but this growth rate is slightly lower, although in the same range for isolate 282 on PDA at 17 or 25°C. The isolate of *E.nigum* used by Zhou *et al.* (1996) for

biological control of *Sclerotinia sclerotiorum* had a similar growth rate, in this case slightly higher: 3.9 mm d^{-1} on PDA at 20°C.

Temperature giving maximum growth rates of *E.nigrum* was 17°C in the three culture media tested. This optimal temperature for growth is characteristic of mesophilic fungi (Cooke and Whipps, 1993). The growth rate of *E.nigrum* was close to zero at 35°C. Other BCAs, such as *Trichoderma* spp. were also unable to grow at 35°C (Jackson *et al.*, 1991). The minimum temperature for growth was not determined, but at 10°C *E.nigrum* was still able to grow.

Decreasing a_w produced a decrease in growth rates in all cases. According to the classification proposed by Griffin, D.M. (1981), *E.nigrum* is a species included in group 2 (sensitive species), with an optimum growth response at *ca.* -1MPa (0.993 a_w at 25°C) and little growth at *ca.* -5 MPa (0.964 a_w at 25°C). Magan and Lacey (1984b) reported a minimum a_w for linear growth of *E.nigrum* isolated from cereal grain of 0.89, at 25°C when using a glycerol-wheat extract medium. In the present study the lowest a_w tested for radial growth of *E.nigrum* isolate 282 was 0.95, and the fungus showed very poor growth and in many cases did not grow at this level of water stress. Sensitivity to low water availability varies depending on the solute used. Fungi are more sensitive to matric than to osmotic potential (Griffin, D.M., 1981). In this work a_w was modified with PEG200, which modifies water potential predominantly by solute forces. For higher molecular weight PEG (>6000) the major component of water potential are matric forces, although osmotic forces are present (Steuter *et al.*, 1981). Glycerol is a non-ionic solute which also modifies the osmotic potential. Therefore, it seems that the isolate

used here is more sensitive to low water availability than the one studied by Magan and Lacey (1984b). Pelhate (1968) also reported a minimal a_w for growth of *E.nigrum* at 22°C of 0.90 a_w. Although growth rates on Petri dishes were almost zero at 0.95 a_w, *E.nigrum* was able to form germ tubes on water agar at 0.935 a_w. This indicates that growth at early stages is not limited by water stress as much as subsequent mycelial extension.

Colonies of *E.nigrum*98 grew faster than *E.nigrum*996 at reduced a_w ($\leq$ 0.990 a_w) in some of the media and temperatures tested. Osmotic adjustment in *E.nigrum* when grown at reduced a_w probably results in a reduction of cell water potential by accumulation of compatible solutes (Section 3.5.3). Inoculum produced in this way may grow better at reduced a_w because a reduction in the imbalance with the external water potential. Even at high a_w, *E.nigrum*98 had higher growth rates than *E.nigrum*996 in some cases. However, *E.nigrum*996 grew faster in others, indicating that at high a_w the advantage of one or another inoculum type depends on other factors and not only on the cell water potential.

Generally, *E.nigrum*98 grew faster at lower temperatures and on rich medium (PDA), while *E.nigrum*996 grew faster at higher temperatures and on poorer media (1/10 PDA and MM). This indicates that complex interactions may be occurring between the factors studied and this area needs further investigation.

It is interesting to note that when water was fully available (high a_w) the optimum temperature for growth was always 17°C. However, at reduced a_w the optimum temperature was in some cases 25°C, and this was observed more frequently for

*E.nigrum*98. This shift in optimum temperature for growth can provide an ecological advantage because usually interacting conditions of high temperature and dryness are commonly encountered in natural environments.

Other interesting features, apart from growth rates of the two inoculum types, were observed. A more intense yellow pigmentation in the culture medium and stronger sporulation were produced in *E.nigrum*98 colonies in low nutrient media (1/10 PDA and MM). The presence of yellow pigmentation indicates that secondary metabolism and metabolite production is more active in *E.nigrum*98 than in *E.nigrum*996. This has been shown for other fungi: maximum amounts of a water diffusable antibiotic were produced by a streptomycete at between -2.5 to -3.5 MPa (0.983-0.976 a_w) (Wong and Griffin, 1974). Antibiotic production by *Cephalosporium gramineum* was minimal under optimum conditions for growth, and highest when the fungus was under moderate water stress (-2.7 -5.5 MPa (0.98-0.965 a_w) (Bruehl *et al.*, 1972). This is very important because *E. nigrum* produces several antibiotic substances, and one of them (flavipin) has been proposed to be involved in biocontrol of *M. laxa* by *E.nigrum* (Madrigal *et al.*, 1991). Biocontrol of *S.sclerotiorum* by *E.purpurascens* is also related to pigmented antifungal compounds (Mercier and Reedler, 1987). Pigment production has also been suggested as a preliminary screen for production of antifungal products by *E.purpurascens* (Zhou and Reeleder, 1990).

The denser sporulation by *E.nigrum*98 observed in some cases at reduced a_w is another feature that can confer this inoculum type with an ecological advantage, providing more opportunities of distribution in natural environments. Improved

sporulation was obtained by Zhou and Reeleder (1990) in mutants tolerant to fungicides. Schol-Schwarz (1959) observed that transfers from very dry cultures of 8 or more months old gave profusely-sporulating cultures. Also, transfers kept at a few degrees above zero for some weeks began to fructify soon after being returned to about 20°C.

4.5.3. Analysis of endogenous reserves in biomass of *Epicoccum nigrum* and *Penicillium frequentans*

Temporal changes in endogenous sugars and sugar alcohols in relation to water stress has never been examined previously for *E.nigrum* and *P.frequentans*.

(a) Endogenous reserves of *Epicoccum nigrum* and *Penicillium frequentans* grown on culture medium

The two fungi studied belong to the P_2-type according to the types of polyols produced (mannitol and other polyols) (Pfyffer and Rast, 1988). *E.nigrum* accumulates glycerol, arabitol and mannitol; *P.frequentans* accumulates these three and also erythritol. When water availability of media was reduced by addition of glycerol there was an increase in glycerol in both *E.nigrum* and *P.frequentans*. A small increase in arabitol in mycelium and conidia of *E.nigrum* was also detected. Accumulation of progressively lower molecular weight polyols in fungi grown over a range of decreasing a_w levels has been found in other fungal species (Van Eck *et al.*, 1993; Hallsworth and Magan, 1994 a, c). Glycerol has been reported as the sole osmoregulatory solute under salt-stressed conditions in yeasts, and arabitol to be present in most of the osmotolerant species (Meikle *et al.*, 1991). Arabitol was the compatible solute in the

xerotolerant yeast *Saccharomyces rouxii*; while the non-xerotolerant yeast *Saccharomyces cerevisiae*, which does not produce arabitol, responded metabolically to increased salinity by synthesising much more glycerol (Brown, 1978). Accumulations of low molecular weight polyols have also frequently been reported in xerophilic fungi at reduced a_w (Adler *et al.*, 1982; Luard, 1982; Hocking and Norton, 1983).

Unsaturated solutions containing an equal amount of mannitol, arabitol, erythritol and glycerol (in g l^{-1}) have a different a_w (Figure 4.1). The capacity for a_w depression is the consequence of two properties: relatively small molecular weights and/or high solubility in water (Chirife *et al.*, 1984). A saturated solution of mannitol has a a_w value of 0.978 at 25°C, due to its limited solubility and suggests that it would only play a minor role as an osmoregulator (Chirife *et al.*, 1984). The solubility of erythritol is limited; a saturated solution has a a_w of 0.914 (Hallsworth, 1995). Arabitol is freely soluble and a saturated solution has a a_w of 0.819; glycerol has infinite solubility in water (Chirife *et al.*, 1984). Therefore, it seems likely that accumulations of compounds such as glycerol and arabitol can incur greater osmotic tolerance than polyols such as erythritol or mannitol. Glycerol is found not only in fungi, but also in some algae, insects, crustaceans and vertebrates, showing convergent evolution in osmotically active solutes (Yancey *et al.*, 1982). Glycerol and arabitol were found to be the main compatible solutes acting for salt and sugar tolerant yeasts (Chirife *et al.*, 1984). However, mannitol is known to act as a compatible solute (Brown, 1978), as well as erythritol (Beever and Laracy, 1986; Hallsworth, 1995).

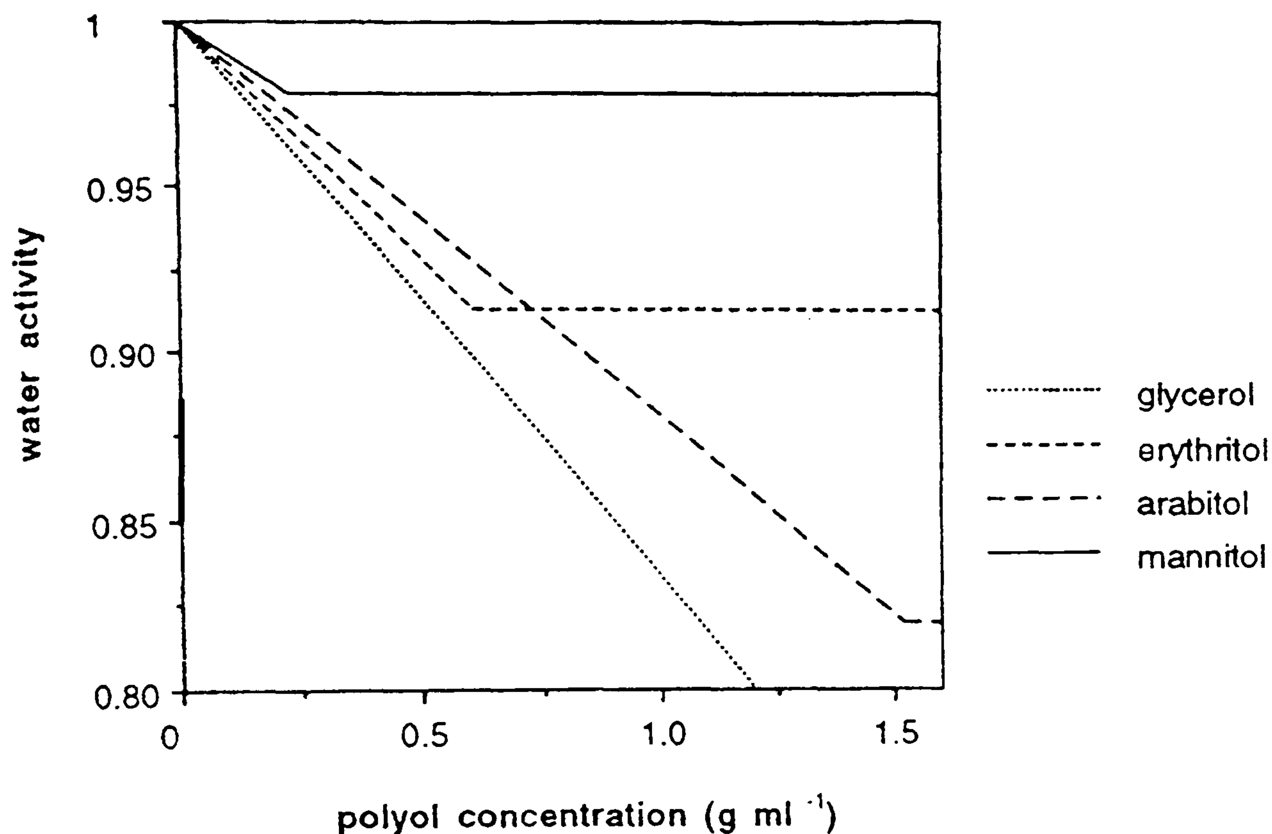


Figure 4.1. Mean water activity of polyol solutions over a range of concentrations, at 25°C. Data taken from Chirife *et al* (1984) and Hallsworth (1995).

