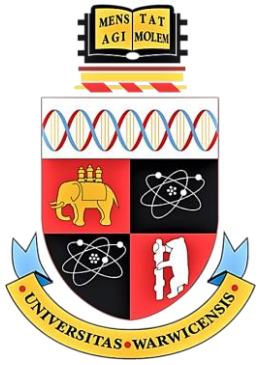


Developing a strain improvement system for the entomopathogenic fungus *Beauveria basssiana*: a way to get better biocontrol agents?

Laura Estefanía Reyes Haro

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant and Environmental Sciences

University of Warwick
Department of Life Sciences

November 2018

Supervisor: Dr David Chandler

I. Table of Contents

II. List of Figures

III. List of Tables

IV. List of Abbreviations

AIC.....Akaike information criterion

Anova.....Analysis of variance

Bt.....*Bacillus thuringiensis*

BCA.....Biocontrol agents

CZA.....Czapek Dox agar

DBM.....Diamondback moth

EPF.....Entomopathogenic fungi

EU..... European Union

IPM.....Integrated pest management

ITS.....Internal transcribed spacer

LD..... Light Dark

MAT.....Mating type

MEA.....Malt extract agar

Nit.....nitrate non-utilizing

OA.....Oatmeal agar

PCR.....Polymerase chain reaction

RO.....Reverse osmosis

SDA.....Sabouraud dextrose agar

SE.....Standard error

T0.....Thermal minima

Tmax.....Thermal maxima

Topt.....Thermal optima

Tukey's HSD.....Tukey's honest significance test

US EPA.....United States Environmental Protection Agency

UV-B.....Ultraviolet radiation (type B)

VCG.....Vegetative compatibility groups

V. Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Dave Chandler, for giving me the chance to work on such an interesting project, for the continuous support of my Ph.D research, for his patience, motivation and encouragement when things weren't going so well. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor and friend for my Ph.D study.

Besides my supervisor, I would like to thank those who helped me during my PhD. Gill Prince, for teaching me all the laboratory techniques that I used, for being not only a friend but also family, for helping me all the way through to succeed in my objectives in the lab. Alison Jackson, for teaching me molecular biology techniques and make my life easier in the lab. Declan Perry, Victoria Wooley and Scott Dwyer for helping me with data and statistical analysis. Marian Elliot for helping me with insect rearing. Andy Taylor and Jessica Fannon for teaching me the use of software for my phylogenetic analysis.

My sincere thanks also goes to SENESCYT (Secretaria de Educación Superior, Ciencia, Tecnología e Innovación), Ecuadorian entity which sponsored my research in this prestigious university and Rafael Correa, ex-president of Ecuador, without their support it would not be possible to conduct this research.

I thank my fellow labmates for the stimulating discussions and for making coming into work every day such a pleasure.

I would like to thank my family: my parents and my brother for supporting me throughout all my research and my life in general. All of you inspire me to keep going and achieve my goals in life. I dedicate this work to my three lovely children, Dary, Joaqui and my little Julia, who are the pride and joy of my life. I love you more than anything and I really, really appreciate all your patience and support during

mommy's Ph.D. studies. Last but not the least, I have not enough words to say thanks to you, my soul mate, my beloved husband and my best friend, Rommel. I love you with all my heart, thank you for being so understanding and supportive in all this crazy adventure we decided to have together. For being my labmate on the weekends and for helping me during the writing of this thesis. For being the one to help me get up in the toughest moments of my life and be the light at the end of the tunnel. I cannot be more grateful with life to put you on my way, you are the best thing that has ever happened to me.

VI. Declaration

This PhD thesis is presented according to the Guide to Examinations for Higher Degrees by Research provided by the Graduate School, University of Warwick. It has been written by myself and has not been submitted for any other degree. All experimental work, analysis, and written work presented here was completed by myself unless otherwise stated.

Laura Estefanía Reyes Haro

VII. Abstract

Biocontrol agents (BCAs) based on entomopathogenic fungi (EPF) are playing an increasing role in Integrated Pest Management programmes. At present, the commercially available EPF consist of wild type strains isolated from nature, however, there is potential to breed more effective strains by recombining wild types with complementary characteristics. EPF *Beauveria bassiana* represents one of the most important organisms used to kill arthropod pests, as they occurred naturally in the environment, do not leave residual activity, are safer for human manipulation and they usually show a high virulence. The aim of this research was to develop a system to improve strains of the entomopathogenic fungus *B. bassiana* through genetic recombination. A group of 50 *Beauveria* strains were genotyped using multi-locus sequencing and mating gene analysis, and then phenotyped with respect to their virulence against Diamondback moth (DBM), thermal biology, tolerance of UV light, and conidial production. A phylogenetic analysis identified two different *Beauveria* species within the fungal collection: *B. bassiana* (78%) and *Beauveria pseudobassiana* (16%). Seven strains from different places of origin were selected with phenotypes such as tolerance to UV-B radiance, thermotolerance, virulence and potentially compatible mating types for parasexual recombination studies. Spontaneously generated nitrate non-utilizing (nit) mutants were produced from these strains using a potassium chlorate-amended selective medium and 35 vegetative compatibility groups were determined within the 50 isolates of *Beauveria spp*. Recombination by hyphal fusion and protoplast fusion proved to be feasible and was observed in two out of three crosses. Only one cross (X2) showed higher radial growth than the parental strains between 20 and 30 °C. Nine fungal strains were selected to investigate the potential for inducing sexual recombination by pairing complementary mating types on three different media that have probed work well in other fungal species (oatmeal agar, malt extract agar, and Czapek Dox agar +/- biotin). After six months, both *in vitro* and *in vivo* assays led to the generation of structures resembling synnemata, however no fruiting bodies nor other clear sexual structures were observed. No relationship was found between the geographical origin of the strains and their tolerance to temperature or UV-B light, suggesting that micro-environmental conditions can play a more important role in the development of determined traits of organisms sourced from specific ecosystems than the latitude or altitude of sampling locations. This study provides a significant amount of information describing several methodologies for parasexual and sexual recombination of this fungus, expanding the current knowledge of this valuable EPF.