The main compatible solute both in *E.nigrum* and *P.frequentans* was glycerol. In the time course experiment it was seen that maximum accumulation of glycerol occurred in both fungi in 5-d-old cultures, and then decreased slightly. This has been observed in other cases (Hocking, 1986). However the two fungi are different in their degree of tolerance to water stress: *E.nigrum* is sensitive, while *P.frequentans* is xerotolerant, according to Griffin, D.M. (1981). This situation is similar to that described by Brown (1978) for two yeasts with variable tolerance to water stress; the major relevant difference between the two species lying in the mechanisms employed to achieve the intracellular concentration of glycerol. *Saccharomyces rouxii*, the xerotolerant species, achieves this largely through a conservative mechanism, namely retention of glycerol, without a diversion of a major proportion of its normal biosynthetic activities to producing additional

compatible solute. On the other hand, *Saccharomyces cerevisiae* has a wasteful production of glycerol in response to water stress and diverts biosynthetic capacity to this end. The fundamental difference in glycerol conservation between the two species is related to substantial differences in permeability or transport mechanisms. This probably accounts for the difference between the two fungi used in this study. Adler *et al.* (1982) found that for the xerotolerant *Penicillium chrysogenum,* only a minor portion of the total polyols were excreted into the external medium, indicating capacity for maintenance of a sharp polyol gradient across the plasma membrane. It is possible that *P.frequentans* is more capable of doing this than *E.nigrum* because it is xerotolerant. Woods and Duniway (1986) also found that the increase in respiration rates at low water potential was higher for *Phytophthora cryptogea* than for the more xerotolerant fungus *Fusarium moniliforme*, suggesting a higher metabolic cost for growth at low water potential.

Another difference between *E.nigrum* and *P.frequentans* is that in the former, contents of glucose, erythritol and mannitol were in general higher in biomass produced at high a_w. These solutes, less useful for osmotic adjustment are probably being consumed parallel to the accumulation of glycerol. For *E.nigrum* glucose content is higher in mycelium produced at high a_w, but the difference is smaller compared to *P.frequentans*, and it is not registered in spores. This indicates different patterns of solute utilisation and could be related to differences in xerotolerance.

In the present study there was no clear pattern in the accumulation of trehalose. A small increase was detected in 15-d-old biomass of *P.frequentans* and 20-d-old

conidia of *E.nigrum* produced at low a_w. On the other hand, trehalose content was higher in 10-d-old cultures of *P.frequentans* produced at high a_w. This could indicate that the method used to analyse trehalose content is not the most suitable to detect differences in this solute. Trehalose is associated with membranes and it is possible that it is released irregularly into the extraction medium. The accumulation of trehalose has been detected in a variety of rhizobial strains in response to osmotic stress, and trehalose is a major compatible solute in a variety of pseudomonad species (Miller and Wood, 1996). It would be very interesting to have an enhanced accumulation of trehalose in biomass produced at reduced a_w, because trehalose confers desiccation tolerance and improves viability. Harman *et al.* (1991) showed that addition of PEG 8000 to media could increase trehalose concentration in fungal mycelium and spores of the biological control agent *Trichoderma harzianum*. On the other hand, Hallsworth and Magan (1994c) found that trehalose content decreased at lowered a_w in three species of entomopathogenic fungi.

When the two biocontrol fungi were used in field trials they were subjected to a homogenisation treatment before spray application. During homogenisation a large amount of the accumulated glycerol (and the other solutes) may be released into the medium. However, it could still help in the osmotic adjustment of the fungi in the phyllosphere as the biomass is applied with the released materials. However this could reduce the advantage of a high internal concentration of glycerol. In *P.frequentans* trehalose, glucose, arabitol and mannitol are released during homogenisation in greater amounts in biomass produced at high a_w, than

that produced under water stress. This did not happen with *E.nigrum*. This seems to indicate that homogenisation has different effects on the two types of cultures.

(b) Endogenous reserves of *E.nigrum* grown on solid grain substrates

The types of polyols accumulated by *E.nigrum* spores produced by solid fermentation were the same as those accumulated by the spores produced on agar culture media, indicating a consistent pattern of accumulation for this fungal species. When the a_w of the substrate was reduced, an enhanced accumulation of the compatible solute glycerol was observed, as occurred in culture medium. Arabitol accumulated in spores produced at reduced a_w only when the cv. Rendeveau was used. In cv. Brigadier arabitol was washed out from the spores. However, glycerol was again the main compatible solute in *E.nigrum*, as found for many filamentous fungi (Griffin, D.M., 1981), followed by arabitol. In xerotolerant yeasts, glycerol is the primary compatible solute, that can be substituted by arabitol as a "second line" compatible solute under conditions of water stress (Brown, 1978). However, for the biocontrol yeast *Candida sake*, glycerol was accumulated at reduced a_w and arabitol was mainly present under unstressed a_w conditions (Teixidó, 1997).

Accumulation of glycerol was not increased when the solute potential of the substrate was modified with a mixture of water/glycerol, when compared with the solid substrate adjusted with water only. The fungus may be utilising some of the glycerol from the substrate as a carbon source rather than a compatible solute. However, potential may exist for the addition of other water/solute mixtures to manipulate endogenous reserves and improve quality and production of conidia.

Other differences when comparing culture medium and solid substrates were observed. In culture medium the content of mannitol in spores produced at 0.996 a_w decreased with time, while in solid substrates an increase occurred. A temporal increase in glucose was observed on solid substrates, in contrast to that observed on solid culture media. Differences in the nutrient status of the medium may account for these differences in accumulation. The increase in glucose observed in spores produced at 0.98 a_w was higher when the substrate was modified with glycerol/water. If glycerol is being used as a carbon source it will be converted to glucose prior to utilisation.

Although the pattern of accumulation of solutes was similar for both wheat cultivars tested, an important difference was observed: trehalose was present in *E.nigrum* spores from cv. Rendeveau but it was only found in trace amounts from cv. Brigadier. This points out the importance of choosing the right substrate when using solid substrate fermentation systems, to produce spores with the highest possible quality. In this case, cv. Rendeveau would be better because trehalose improves desiccation tolerance (McBride and Ensign, 1987; Jin *et al.*, 1991).

Amounts of endogenous reserves accumulated in spores produced by solid substrate fermentation were lower than those in spores produced in culture medium. This can be a consequence of the method used to separate the spores from the solid substrate. Indeed, a significant amount of endogenous solutes were detected in the washing liquid used for the separation of spores. Usually, the amount of solutes washed paralleled those in the spores. However this was not always the case: glycerol was not washed out in higher quantities from spores

produced at 0.98 a_w adjusted with water (while spores contained higher amounts of glycerol). This may indicate that glycerol in spores produced at 0.98 a_w is less easily washed out than in spores produced at 0.996 a_w. On the other hand, arabitol was easily washed from spores produced at 0.98 a_w, and this may be responsible for the low amounts of arabitol detected in this type of spores.

4.5.4. Turgor pressure in *Epicoccum nigrum* mycelium

Turgor pressure of *E.nigrum* mycelium produced at standard and reduced a_w was determined as a part of the characterisation phase of this BCA. Mycelium was used because it is more easily handled than spores for the determination. Turgor pressure (or potential) was determined indirectly by thermocouple psychrometry. The total water potential of the samples was determined and then the samples were frozen rapidly in liquid nitrogen. Turgor potential (Ψ_p) is reduced to zero after freezing, due to rupturing of the hyphae as a result of ice-formation in the cell, and this allows the turgor pressure of the intact hyphae to be calculated (Thompson *et al.*, 1985). In several studies it has been assumed that the water potential of the culture medium equals the water potential of the mycelium (Luard and Griffin, 1981; Blomberg and Adler, 1992). However, according to Eamus and Jennings (1986) this is a wrong assumption because for water uptake to occur, a gradient of Ψ between the culture medium and hypha is necessary. Therefore a measurement of total water potential was needed. The results showed that for *E.nigrum* mycelium it can be assumed that the mycelium water potential is in equilibrium with the culture medium. Total water potential (Ψ_w) of *E.nigrum*98 mycelium (-2.71 MPa) was almost the same as the water potential of PDB modified with glycerol to 0.98 a_w (-2.78 MPa). Also, water potential of

*E.nigrum*996 mycelium (-0.611 MPa) is similar to the water potential of PDB (0.996 a_w = -0.551 MPa).

Osmotic potential (Ψ_π) was lower in *E.nigrum*98 colonies, and this supports the significant increase in accumulation of compatible solutes observed (Section 3.5.3). Although water potential has two major components (solute and matric), matric potential has been assumed to be negligible in fungal cells, as in plant cells, although this is arguable (Luard and Griffin, 1981). The difference between total water potential and osmotic potential gives turgor pressure. The values obtained for the two types of mycelium of *E.nigrum* were almost identical (0.46 MPa for *E.nigrum*996, and 0.48 MPa for *E.nigrum*98). These values are lower than those obtained by Luard and Griffin (1981) by thermocouple psychrometry, for fungi with different tolerances to water stress. According to Jennings (1995), fungal turgor potentials can vary between 0.1 and 3.25 MPa. However, there can be marked differences in the value for turgor potential obtained by different methods with the same fungus. From data obtained by psychrometry, the turgor pressure of *E.nigrum* is similar to that of *Phellinus noxious* (0.6 MPa), *Phallus impudicus* (0.41 MPa) or *Serpula lacrymans* (0.43 MPa).

In the present study, growth of *E.nigrum* was reduced as medium a_w decreased. However, it is difficult to correlate these changes with a reduction in turgor pressure. Luard and Griffin (1981) found that large positive turgor potentials were maintained in xerophytes and drought-sensitive fungi even when the external potential severely inhibited growth and observed a tendency for turgor to increase at low water potentials. However, in the oomycete *Saprolegnia ferax*, hyphae

subjected to water stress had diminished turgor pressure, although hyphae continued to extend (Money and Harold, 1996). Woods and Duniway (1986) also found that *Phytophthora cryptogea* and *Fusarium moniliforme* maintained large turgors throughout the entire water potential range that permitted growth. According to Eamus and Jennings (1986b) the results obtained by Luard and Griffin, (1981) were produced by assuming wrongly an equilibrium between hyphae and medium water potential. Eamus and Jennings (1986a) found a linear correlation between the turgor potential at the mycelial front and the linear growth rate for *Serpula lacrimans* and *Phallus impudicus*. Jennings (1995) recently analysed the results obtained by different authors, but was unable to draw any general conclusion about the relationship between growth and turgor pressure. For *E.nigrum*, the parameters do not appear to be directly related.

4.5.5. Niche overlap index for *Epicoccum nigrum* and *Monilinia laxa*

Niche overlap indices (NOIs) were calculated as a measure of ecological similarity (Wilson and Lindow, 1994) between the pathogen *M.laxa* and the antagonist *E.nigrum* in different water stress conditions, to assess the competitive ability of *E.nigrum* produced at standard and reduced a_w. At high a_w, the two types of *E.nigrum* spores were able to utilise a high percentage (88%) of the carbon sources utilised by *M.laxa* (NOI=0.88), indicating a high ecological similarity between the antagonist and the pathogen. Overlap measures vary from zero to one. Zero overlap indicates two species are completely dissimilar, and a value of one indicates complete overlap (Lawlor, 1980). Occupation of the same sites in the phyllosphere and utilisation of similar resources would be of primary importance in pre-emptive exclusion (Wilson *et al.*, 1995) of a pathogen by an

antagonist. Therefore the values obtained may indicate a high ability of *E.nigrum* to compete with *M.laxa* for carbon sources in the phyllosphere. At reduced a_w (0.985) the NOI for *E.nigrum*996 decreased to 0.79, while that for *E.nigrum*98 remained at about the same value (0.89). This indicates that the modified inoculum is still able to compete with *M.laxa*, while this ability is reduced for the standard inoculum. An improved biological control by *E.nigrum*98 could then be expected if this enhanced competitive ability is also occurring in the phyllosphere, where water availability is limited. The carbon sources used to determine the NOIs were those present in BIOLOG plates. These carbon sources cover a wide range of different substances, such as sugars, polysaccharides, polyols, organic acids, aminoacids and polyamines. The usefulness of NOIs for examining epiphytic communities would be substantially improved if they were based on only those carbon sources actually present in the phyllosphere under examination (Wilson and Lindow, 1994). Previously, no attempts have been made to examine the influence of water availability on the NOIs of plant pathogens and fungal antagonists.

4.5.6. Viability of *Epicoccum nigrum* spores after storage

E.nigrum spores stored fresh at 4°C survived well for 27 weeks, but *E.nigrum*98 spores survived better (92 % survival) than *E.nigrum*996 spores (82 % survival). This difference between the two spore types was also observed for fresh spores stored at 25°C, but in this case percentages of survival were much lower. *E.nigrum*98 spores have an enhanced accumulation of the compatible solutes glycerol and arabitol (Section 3.5.3) and this could be related to their higher survival ability. Other endogenous reserves are known to affect survival of fungi.

Spores of *Colletotrichum truncatum* produced in nitrogen-limited batch cultures have been shown to contain greater amounts of lipids and less proteins (Jackson and Schisler, 1992) and to survive longer compared to carbon-limited spores (Lane *et al.*, 1991a). It is also possible that *E.nigrum*98 spores are in a more dormant state than *E.nigrum*996 spores. This would contribute to their improved survival during storage.

Freeze-drying affected severely the survival of both spore types, which was zero after 12 weeks storage. Preservation of freeze-dried microorganisms can be assisted by the inclusion of glycerol or a sugar, this reduces mortality during dehydration, storage and rehydration. These molecules function by directly substituting as a "solvating" molecule for the water that is removed on dehydration, and provide an alternative source of H bonds that can prevent the formation of the intersite bonds and thereby prevent protein inactivation (Brown, 1976). However, in the case of *E.nigrum*, the enhanced accumulation of glycerol did not result in a higher survival after drying.

When comparing the effect of freeze-drying on *E.nigrum* and *P.oxalicum* (Section 4.2.3.) it was observed that *P.oxalicum* survived much better than *E.nigrum*. As pointed out earlier, damage during dehydration is produced by the formation of intra or intermolecular H bonds (Brown, 1976). Whether this process occurs to a larger extent in the case of *E.nigrum* is not clear. Berny and Hennebert (1991) studied viability of different fungal species during freeze-drying and found that *Trichoderma viride* was much more resistant to the freeze-drying process than *Saccharomyces cerevisiae, Brettanomyces bruxellensis* or *Arthrobotrys*

arthrobotryoides, but no explanations were given for the differences. Viability and stability during freeze-drying can be improved not only by protective endogenous/exogenous substances but also by controlling the cooling rate. According to Berny and Hennebert (1991) the most favourable cooling rate is 3 °C/min.

Storage temperature affected the survival of spores, which was higher at 4°C. *E.nigrum* was more sensitive to temperature than *P.oxalicum* (Section 4.2.3.). This may indicate a difference in metabolic activity/dormant state compared to *P.oxalicum*. Germination of *E.nigrum* spores on PDA is faster than germination of *P.oxalicum* spores, indicating a lower degree of dormancy, which may be related to lower survival ability.

4.6. ASSESSMENT OF THE EFFICACY OF *Epicoccum nigrum* AND *Penicillium frequentans*

4.6.1. Efficacy of *Epicoccum nigrum* as a post-harvest BCA for brown rot of fruit

E.nigrum successfully controlled brown rot of cherries produced by *M.laxa* in conditions very favourable for disease development (25°C and %RH as high as 100%). Disease suppression was almost complete 6 days after inoculation. After 10 days percentage control varied between 80 and 99%. This same isolate of *E.nigrum* has been shown to reduce brown rot of peaches, with percentage disease control ranging between 40 and 80% (Foschi *et al.*, 1994). In field experiments *E.nigrum* was the most effective fungus in reducing the number of

fruits affected by *M.laxa*, compared to other fungal antagonists (*P.frequentans*, *P.purpurogenum* and *Trichoderma pseudokoningii*) and the level of disease control before harvest was equivalent to that obtained with the fungicide procymidone (Foshi *et al.*, 1994). No other fungal antagonists have been tested for the biocontrol of brown rot. However, several bacteria have been shown to effectively control the disease. *B.subtilis* was able to prevent brown rot (*Monilinia fructicola*) of peaches and apricots and substantially reduce decay of plums and nectarines (Pusey and Wilson, 1984). Utkhede and Sholberg (1986) obtained some *B. subtilis* isolates antagonistic to *A. alternata* and *M. fructicola* in cherry fruits. Control of rots caused by these fungi was similar to that obtained with the standard fungicides benomyl or iprodione. Endophytic bacteria have been also tested, with control levels greater than 90% (Pratella *et al.*, 1993). Control of brown rot of peaches and nectarines by *Pseudomonas corrugata* and *Pseudomonas cepacia* was in some cases better than that obtained by fungicides when fruits were artificially inoculated (Smilanik *et al.*, 1993).

Disease development was faster at higher relative humidities (75 and 100%). Only a very drastic reduction in %RH (33%) resulted in a reduction in disease percentage. However, storage of fruits at this low %RH is not realistic, because it affects the water content of the fruits. On the other hand, disease control by *E.nigrum* was equally effective at any of the %RH tested, and efficacy of the antagonist produced at normal or reduced a_w was similar. It has been seen earlier (Section 3.5.2.) that *E.nigrum*98 has certain advantages compared to *E.nigrum*996 on culture media at reduced a_w: in many cases growth rates were higher or a more intense sporulation was observed. However, in this experiment

the fungus probably takes water from the fruits, because it was placed on a wounded tissue, at the insertion point of the peduncle, and in this way the influence of water stress produced by the low relative humidity in the chambers is reduced.

The efficacy of modified inoculum as post-harvest BCAs has also been studied by Teixidó (1997). This showed that biocontrol of *P.expansum* in apples by *Candida sake* (strain CPA-1) was conserved or improved when the yeast cells were modified to contain enhanced amounts of sugar alcohols and sugars. Apart from the efficacy it was pointed out that viability of *C.sake* cells was improved when produced at reduced a_w. Viability of *E.nigrum* was also improved in this way (Section 3.5.6).

The effect of RH has not been previously tested for other post-harvest BCAs of brown rot of fruit. However, the effect of temperature on disease control was studied by Pusey and Wilson (1984). They found that very little decay of fruit treated with *B.subtilis* (B-3) was observed at temperatures ranging from 5 to 30°C. It seems therefore that environmental storage conditions of temperature and %RH are not limiting to an efficient biocontrol of brown rot of fruit, which is an advantage in the practical use of microbial antagonists for the control of this disease.

4.6.2. Control of peach twig blight by *Epicoccum nigrum* and *Penicillium frequentans*

In this study, both fungi tested were able to reduce the disease induced by *M.laxa* on peach twigs to similar levels as the fungicide captan. Previously, the potential of several fungal antagonists (*Penicillium frequentans*, *P.purpurogenum*, *Aspergillus flavus* and *Epicoccum nigrum*) for biocontrol of *M.laxa* twig blight on peach has been demonstrated in field experiments (Melgarejo *et al.*, 1986), but using a different method for the application of antagonists. This consisted of inoculation of shoots with mycelial plugs from agar cultures. Disease was also reduced by spray application of spore suspensions of *P.frequentans* (De Cal *et al.*, 1990) and *E.nigrum* (Madrigal *et al.*, 1994) in orchard trials. *E.nigrum* has also been evaluated in biocontrol studies for other pathogens, such as *Sclerotinia sclerotiorum* (Inglis and Boland, 1992).

The parameters used to assess disease development showed different results: reduction of lesion length was significant 35 and 46 days after inoculation, disease index was smaller for treated twigs already by the 26th day, and no differences in the extent of pathogen colonisation were found at the end of the experiment. In other field trials with these same fungi as BCAs, disease reduction was recorded at different dates of evaluation with degree of control being variable depending on the year. However, in most cases reduction of the extent of colonisation of the pathogen by biological treatments was recorded (Melgarejo *et al.*, 1986; De Cal *et al.*, 1990; Madrigal *et al.*, 1994). These differences could be due to variations in disease development in different years. In this trial in 1995 disease development in the untreated control in the first 2-3 weeks after infection

was not very rapid and this may have contributed to the degree of control obtained with *E.nigrum* (Madrigal *et al.*, 1994) and *P.frequentans* (De Cal *et al.*, 1990). On the other hand, this slow disease development in the untreated control resulted in an average maximum lesion length of 56 mm, making it very difficult to detect differences in pathogen extension following the procedure used, where twigs where cut into 5 cm long pieces.

The degree of disease control by *Epicoccum nigrum* was higher when the fungus was produced in media at reduced a_w. This was shown by a reduction in both lesion length and disease index. *E.nigrum* is a species very sensitive to conditions of low water availability. The strain of *E.nigrum* used in this study grows very poorly in culture medium at 0.95 a_w (Section 3.5.2), with the minimum a_w value producing enough biomass for treatment application being 0.98, which was the conditions used to produce inoculum. This sensitivity parallels the high dependence of *E.nigrum* on conditions of high relative humidity for successful control of peach twig blight (Madrigal *et al.*, 1994). On the other hand, Hannusch and Boland (1996a, b) found that efficacy of *Epicoccum purpurascens* in controlling *Botrytis cinerea* and *Sclerotinia sclerotiorum* was not affected by temperature or relative humidity (100, 95 and 90%). They suggested that the behaviour of *E.purpurascens* may be attributable to its ecological competence or to a mechanism of biocontrol action, such as antibiosis, which might be independent of the atmospheric environment. The independence of antibiosis from the prevailing environmental conditions is however difficult to accept. It is possible that the isolate used by these authors is more tolerant to low water

availability than the one used in the present study and more competent in drier conditions.

When grown at reduced a_w, osmotic adjustment in *E.nigrum* probably results in a reduction of cell water potential by accumulation of compatible solutes (Section 3.5.3). When the fungus is then sprayed onto peach twigs the imbalance in water potential with the phyllosphere is reduced compared to the inoculum produced at high water availability. *E.nigrum* becomes in this way less dependent on high atmospheric relative humidity, and an advantageous colonisation may occur on the twigs resulting in improved disease control. No previous studies have reported improved disease control in the phyllosphere by physiological manipulation of the antagonists. This is not the case for the efficacy of biocontrol fungi against pests. Hallsworth and Magan (1994 b, c) demonstrated that conidia of entomopathogenic fungi with enhanced levels of low molecular weight polyols are more pathogenic than unmodified conidia over a range of water availabilities, this enhanced pathogenicity being related to improved germination at reduced a_w (Hallsworth and Magan, 1995). Other authors have also shown that mutants of entomopathogenic fungi that germinate and grow at reduced relative humidities were more virulent than their parental strains (Matewele *et al.*, 1994) although polyol and sugar contents were not determined. In the present study it has been shown that no improved germination is obtained by growing *E.nigrum* at reduced a_w. However, improved germ tube length and growth was obtained in some of the conditions tested (Sections 3.5.1 and 3.5.2). This could contribute to the improved biocontrol obtained. On the other hand, improved biocontrol could be related not

only to an improved colonisation of peach twigs but also to a more active production of flavipin when the fungus was grown at 0.98 a_w, as discussed earlier.

Penicillium frequentans was also able to reduce peach twig blight. Reduction in lesion length was similar when the fungus was grown at high or reduced a_w. On the other hand, disease index was smaller in trees treated with *P.frequentans* produced at reduced a_w. These results indicate that with *P.frequentans* it is more difficult to improve disease control using the strategy proposed. This is not surprising considering that species of *Penicillium* are xerotolerant, with a minimum a_w for growth ranging between 0.90 and 0.78 (Corry, 1987). The strain used in this study grows abundantly in culture medium at 0.95 a_w, which was the chosen value to produce inoculum. *P.frequentans* produced on medium at high a_w and applied to peach twigs becomes rapidly adapted to the dry conditions of the phyllosphere and it is able to colonise the twigs successfully. This counteracts rapidly the possible initial advantage of the inoculum produced at reduced a_w. On the other hand, it has been shown that efficacy of *P.frequentans* depends on the establishment of a high population of the fungus on the twigs (De Cal *et al.*, 1990). Other factors apart from water availability are probably negatively affecting this establishment, especially the presence of competitors. Application of the adequate nutrients has been shown to be effective in improving biocontrol of the disease by *P.frequentans*, probably by eliminating the competition effect (De Cal and Melgarejo, 1992).

Differences in disease suppression by *E.nigrum* were not related to the nutrients added to the inoculum. Only disease index was different 35 days after inoculation

between treatments with and without nutrients, and the value of disease index was higher for treatments with nutrients. The importance of nutrients in successful biological control have been shown for *P.frequentans* in the control of peach twig blight. The nutrients proposed by De Cal *et al.* (1990) were used in this study. Nutrients are also important in other biocontrol systems (Bashi and Fokkema, 1977; Cullen *et al*,. 1984). The nutrients used in this study applied with *E.nigrum* have been selected *in vitro* from those giving maximum growth of the antagonist without favouring growth of *M.laxa* (De Cal *et al.*, 1993), but this is not reflected in improved biocontrol *in vivo*, indicating that nutrient limitation in the phyllosphere is not a specific factor limiting biocontrol by *E.nigrum*. This is in agreement with results obtained by Zhou and Reeleder (1989), who showed that addition of nutrients to spore suspensions of *E.purpurascens* had no significant effect on the control of white mould of snap bean. In this case water relations should play a more important role in the biocontrol, as shown before.

CHAPTER 5

CONCLUSIONS

AND

FUTURE WORK

CONCLUSIONS

1. A method has been developed for the induction of sporulation of *Penicillium oxalicum* in submerged culture. Sporulation occurred in two situations: a) after transfer of 24 h cultures to nitrogen-free medium, and b) in medium with relatively high C:N ratio amended with calcium.

2. *P.oxalicum* is able to colonise sterile soil. Colonisation was favoured by low water potential and nutrient addition.

3. Surface hydrophobicity of aerial and submerged conidia of *P.oxalicum* is similar, but slight differences were observed depending on the method used.

4. Observations under the SEM showed that submerged spores of *P.oxalicum* were covered by a filamentous matrix, which was absent in aerial ones. Otherwise, appearance of both spore types was similar in size and shape.

5. The minimum concentration of *P.oxalicum* aerial spores to reduce *Fusarium* wilt of tomato is $6x10^4$ spores ml^{-1} substratum.

6. Aerial spores were slightly more effective in controlling *Fusarium* wilt of tomato than submerged ones. Mycelium was ineffective.

7. Viability of aerial and submerged *P.oxalicum* spores was high after 27 weeks when stored fresh at either 4 or 25°C, but aerial spores survived slightly better. Freeze-drying severely affected viability, especially of submerged spores.

8. Formulation of aerial spores of *P.oxalicum* with tomato seeds enhanced the growth promotion effect of the antagonist *in vitro*, which may be related to the ability of the fungus to control the disease.

9. A method has been developed for spore production of *Epicoccum nigrum* by solid substrate fermentation (SSF). Maximum levels of sporulation were obtained at high a_w (0.996) or reduced a_w (0.98) adjusted with a mixture of glycerol/water.

10. *E.nigrum* produced at reduced a_w showed improved germ tube extension and in some cases colony growth rate under water stress conditions, showing the acclimatisation capability of the fungus.

11. When grown in culture medium at reduced a_w, *E.nigrum* and *Penicillium frequentans* accumulated glycerol as the main compatible solute. However, the mechanism of retention must be different, as both fungi have different tolerance to low water availability.

12. Glycerol was also the main compatible solute accumulated by *E.nigrum* when grown at reduced a_w by SSF.

13. Accumulation of solutes produced differences in osmotic potential of *E.nigrum* mycelium, but not in turgor pressure. No correlation between turgor pressure and growth rate was established for this fungus.

14. *E.nigrum* produced under water stress conditions showed enhanced competence against the pathogen *Monilinia laxa*, shown by a higher Niche Overlap Index under water stress conditions, compared to the inoculum produced at high a_w.

15. Production of *E.nigrum* spores at low a_w resulted in an improved survival when stored fresh. Freeze-drying severely affected the viability of spores produced either at high or reduced a_w.

16. *E.nigrum* was able to control brown rot of cherries (*M.laxa*) at optimum conditions for the development of the disease. Control by *E.nigrum* produced at high or reduced a_w was similar.

17. Biocontrol of peach twig blight (*M.laxa*) was improved by producing *E.nigrum* at reduced a_w, probably because of better colonisation of the phyllosphere. However, this did not occur for *P.frequentans*.

SUGGESTIONS FOR FUTURE WORK

1. Studies to identify the factor(s) responsible for the differences in efficacy between aerial and submerged spores of *Penicillium oxalicum* in the control of *Fusarium* wilt of tomato. These include a more detailed analysis on the assemblage of hydrophobins on the spore surface, or a determination of the production of plant hormones by the fungus.

2. Experiments to determine the relationship between the growth promotion effect produced by *P.oxalicum* in the tomato plant and its ability to reduce the disease.

3. Studies on practical aspects for the application of *P.oxalicum*: determination of duration of resistance and other aspects related to timing and frequency of applications, scaling-up the production of the fungus in fermenters, and testing of industrial methods for coating tomato seeds.

4. Production of improved strains of *Epicoccum nigrum*: Examine subculturing of the fungus under conditions of decreasing water availability and mutagenesis (UV and chemical) to obtain strains more tolerant to water stress.

5. Competition studies between *Monilinia laxa* and *Epicoccum nigrum* simulating as much as possible real environmental conditions, especially fluctuations of temperature and humidity. Development of methods to apply tools such as the de Wit replacement series to this system.

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APPENDIX

PUBLISHED PAPERS

Pascual, S., Magan, N. and Melgarejo, P. (1996). Improved biological control of peach twig blight by physiological manipulation of *Epicoccum nigrum*. Proceedings of the Brighton Crop Protection Conference. Pests and Diseases 411-412.

Pascual, S., Melgarejo, P. and Magan, N, (1997). Induction of submerged conidiation of the biocontrol agent *Penicillium oxalicum*. Appl. Microbiol. Biotechnol. 48, 389-392.

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IMPROVED BIOLOGICAL CONTROL OF PEACH TWIG BLIGHT BY PHYSIOLOGICAL MANIPULATION OF *EPICOCCUM NIGRUM*

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ABSTRACT

The performance of *Epicoccum nigrum* as a biocontrol agent of peach twig blight (*Monilinia laxa*) depends on conditions of high relative humidity. Production of *E.nigrum* in medium at reduced water availability (a_w = 0.98) resulted in improved biocontrol, compared with inoculum produced at high water availability (a_w = 0.995). The fungus accumulated compatible solutes when grown at reduced water availability. This accumulation probably contributes to a more efficient use of water when the fungus is placed in the phyllosphere.

INTRODUCTION

Epicoccum nigrum, a component of the resident mycoflora of peach twigs and flowers, reduces twig blight caused by *Monilinia laxa*. However, biocontrol obtained is variable, depending on disease severity and humidity conditions (Madrigal *et al.*, 1994). When exposed to conditions of low water availability, fungal cells accumulate compatible solutes, generally polyols, glycerol being the most important. Recently, it has been shown that conidia of entomopathogenic fungi with elevated concentrations of polyols tolerated lower water potentials and were more pathogenic than unmodified conidia (Hallsworth & Magan, 1994). The present investigation attempted to use this approach to improve biological control by aclimatization of *E.nigrum* to conditions of low water availability in the phyllosphere.

METHOD

Biomass of *E.nigrum* was obtained from 10 d old cultures on potato dextrose agar (PDA) (mainly spores) and potato dextrose broth (PDB) (mainly mycelium). PDA and PDB were unmodified (water activity = 0.995) or modified with glycerol (water activity = 0.98). Polyols were quantified by HPLC, with a Hamilton HC-75 Ca^{2+} column; a refraction index detector; mobile phase of acetonitrile:water (40:60). Mycelium and spores were separated from the culture media, washed and freeze dried. Samples of 50 mg of freeze-dried material were mixed with 1 ml Analar water, sonicated for 2 min, boiled for 5.5 min and filtered through 0.2 μm filters. Samples were then injected in the HPLC. For the field trial, mycelium and spores of *E.nigrum* were separated from the culture media, homogenized in a 0.06 % Nu-Film-17 solution and filtered through cheesecloth. The fungal suspensions were adjusted to 10^6 conidia (plus mycelial fragments)/ml. Treatments were first applied the day before artificial inoculation of peach twigs with *M.laxa*, and applications were then repeated four times at 7-day intervals. A control treatment with captan was set up. Disease was

assessed by measuring the length of lesion induced by *M.laxa* in the shoots.

RESULTS AND DISCUSSION

Disease control by *E.nigrum* was higher when the fungus was produced in media at reduced water activity compared to normal water activity, although neither differed from captan ($p=0.05$) (Figure 1). In these conditions osmotic adjustment probably results in a reduction of cell water potential by accumulation of compatible solutes. Glycerol and arabitol were the solutes accumulated (Table 1), with an ability to depress water activity greater than others such as erythritol or mannitol. When the fungus is then sprayed, the imbalance in water potential with the phyllosphere is reduced compared to the inoculum produced at high water availability. *E.nigrum* becomes less dependent on high atmospheric relative humidity, and an advantageous colonization occurs on the twigs resulting in improved disease control. This demonstrates that quality of inoculants can be modified during the production process by physiological manipulation of antagonists.

Figure 1. Evolution of lesions induced by *M.laxa*.

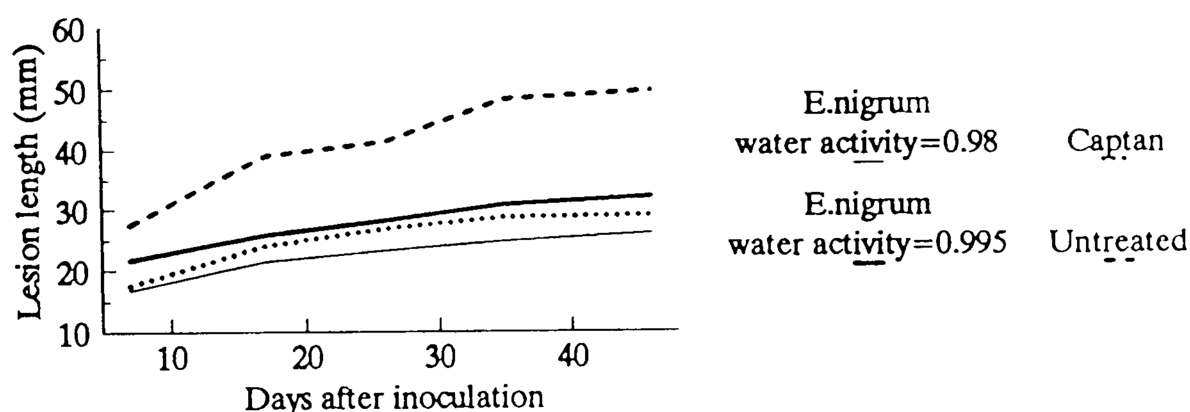

Table 1. Contents of polyols in spores (S) and mycelium (M) of *E.nigrum*. Data are means of three replicates and indicate mg/g freeze-dried biomass. Asterisks indicate a significant difference in the solute analyzed.

water activity of medium (a_w)	glycerol		arabitol		mannitol	
	S	M	S	M	S	M
0.995	15.84	2.38	4.45	22.68	99.93	123.89
0.98	149.31*	382.29*	16.37*	25.41	66.79	82.04

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Ecophysiological factors affecting growth, sporulation and survival of the biocontrol agent *Penicillium oxalicum*

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Abstract

The effect of temperature, pH, water potential and sources of nitrogen and carbon on the biocontrol agent *Penicillium oxalicum* were studied in vitro. The fungus is xerotolerant, mesophillic and has a wide pH tolerance. The parameters evaluated (germination, germ tube length, growth rate and sporulation) showed different sensitivities to the environmental factors. Peptone and free amino acids gave the highest growth rates and high levels of sporulation. Xylose, mannose and fructose gave the highest growth rates and mannose induced strong sporulation. The effect of nutrients (mannose + arginine) and water potential was also studied in vivo. The xerotolerant character of the fungus was confirmed. From this study we consider *Penicillium oxalicum* ecologically competent to perform effectively as a biocontrol agent in the soil environment.

Introduction

Penicillium oxalicum is a promising fungal agent for biological control of soil-borne diseases [1–5]. Recently, De Cal et al. (6) reported that *P. oxalicum* (isolate 121) induced resistance to *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants. Generally, the application of a conidial suspension of *P. oxalicum* to seedbeds before or at the time of transplantation resulted in a disease reduction of 20–50% [6, 7]. This may be due, at least in part, to a lack of understanding of the environmental factors affecting conidia germination and growth of *P. oxalicum*. Environmental factors such as water availability (water potential), temperature and pH can have important influences on the ability of fungi to use soil nutrients and grow in soil [8, 9].

Little information is available on the nutritional and environmental requirements of *P. oxalicum*. As such knowledge is needed to understand and to improve the biocontrol efficacy of *P. oxalicum* in the field, a study of the nutritional and environmental requirements of the fungus was carried out. This study reports on the effect of (a) carbon and nitrogen sources, (b) temperature, (c) pH and (d) water potential on parameters such as germination, germ tube length, growth rate, sporulation and soil populations of *P. oxalicum*. This approach has already provided useful information for obtaining optimal production of biomass and conidial inoculum.

Materials and methods

Fungus

Isolate 121 of *Penicillium oxalicum* was kindly provided by Dr. F. Reyes (Consejo Superior de Investigaciones Cientificas) and used in all experiments. This isolate of *P. oxalicum* was selected in our laboratory for its ability to biocontrol *F. oxysporum* f. sp. *lycopersici* in tomato plants [6]. *P. oxalicum* cultures were stored on potato dextrose agar (PDA) slants at 4 °C and grown in the dark at 20–25 °C on PDA for conidial and mycelial production.

Media

(a) Basal medium (BM). The BM contained per litre: 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, and 1 ml of a trace element solution containing per litre: 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 70 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, 10 mg $(NH_4)6MoO_{24} \cdot 4H_2O$ and 10 mg $CuSO_4 \cdot 5H_2O$ [10]. The BM was amended with glucose (10 $g \cdot l^{-1}$) or with a mixture of arginine-glycine (1 :

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1; w/w) (2 g·l^{-1}), when testing nitrogen sources (NS) and carbon sources (CS) (Table 1), respectively. NS and CS were filter-sterilized and added to autoclaved medium at a rate of 2 g·l^{-1} and 20 g·l^{-1}, respectively. To obtain solid BM, 2% agar was added to the medium, which was dispensed in 9 cm diameter Petri dishes.

(b) Potato Dextrose Agar (PDA) and Czapek-Dox Broth (both from Difco, Difco Laboratories, Detroit, MI, USA). For water activity studies, the media were osmotically modified to −0.8, −2.3, −3.8, −6.8, −9.8, −12.8, −15.7 and −18.7 MPa using NaCl [11], and matrically to −0.8, −1.9, −3.0, −5.1, −6.2, −7.2, −9.4 and −11.7 MPa with polyethylene glycol 8000 (PEG 8000) [12]. Media and PEG solutions were autoclaved separately. For pH studies, the pH of media was adjusted after autoclaving with sterile solutions of 1 N HCl or 2 N NaOH to obtain the following levels: 4, 5, 6, 7, 8, 9 and 10.

In vitro assays

(a) Germination and germ tube extension. The effect of temperature, pH and water potential on spore germination and germ tube length was assessed in Czapek Broth as indicated by De Cal et al. [13], with slight modifications. Microscope slides were placed in 15 mm diameter Petri dishes containing damp filter papers. A 45 μl drop of a *P. oxalicum* spore suspension (1×10^6 spores·ml^{-1}) in Czapek or Czapek modified with HCl, NaOH, NaCl or PEG 8000, was placed on each microscope slide. Filter papers were wetted with solutions of NaCl or PEG 8000 when the effect of water potential was studied. Incubation conditions were 17 h at 25 °C or 17 h at temperatures ranging from 4° to 35 °C in the experiment to examine the effect of temperature. Four replicates were used for each treatment and factor studied. Fifty conidia and 25 germ tubes were examined for each replicate. The experiments were repeated at least twice

(b) Mycelial growth rate (mm·day^{-1}). The effect of nutrients on mycelial growth rate was assessed in 90 mm Petri dishes containing solid BM. Plates containing PDA were used to test the effect of temperature (4, 10, 15, 20, 25, 30 and 35 °C), pH and water potential. Plates were inoculated with a drop of a heavily concentrated spore suspension of *P. oxalicum* in water + 0.2% agar + 0.05% Tween 80, from 7-day-old cultures and were incubated in the dark at 25 °C for 10 days, except for nitrogen and carbon sources which were incubated for 12 days. Colony diameter was recorded daily. Ten replicate plates were inoculated for each treatment and factor studied. For each factor the experiment was repeated at least twice.

(c) Sporulation. To study the effect of nutrients, temperature, pH and water potential on sporulation of *P. oxalicum*, the same plates used in the mycelial experiment were used (five plates for each treatment and factor). The colonies were flooded with 20 ml of water + Tween 80 and the conidia dislodged with a glass rod. The suspension was then transferred to 100 ml flasks and sonicated for 5 min. Serial dilution was carried out when needed to be able to measure absorbance in a spectrophotometer at 415 nm. A calibration curve was prepared ($y = 0.0009825 + 0.0515234x$; where y was absorbance and x was spore concentrations) from suspensions of 11 different spore concentrations estimated with a haemocytometer.

(d) Mycelial dry weight. The effect of NS and CS on mycelium dry weight was assessed in BM as described by De Cal et al. [10]. Five 100 ml flasks were inoculated for each nutrient source. After 13 days incubation, the mycelium was harvested by filtration through Whatmann No. 1 filter paper and the dry weight (mg) was recorded.

In vivo assays

The effect of soil water potential on *P. oxalicum* population was assayed in two experiments:

(a) Pot experiment. Pots (12 × 12 × 12 cm) containing sterile soil with pH = 7.3, total organic matter = 45%, initial water content = 3.35% and field capacity = 42.8% were inoculated with 50 ml of a *P. oxalicum* spore suspension (0.5×10^8 spores·ml^{-1}) in water +0.1% Tween 80. The soil characteristic curve, to relate the water potential of the soil to the actual water content, was determined by psychometry. For the adsorption curve, different amounts of water were added to 10 g samples of soil and allowed to equilibrate for 24 h with regular mixing. Subsamples were then placed in an automated psychrometer (Wescor HT33 Psychrometer) and subsequently dried in an oven at 80 °C for 24 h to determine actual water content. Water potential of soil was adjusted by adding sterile distilled water to dry soil to bring the final moisture content to a water potential extrapolated from the moisture-release curve. Soil moistures were adjusted to give water con-

tents of 48, 40.7, 31.5 and 30%, equivalent to water potential values of 1.83, 2.83, 7.23, and 7.66 (−MPa), respectively. Pots were maintained at room temperature and water content was readjusted daily using distilled water. Five pots were inoculated for each humidity value tested.

(b) Test tube experiment. Ten gram aliquots of soil were placed in test tubes and autoclaved for 1 h at 120 °C; water losses during the sterilization were taken into account when adjusting the soil water potential. Water potential of soil was adjusted by adding sterile distilled water to autoclaved soil to bring the final moisture content to a water potential extrapolated from the adsorption curve. Soil moistures were adjusted to give water contents of 45.3, 40, 28 and 19%, equivalent to water potential values of 2.1, 3.2, 8.5, and 14.9 (−MPa), respectively.

Soil in test tubes was allowed to equilibrate for 24 h in separate hermetic containers with relative humidities of 98.5, 97.7, 94.0 and 89.7% (equivalent to 2.1, 3.2, 8.5, and 14.9 −MPa) relative humidities were in equilibrium with glycerol solutions of the corresponding water activities. Each tube was inoculated with 0.5 ml of a spore suspension of *P. oxalicum* containing 1×10^8 spores ml^{-1}; this was taken into account when calculating the water needed to adjust the water content. The hermetic containers were placed in an incubator at 25 ± 1 °C.

The effect of nutrients was studied in experiment (b) at −2.1 MPa water potential. Water content was adjusted in this case by adding sterile nutrient solution to soil to bring the final moisture content to 45.3%. The sterile nutrient solution contained 0.8% mannose, 0.08% arginine, and 0.1% Tween 80, as these nutrients performed best in the in vitro tests.

P. oxalicum populations were estimated 1, 10, 20, 30, 40, 50 and 60 days after inoculation in experiment (a), and 1, 5, 10, 20, 30, 40, 50 and 60 days after inoculation in experiment (b). In both cases, three 1 g samples were taken from each treatment on each date. Samples were placed in 250 ml flasks containing 150 ml buffer (pH = 7) and shaken for 30 mm at 150 rpm. After 100- and 1000-fold dilution, three 0.1 ml aliquots per dilution were spread on Petri dishes containing *P. oxalicum* selective medium. This medium is based on Nash & Snyder medium [14] modified. to contain 1 molal concentration of NaCl. Plates were incubated in the dark at 25 ± 1 °C for 5 days before the colonies were counted. Plates containing fewer than 5 or more than 150 colonies were discarded [15, 16]. The number of colony forming units (cfu) per gram of soil dry weight was estimated for each sample.

In experiment (b), microscopy was also carried out to observe growth of *P. oxalicum* in soil, at the same dates the population was estimated. Three 1 g samples were taken per treatment and processed as described by Couteaudier and Steinberg [17] with slight modifications. Each sample was incubated for 2 h with 0.5 ml of a 0.3% calcofluor solution. After incubation, 100 ml of phosphate buffer (0.06 M, pH = 7.6) were added to each sample and 3 ml were taken and filtered through a Millipore membrane (GSWP, 0.2 μm pore diameter). The filter was mounted with Entellan (Merck) and the preparation was observed using a Nikon microscope equipped with an epifluorescence illuminator and UV excitor filter. Three slides were mounted per sample. Three photographs were taken per slide and percentage germination and germ tube length were estimated for each sample.

Statistical analysis

The data were analyzed by analysis of variance. Prior to analysis, data of percentage germination in the in vitro assay were transformed by an arcsin function, data of cfu in the in vivo assay (a) were log transformed, and data of cfu in the in vivo assay (b) were transformed by an sqrt-log function. Means were compared by a Least Significant Difference test ($P = 0.05$) when significant effects were found.

Results

In vitro assays

Addition of all of the carbon sources produced a significant increase ($P = 0.05$) in growth rate and dry weight of *P. oxalicum* compared to that of the basal medium, except for arabinose growth rate (Table 1). Xylose, mannose, sucrose, and fructose were the carbon sources which produced the highest ($P = 0.05$) growth rates of *P. oxalicum* (Table 1). Xylose, sucrose and cellobiose were the best ($P = 0.05$) for increases in dry weight. In contrast, only two carbon sources, arabinose and mannose stimulated sporulation of *P. oxalicum* when they were added to the basal medium.

Peptone, tryptone, ammonium tartrate, malt extract and free amino acids produced a significantly higher ($P = 0.05$) growth rate for *P. oxalicum* than basal medium (Table 1). The dry weight of *P. oxalicum* was signif-

Table 1. Effect of different carbon[a] (C) or nitrogen[b] (N) sources on growth rate, sporulation (12-day-old cultures) and mycelial dry weight (13-day-old cultures) of *Penicillium oxalicum.*

C/N source	Growth rate (mm/d)[c]	Sporulation (10^6/cm^2)[d]	Dry weight (mg)[d]
Glucose	70	–	12
Arabinose	–	674	164
Xylose	107	–	639
Fructose	121	–	528
Mannose	121	441	316
Galactose	78	–	573
Maltose	99	–	547
Sucrose	105	–	601
Lactose	22	–	146
Cellobiose	26	–	705
Trehalose	65	–	395
Sorbitol	94	–	354
NH_4NO_3	–	618	362
NH_4HPO_4	–	–	807
$(CHOHCOONH_4)_2$	8	–	706
KNO_3	–	384	385
$NaNO_2$	–	1943	89
$NaNO_3$	–	426	471
Asparagine	29	1548	971
Arginine	26	1291	885
Glycine	26	1006	766
Serine	9	1434	836
Glutamine	11	–	661
Peptone	10	1520	883
Tryptone	10	–	568
Yeast extract	–	828	465
Malt extract	9	–	227
NH_4Cl	–	589	556

[a] All carbon sources were added to basal medium at a rate of 20 g·l^{-1}. The basal medium was amended with a mixture of arginine-glycine (1 : 1; w/w) (2 g·l^{-1}). To assess growth rate and sporulation 20 g agar per litre was added to basal medium.
[b] All nitrogen sources were added to basal medium at a rate of 2 g·l^{-1}. The basal medium was amended with glucose (10 g·l^{-1}). To assess growth rate and sporulation, 20 g agar per litre was added to basal medium.
[c,d] Data are means of [c]ten or [d]five replicates. Data were transformed by $y = (p \times 100/q) - 100$ where p = growth rate, sporulation or dry weight in medium plus the tested carbon or nitrogen source; q = growth rate, sporulation or dry weight in basal medium. Only data significantly ($P = 0.05$) different from data in basal medium are presented. Values obtained in basal medium amended with arginine-glycine were 2.46 mm/d, 2.7×10^6/cm^2 and 14.6 mg for growth rate, sporulation and dry weight, respectively. Values obtained in basal medium plus glucose were 4.02 mm/d, 0.999×10^6/cm^2 and 7.78 mg for growth rate, sporulation and dry weight, respectively.

Table 2. Effect of temperature on growth rate (GR), sporulation (S) (10-day-old cultures) and germination (G) and germ tube extension (GTE) (17 h incubation) of *Penicillium oxalicum.*

T(°C)	GR (mm/d)	S ($\times 10^6$/cm^2)	G (%)	GTE (μm)
4	0.4	43	0(0.000)	0
10	2.1	106	5(0.010)	2
15	3.2	157	49(0.105)	6
20	4.7	140	90(0.196)	52
25	5.1	133	95(0.207)	105
30	4.2	161	80(0.174)	48
35	0.4	129	1(0.002)	2
LSD	0.2	47	(0.023)	16

Data are means of ten (for GR), five (for S) or four (for G and GTE) replicates (data in brackets are arcsin transformed). Growth rate and sporulation were tested in potato-dextrose agar. Germination and germ tube extension were tested in Czapek broth.

icantly higher ($P = 0.05$) in all media tested when compared to the basal medium. *P. oxalicum* growing on an asparagine medium gave the maximum growth rate (5.2 mm·day^{-1}) and mycelium dry weight (83.37 mg). However, when nitrogen was supplied as nitrate, nitrite or yeast extract, the growth rate was lower ($P = 0.05$) than in the basal medium. Maximum sporulation ($P = 0.05$) was recorded with $NaNO_2$ (2×10^7 conidia cm^{-1}). Good sporulation ($P = 0.05$) was also obtained in media containing free amino acids and peptone (Table 1).

The optimum growth temperature was 25 °C (Table 2), where growth rates, germination and length of germ tubes, were significantly greater ($P = 0.05$) than at other temperatures. Growth rate and germination was reduced by almost half at 10 °C and to minimum values below 10 °C and above 30 °C. The sporulation range was between 15 ° and 30 °C, with temperatures outside this range inhibiting sporulation, particularly below 10 °C. Germ tube extension was the most temperature sensitive parameter, being reduced by half (Table 2) below and above 25 °C.

Growth was significantly ($P = 0.05$) reduced on alkaline medium (pH $\geq$ 9), and the optimum values were obtained on acid media (pH 4–6) for all growth parameters. In contrast, sporulation was stimulated at pH 7–8 (data not shown).

When the osmotic potential was decreased below −15.7 MPa, growth rate and germination were significantly ($P = 0.05$) reduced compared to the unmodified

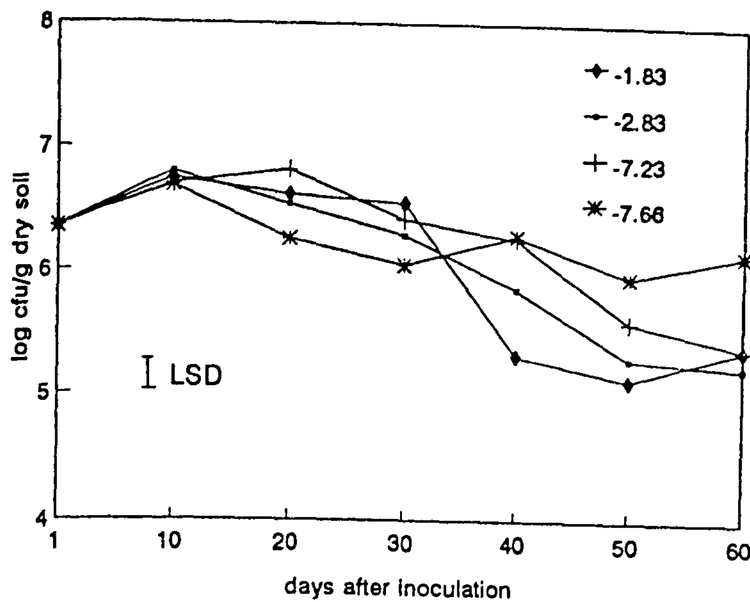

Figure 1. Survival of recovery of colony forming units (cfu) of *Penicillium oxalicum* in soil with different water potentials (MPa) for 60 days in the pot experiment. Data are means of three replicates.

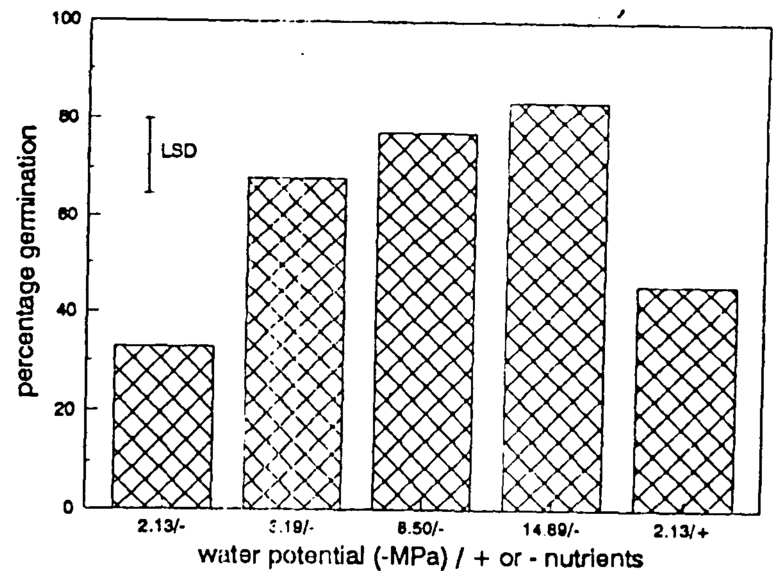

Figure 2. Effects of soil water potential and nutrient (0.8% mannose, 0.08% arginine, and 0.1% Tween 80) addition on percentage germination of *Penicillium oxalicum* at 25 ± 1 °C, 5 days after inoculation. Data are means of three replicates.

medium (Table 3). The highest growth rate occurred at −6.8 MPa. Germination rates were similar regardless of osmotic potential of medium until −12.8 MPa. Sporulation was only slightly reduced by decreasing osmotic potential. In contrast, germ tube length was strongly affected by changes in osmotic potential (Table 3).

Growth rate was also influenced by changes in matric potential (Table 3). At −5.1 MPa or lower, growth rate, sporulation and germ tube length were all significantly (P = 0.05) reduced.

In vivo assays

(a) Pot experiment. The highest number of colony-form units (cfu) of *P. oxalicum* isolated from soil were at the lowest water potential (−7.7 MPa) (P = 0.05), 60 days after inoculation and this incubation was maintained throughout the experiment (Figure 1). At −1.83 MPa, the *P. oxalicum* population decreased significantly from 2.2×10^6 to 2×10^5 cfu·g^{-1} dry soil (P = 0.05) after 40 days. Lower water potentials (−2.8 and −7.2 MPa) showed a significant decrease (P = 0.05) in cfu after 50 days.

(b) Test tube experiment. Data of colony forming units (cfu) are shown in Table 4. The population level of *P. oxalicum* was affected both by soil water potential and nutrient addition. From day 5 onwards the effect of water potential was observed (P = 0.05): the lower the water potential the higher the cfu number. An effect of nutrients was first observed (P = 0.05) 20 days after incubation: cfu value was higher for the treatment where nutrients were added.

Growth of *P. oxalicum* in soil was also monitored by fluorescence microscopy. Percentage germination and germ tube length were estimated at 1 and 5 days after inoculation. The highest (P = 0.05) percentage germination was obtained in soil at −14.89 MPa (Figure 2), then there was a progressive decrease parallel to the increase in water potential, with minimum value for soil at −2.13 MPa, regardless of the presence of nutrients. No significant differences (P = 0.05) in percentage germination were found when comparing data from 1 and 5 days after inoculation. Neither soil water potential nor nutrient addition had any significant (P = 0.05) effect on germ tube length. On the other hand, germ tube length increased slightly from the first day (21 μm) until the 5th day (28 μm).

Discussion

P. oxalicum (isolate 121) was adapted to a wide pH range, and to dry and warm conditions. The minimum, maximum and optimum temperatures for growth of *P. oxalicum* were 4 °C, 35 °C and 15–30 °C, respectively. These values are slightly different to those reported for other *P. oxalicum* isolates: in a study by Domsch et al. [18], minimum, maximum and optimum temperatures were 5 °C higher than 37 °C and 20–25 °C, respectively. In other study reported by Mislivec and Tuite [19], *P. oxalicum* did not grow below 8 °C, but grew optimally near 30 °C and well at 35 °C. Fungi with

Table 3. Effect of osmotic potential (OP) (MPa) (modified with NaCl) and of matric potential (MP) (MPa)(modified with PEG 8000) on growth rate (GR) (mm/d), sporulation (S) ($\times 10^6$/cm^2)(10-day-old cultures), germination (G) (%), and germ tube extension (GTE) (μm) (17 h incubation) of *Penicillium oxalicum.*

OP	GR	S	G	GTE	MP	GR	S	G	GTE
−0.8	5.1	97	90(0.196)	73	−0.8	5.1	120	90(0.199)	73
−2.3	6.0	84	93(0.202)	47	−1.9	7.8	26	84(0.186)	26
−3.8	6.6	65	92(0.200)	39	−3.0	6.5	49	93(0.207)	21
−6.8	7.0	60	87(0.190)	20	−5.1	3.6	54	93(0.206)	19
−9.8	6.8	61	89(0.194)	14	−6.2	3.4	0	88(0.196)	18
−12.8	6.2	71	86(0.186)	10	−7.2	2.6	0	90(0.200)	16
−15.8	5.2	84	58(0.125)	8	−9.4	1.7	0	48(0.106)	9
−18.7	4.7	83	37(0.080)	5	−11.7	0.0	0	5(0.011)	4
LSD	0.1	17	(0.027)	12	LSD	0.2	18	(0.035)	10

Data are means of ten (for GR), five (for S) or four (for G and GTE) replicates (data in brackets are arcsin transformed).
Growth rate and sporulation were tested in potato-dextrose agar. Germination and germ tube extension were tested in Czapek broth. Temperature of incubation was 25 ± 1 °C and pH was 5.6. Osmotic and matric potential of control media was −0.8.

Table 4. Effect of soil water potential (Ψ_w) and nutrient addition on recovery of *Penicillium oxalicum* population (log cfu per gram dry soil) for 60 days in the test tube experiment.

Ψ_w (−MPa)/ ± nutrients	Day after inoculation 1	5	10	20	30	40	50	60
2.1/−	6.18	5.06	3.60	3.66	1.49	6.24	4.32	2.77
	(2.49)	(2.25)	(1.90)	(1.91)	(1.22)	(2.50)	(2.08)	(1.66)
3.2/−	4.39	4.50	6.61	6.91	5.86	4.75	10.56	6.67
	(2.10)	(2.12)	(2.57)	(2.63)	(2.42)	(2.18)	(3.25)	(2.58)
8.5/−	5.53	8.98	10.74	14.03	9.45	8.13	13.40	14.47
	(2.35)	(3.00)	(3.28)	(3.75)	(3.07)	(2.85)	(3.66)	(3.80)
14.9/−	3.43	10.94	13.78	16.47	21.40	13.58	16.57	12.98
	(1.85)	(3.31)	(3.71)	(4.06)	(4.63)	(3.69)	(4.07)	(3.60)
2.1/+	5.49	6.76	6.61	9.17	9.20	8.56	16.40	14.49
	(2.34)	(2.60)	(2.57)	(3.03)	(3.03)	(2.93)	(4.05)	(3.81)
LSD for transformed data = (0.86)								

Data are means of three replicates (data in brackets are sqrt transformed). Nutrients were 0.8% mannose, 0.08% arginine, and 0.1% Tween 80.

these characteristics are considered to be mesophyllic, according to Griffin [8]. In relation to pH, the range of pH values giving optimal growth of *P. oxalicum* was wide (from 4.0 to 8.0). This is in accordance with the results reported by Frisvard [20] in which some isolates of *P. oxalicum* show no growth on his alkaline creatine-sucrose agar.

Germination of this isolate 121 was not reduced until the water potential reached very low values. For spores to germinate they have to absorb water from the medium and water enters following a water potential gradient. The spore water potential of isolate 121 is low enough to allow water to enter until levels of water potential in the medium of −9.4 and −15.7 MPa, matric and osmotic, respectively. Hocking and Pitt [21] reported 0.88 as the minimum water activity permitting germination of *P. oxalicum* in glycerol-based media (equivalent to −17.7 MPa), and 0.86 in NaCl based medium (equivalent to −20.7 MPa). For other *Penicillium* species, the value ranged from 0.78 (−34.1 MPa) to 0.90 (−14.5 MPa). Gervais et al. [22] observed that complete germination of *Penicillium roqueforti* occurred for water activity values ranging from 0.98 (−2.8 MPa) to ca. 1 (0 MPa); germination was reduced

outside this range; they attributed this reduction to lack of diffusion of self-inhibitors out of the spore. In our study, germ tube extension was more affected than germination by reductions in water potential; germ tube extension was reduced at -1.9 and -2.3 MPa matric and osmotic potential, respectively, but this behaviour was not maintained over time: growth rate data indicate that in later stages the fungus had a preference for media with slightly reduced water potential. Maximum radial growth rates for *Penicillium* species varied widely with water activity (a_w) [21]. The majority showed well-defined optima at 0.98–$0.97 a_w$ (-3.0, -4.2 MPa water potential). In almost all cases, growth was observed at all a_w levels at which germination occurred. In relation to sporulation, matric potentials below -1.9 MPa significantly reduced sporulation, and below -6.2 MPa cultures did not sporulate [21]. Mislevic and Tuite [19] also observed that several species of *Penicillium* including *P. oxalicum*, often required a higher relative humidity to sporulate than to germinate.

The matric potential had more inhibitory effect on the growth of this isolate than the osmotic potential. This effect has been reported for other fungi [23]. Polyethylene glycol cannot enter the cell, but membranes are not impermeable to NaCl. In this way, NaCl can contribute to reduce water potential inside the cells, facilitating water entrance and growth. Behaviour of *P. oxalicum* in relation to water potential was different in soil, where matric forces are of primary importance [24].

The relatively zero tolerant character of this isolate was confirmed in the two in vivo assays. The zero tolerant character is important for an effective introduction of the antagonist into soil; soil microbial populations are controlled more by humidity than temperature [25, 26]. Colony forming units of *P. oxalicum* in soil stayed unchanged in the pot experiment and increased slightly with time in the test tube experiment when soil water potential was -3.9 MPa. In the test tube experiment, the increase in population was higher for lower water potentials up to the 20–30th day and then the increase stopped. Differences in cfu values could be thought to be due to the soil washing method, and the isolation procedure favouring conidia above mycelium [27], could lead to underestimating mycelial growth of the fungus. It is reasonable in this case to consider that the germination differences observed in the microscope study (in the test tube experiment) are the cause of the differences in cfu values, as in all the treatments, mycelial growth was observed from germinated spores. Germination was maximum at the lowest water potential values tested (-14.9 and -8.5 MPa). In the wettest soil, water availability for the fungus was higher, and inhibition in germination could be due to a shortage of oxygen, which is an essential factor for spore germination [8]. However, germ tube extension was not affected by this lack of oxygen at least 24 h after inoculation.

There is a relationship between temperature–water potential and growth, competition and nutrient utilization by fungi [23, 28]. This isolate grew better on organic nitrogen sources, such as peptone or amino acids, and fructose, xylose, mannose, maltose and sucrose stimulated growth. With the exception of mannose, these compounds have been shown to stimulate growth in *Penicillium frequentans* and *Penicillium purpurogenum* [10]. However, populations of *P. oxalicum* in the soil and the rhizosphere did not increase when the fungus was applied in a solution of malt and yeast extracts [6]. In this study, the population increased in sterile soil where nutrients favouring growth in vitro (0.8% mannose, 0.08% arginine) were added. Nutrients were not able to counteract the negative effect of excess water in soil at the first growth stages. However, when there is significant mycelial growth, there is a greater demand on nutrient supply; hence, in treatments in dry soil, growth stops at 20–30 days, the effect of water potential is reduced, and nutrients start having a significant effect on the population of *P. oxalicum*.

The carbon and nitrogen sources encountered in the soil will largely be present in more complex compounds than those considered in this study. However, *P. oxalicum* has been reported to produce a wide range of polysacharide and pectin degrading enzymes [29] as well as glucanases, cellulases, hemicellulases and other enzymes [30].

In this study we have analyzed the effect of some abiotic factors on growth of *P. oxalicum* in sterilized soil. However, in non-sterilized soil, biotic interactions are also important, and competition or even predation may have a significant effect on the survival of *P. oxalicum*. We studied elsewhere [6, 7] the behaviour of a suspension of conidia of *P. oxalicum* (10^9 conidia·ml^{-1}) alone or with *F. oxysporum* f. sp *lycopersici* (10^6 propagules·ml^{-1}) in sterilized soil and in the rhizosphere of tomato plants in greenhouse experiments. The *P. oxalicum* population was 10^6 conidia·ml^{-1} at 7 days and remained unchanged for 30–60 days in both cases. These results suggest that competition between *P. oxalicum* and *F. oxysporum* f. sp *lycopersici* may occur. However, in the rhizosphere of tomato plants in a non-sterilized soil, the population

of *P. oxalicum* decreased from 10^6 cfu·g^{-1} fresh root at 7 days to 10^4 cfu·g^{-1} fresh root at 35 days after fungal application. This decrease is the same range as the variations observed in this study due to water potential/nutrient addition, indicating that both biotic and abiotic factors affect growth of *P. oxalicum* in soil.

The isolate of *P. oxalicum* could be an effective biocontrol fungus, because it has the potential to grow well in a wide range of environmental conditions. The weakest aspect of its performance is probably aeration and nutrients; an appropriate formulation of *P. oxalicum* would improve the implementation of biological control. Research is in progress to optimize the use of *P. oxalicum* for practical disease control.

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SHORT CONTRIBUTION

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Induction of submerged conidiation of the biocontrol agent *Penicillium oxalicum*

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Abstract Induction of submerged conidiation of *Penicillium oxalicum* has been examined using a range of synthetic and complex media and complex media supplemented with by-products of the brewing industry. Only one method (Morton's method), consisting of growth in a glucose/salts-based medium (C:N ratio 62.5, medium A) for 24 h and then transference to the same medium without a nitrogen source (medium B), induced conidiation. Levels of sporulation were significantly ($P = 0.05$) increased by addition of calcium or poly(ethylene glycol) 6000 to medium B. The optimum age for transference of the mycelium was 24 h and the optimum pH was 6. Calcium was an induction factor when added to medium A (C:N ratio 62.5) of Morton's method. It was concluded that nitrogen depletion and calcium addition to a medium with high C:N ratio are the factors inducing conidiation of *P. oxalicum*. Maximum levels of conidiation (35×10^6 spores ml^{-1}) were obtained when the nitrogen level in medium A of Morton's method was further reduced (C:N ratio 142.9) and calcium (20 mM) was added. These results are the essential starting point to investigate liquid fermentation systems for the biocontrol agent *P. oxalicum*.

Introduction

Penicillium oxalicum Currie & Thom has shown potential for biological control of *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & H. N. Hansen in greenhouse experiments (De Cal et al. 1995). In the development of a biocontrol agent it is essential to test the suitability of the microorganism for industrial production. Liquid fermentation is preferred over solid fermentation and conidia are preferred to mycelium as the inoculum form. Studies dealing with submerged sporulation of *P. oxalicum* are needed if it is to be used as a biocontrol agent in practice.

Most filamentous fungi will remain entirely vegetative in submerged culture because of complex nutritional and environmental factors (Smith 1978). Sporulation of *Penicillium* spp. has been induced in submerged culture by different methods (Foster et al. 1945; Hadley and Harrold 1958a, b; Morton et al. 1958; Pitt and Poole 1981). Calcium and nitrogen depletion seem to be the most important factors triggering sporulation, and the role of calcium has been extensively investigated for *Penicillium cyclopium* (Ugalde and Pitt 1983a). In this study these factors were tested on *P. oxalicum*.

It is also important from an industrial point of view to be able to produce microbial biomass inexpensively; a suitable medium should consist of readily available agricultural by-products with the appropriate nutrient balance (Lewis and Papavizas 1991). These types of media were examined in the study, and those inducing heavy sporulation of *P. oxalicum* in solid culture were also tested.

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Materials and methods

Microorganism

The *Penicillium oxalicum* isolate was kindly provided by Dr. F. Reyes (Consejo Superior de Investigaciones Científicas). Cultures were stored in potato dextrose agar (PDA) slants at 4 °C and grown in darkness at 25 °C on PDA amended with 7.5% (w:v) NaCl (PDA NaCl) for conidial production.

Media

A range of synthetic media, complex media and complex media based on by-products were tested, but only when Morton's medium

was used (Morton et al. 1958) was submerged sporulation observed. The composition of this medium was as follows. Medium A contained (per litre) 50 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.3 g KNO_3 and 1 ml trace element solution containing (per litre) 225 mg $ZnSO_4 \cdot 7H_2O$, 200 mg $FeSO_4 \cdot 7H_2O$, 40 mg $(NH_4)_6MoO_{24} \cdot 4H_2O$, 38 mg $CuSO_4 \cdot 5H_2O$ and 24 mg $MnSO_4$. Medium B consisted of medium A without a nitrogen source. The fungus was grown for 24 h in 250-ml flasks containing 50 ml medium A, which were inoculated with 1 ml spore suspension (1×10^7 spores ml^{-1}) from PDA/NaCl plates. Then the culture was filtered through Whatman no. 1 filter-paper and the mycelium was washed with sterile water and transferred to 250-ml flasks containing 50 ml medium B. Flasks were incubated for 6 days (except in the time-course study). The conditions of incubation were 25 °C in culture shaken at 150 rpm in the dark.

Experimental procedure

Different experiments were carried out using Morton's method.

Time course study

Sporulation was quantified (see below) 2, 3 and 6 days after transfer of mycelium to medium B.

Effect of age of mycelium at transference

The fungus was grown in medium A for 24 h or 48 h before being transferred to medium B.

Effect of pH

The pH of the media (A and B) was modified with 1 M HCl or NaOH solution before autoclaving to obtain the following values: 4, 5, 6, 7 and 8. After autoclaving, the pH values were 4.06, 4.6, 5.72, 6.42 and 6.32 (medium A) and 3.89, 4.49, 5.77, 6.25 and 6.33 (medium B).

Effect of Calcium

Medium B was amended with $CaCl_2$ to give a concentration 40 mM.

Effect of poly(ethylene glycol)

Medium B was amended with poly (ethylene glycol) (PEG) 6000 at two rates: 200 g l^{-1} and 400 g l^{-1}.

Finally, attempts were made to obtain sporulation without transference to medium B, by reduction in nitrogen concentration and calcium addition. Medium A was adjusted to pH 6. The levels of nitrogen were (g l^{-1}) 2.3, 1.5, 1.0, and 0.5. The levels of calcium were 0 and 20 mM. Flasks were coated with Sigmacote (Sigma) to prevent accretion on flask walls (Ugalde and Pitt 1983b).

Spore production was quantified after 7 days of inoculation or 6 days after transference to medium B. Cultures were homogenised in a blender operating at 4000 rpm for 2 min, made up to the original 50 ml with water and divided into two 25-ml samples. The first sample was passed through Whatman no. 1 filter-paper and dried to constant weight at 100 C. The second sample was filtered through glass wool after addition of a drop of Tween 80. The conidia were counted in a Thoma counting chamber and spore concentration (millions spores ml^{-1}) and sporulation index (millions spores mg^{-1} dry weight) were determined as described by Pitt and Poole (1981).

Statistical analysis

Three replicate flasks per treatment were used in all experiments. Experiments were carried out twice. The data were analysed by analysis of variance and means were compared by a least-significant difference test ($P = 0.05$) when significant effects were found.

Results and discussion

Complex media and complex media based on by-products were not suitable for the induction of submerged sporulation of *P. oxalicum*. The fungus formed mycelial pellets in all cases. This same behaviour was observed for synthetic media except for Morton's media. In this case the fungus grew in medium A, forming vegetative mycelial pellets with conidiation observed 24 h after transference to medium B.

Spore concentration and the sporulation index increased from the 2nd to the 6th day after transference to

Table 1 Effect of time, culture age, pH, and Ca^{2+} and poly(ethylene glycol)(PEG) concentration on submerged sporulation of *Penicillium oxalicum* at 25 °C. Results are means of three replicates

Variable	Sporulation index (millions spores mg^{-1} dry weight)	Spore concentration (millions spores ml^{-1})
Time (days)		
2	2.01*	3.48*
3	2.40*	4.20*
6	3.75**	6.53**
Culture age (h)		
24	0.10**	20.30*
48	0.013*	14.80*
Medium pH		
4		0.05*
5		1.46*,**
6		3.92***
7		2.62**,***
8		0.58*
Medium additions		
None	4.38*	9.43*
Ca^{2+}(40 mM)	5.47**	12.54*,
PEG (200 g l^{-1})	3.83*	10.60*
PEG (400 g l^{-1})	3.97*	14.39**

*-*** Values within each category having the same superscripts are not significantly different ($P = 0.05$)

medium B (Table 1). With regard to the effect of the age of mycelium at transference it was found (Table 1) that the difference in spore concentration between the two treatments was not significant. However, the sporulation index was higher in the 24-h treatment. This is probably an indirect effect: incubation for a longer time in medium A resulted in an increase in size of the mycelial pellets, but in a reduction of sporulation index, as sporulation occurred only on the surface of the pellets. The pH of the medium affected sporulation, with maximum spore concentration at pH 6 (Table 1). There were no significant variations in the values of the sporulation indices. This is also an indirect effect, with pH affecting growth directly and sporulation indirectly, as a consequence of its effect on growth.

Other stimuli can act synergistically after transference to a medium without nitrogen. We observed an increase in sporulation by the addition of calcium or PEG 6000 at a rate of 400 g l^{-1}. PEG 6000 at 200 g l^{-1} did not have any effect (Table 1). PEG reduces water activity in the culture medium, and water stress is a factor that induces and influences sporulation in other fungi (Jin et al. 1991). PEG could also be acting as a surface activator, as described by Morton (1961) with sporulation in aerial cultures occurring after rapid formation of surface-active material, which does not depend on water loss from the mycelium.

When nitrogen concentration was reduced in medium A this did not result per se in an induction of sporulation, as the mycelium remained vegetative throughout the experiment. When medium A was amended with calcium at 20 ppm there was an induction of sporulation, and this was stronger when the nitrogen concentration was reduced; the maximum values of spore concentration and sporulation index were obtained when nitrogen was added at a rate of 1–1.5 g l^{-1} (9.89–14.83 mM) (Fig. 1). Calcium has been reported to induce sporulation in submerged cultures of different species of *Penicillium* when grown on medium FCa (based on medium F, a synthetic medium with C:N = 8.5, amended with 10 mM Ca^{2+}) (Foster et al. 1945; Pitt and Poole 1981; Ugalde and Pitt 1983a). In this work no sporulation occurred when *P. oxalicum* was grown on medium F modified with different amounts of calcium. The composition of medium F is as follows (per litre): 20 g sucrose, 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 1 ml of a trace element solution containing (per litre): 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 70 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, 10 mg $(NH_4)_6$-$MoO_{24} \cdot 4H_2O$ and 10 mg $CuSO_4 \cdot 5H_2O$. The C:N ratio of medium A, with the nitrogen source reduced to 1.5 g l^{-1} KNO_3, was 92.5. Thus, when no calcium is present in the culture medium, only the total depletion of nitrogen is able to stimulate sporulation. On the other hand, when calcium is present it is still possible to obtain sporulation but only at values determined by the C:N ratio in the medium. Hadley and Harrold (1958a, b) studied sporulation of *Penicillium notatum* and also found interaction between calcium concentration and that of other nutrients in the culture medium. This confirms that calcium and nitrogen concentrations are major stimuli triggering sporulation in *Penicillium* spp., although the response to these stimuli varies slightly amongst the different species.

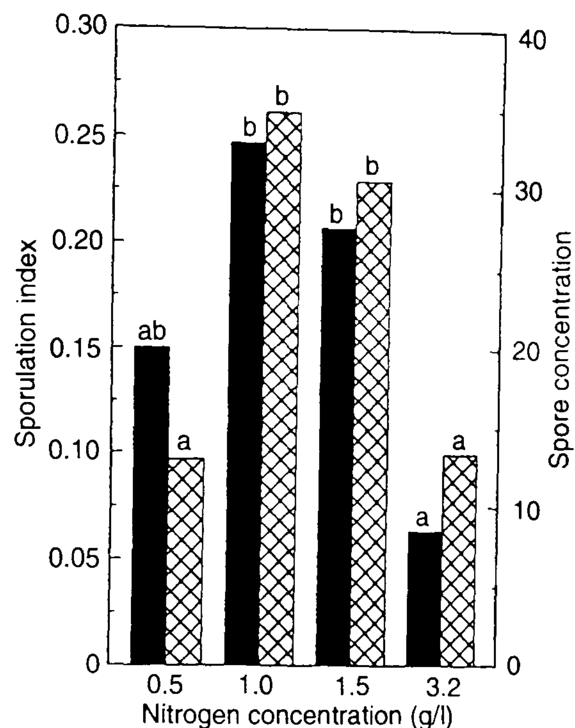

Fig. 1 Effect of reduction in nitrogen concentration in medium A amended with calcium (20 mM), on sporulation of *Penicillium oxalicum* in submerged culture. The medium was modified to pH = 6; sporulation was estimated 7 days after inoculation. ■ Sporulation index (millions spores mg^{-1} dry weight); ▩ spore concentraction (millions spores ml^{-1}). Bars with same letters for each parameter indicate no significant difference by a least-significant-difference test (P = 0.05)

Spores of other fungi produced in nitrogen-limited cultures have improved survival (Lane et al. 1991). Tolerance of heat and desiccation has been improved by using media containing excess glucose (McBride and Ensign 1987). These aspects merit further investigation for *P. oxalicum*. From a practical point of view, it is very important that the method developed in this work avoids the need for transferring the mycelium after 24 h growth, and thereby overcomes a difficult step in the industrial-scale production of inoculum using fermenters.

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BOOK OF ABSTRACTS

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PRODUCTION OF *Epicoccum nigrum* 282 FOR BIOCONTROL OF BROWN ROT (*Monilinia laxa*)

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Epicoccum nigrum isolate 282 is a resident component of peach twig mycoflora. Its antagonistic activity was screened *in vitro* and *in vivo* against the brown rot fungi of fruit, *Monilinia laxa*. The fungus was able to control the disease caused by *M.laxa* in peach twigs in commercial peach orchards and rot of fruits on peaches and cherries.

This isolate is especially suitable for studies on industrial production because it sporulates heavily on culture media. Spore production was tested in a solid fermentation system, using wheat grains as solid substrate. Spore production was tested at 0.996 and 0.98 water activity (a_w). Water or a mixture water-glycerol (0.99 a_w) was used to adjust the substrate to 0.98 a_w. After 21 days incubation maximum sporulation was obtained from the substrate at 0.98 a_w modified with water-glycerol ($7x10^6$ spores/g fresh grain).

Spores produced in culture medium at 0.996 and 0.98 a_w were tested for control of brown rot on cherries. Both spore types controlled the disease at 25 ° C and 70 or 100% relative humidity. Spores produced at 0.98 a_w presented the advantage of an improved survival when stored fresh for twelve weeks at either 4 or 25 ° C. This could be related to an enhanced accumulation of endogenous reserves, glycerol and arabitol were found to accumulate in this type of spores. Freeze-drying of spores reduced viability very quickly: after five weeks viability was almost zero regardless the spore type or the storage temperature.