1 Introduction

1.1 Crop pests and Integrated Pest Management

Invertebrate pests, plant pathogens and weeds are a significant impediment to crop production. Crop plantations create suitable conditions for pest proliferation, either by providing a concentrated food resource for phytophagous invertebrates, an abundance of plants as hosts for pathogens, or by causing ground disturbance through ploughing that creates niche space for weeds (Hassan & Gökçe, 2014). Therefore, it is likely that crop protection has been a key activity for farmers since the beginning of agriculture more than 10,000 years ago (Oerke, 2006). For most of the history of agriculture, crop protection would have been based on physical controls, cultivation methods such as crop rotation, and varietal selection. The systematic development of crop protection technologies goes back as far as the 2500 BC, with the first records of Sulphur compounds and botanical extracts used as pesticides by the Sumerians and in ancient China (Oerke, 2006). However, major advances in crop protection on a global scale arguably did not occur until the 20th Century, with the development of synthetic chemical pesticides, combined with mechanization, crop breeding, synthetic fertilizers and improved farm management systems, which together caused significant increases in yield, particularly during the Green Revolution from the 1940s – late 1970s (Patel, 2013).

There are an estimated of 70,000 different pest species of agricultural crops, of which 9,000 are arthropod pests (insects and mites) responsible for an estimated 20% loss of potential global crop yields (Oerke, 2006). Currently, crop protection against arthropod pests is heavily reliant on routine application of synthetic chemical pesticides (Asi *et al.*, 2013). Pesticides can be grouped based on the types of pests that they target, as follows: insecticides - insects, herbicides - plants, rodenticides – rodents, bactericides - bacteria, larvicides – larvae, and fungicides - fungi; and among insecticides, there are five main groups of synthetic compounds: organochlorides, organophosphates and carbamates, pyrethroids, neonicotinoids and ryanoids (Singh *et al.*, 2015). Global pesticide applications are estimated at 3 million metric tons of pesticides every year, but even with this level of application, crop losses due to pests

are estimated at $2000 billion (Oerke, 2006; Oerke & Dehne, 2004; Pimentel, 2009). While conventional toxicant chemical pesticides are undoubtedly an essential tool for many farmers and growers, the indiscriminate application of these chemicals can result in adverse effects on the environment by lethal and sublethal effects on non-target organisms. This can include effects on aquatic non-targets through contamination of ground and surfaces waters and effects on terrestrial invertebrates. Pesticides based on "old" chemistry, such as DDT, can be detrimental to vertebrates by being concentrated up the food chain. In response to these environmental concerns, many pesticide active substances have been withdrawn from sale as a result of new legislation (Chandler *et al.*, 2010; Isman, 2006). The history of the development of synthetic chemical pesticides has been characterized by the replacement of broad-spectrum compounds, such as organophosphates and organochlorines, with new molecules with increasing potency to target arthropods combined with significantly reduced mammalian and avian toxicity, resulting in safer compounds being put into general use by farmers and growers, albeit other adverse effects remain. The excessive use of pesticides can result in the selection for heritable resistance in target pest populations. In the early 1980s the United Nations Environmental program suggested that pesticide resistance could be one of the top 4 environmental problems in the world and in 2003 resistance was recorded in about 520 species of insects and mites, 150 plant pathogens and 273 weeds (Chandler *et al.*, 2011; Devine & Furlong, 2007). In addition to this, beneficial non-target species that act as natural enemies, pollinators or which provide other ecosystem services can be adversely affected. Natural enemies are estimated to be responsible for up to 90% of pest control in some agroecosystems, and their loss through pesticide action can help to increase the population size of existing pests as well as increase the number of pest species, requiring the use of additional pesticides. Watercourses have also been affected, as in California, USA where 46 out of 100 of these sources of water were contaminated by pesticides (Steinmann *et al.*, 2010). Finally, there are significant concerns about adverse human health effects of some pesticides, either by direct exposure to pesticides of farm workers or exposure of consumers to pesticide residues (Denholm & Rowland, 1992; Lechenet *et al.*, 2014; Steinmann *et al.*, 2010). The impact of pesticides on human health from a study in the USA

considered that about 26 million people are poisoned every year, while globally pesticide poisoning has been estimated to cause the death of 220, 000 people, principally farm workers in developing countries who do not have access to personal protective equipment, training, or safe pesticide storage facilities (Pimentel & Burgess, 2014).

Over time, the use of pesticides has increased, yet crop losses caused by arthropod pests have not decreased. The reasons for this are thought to include the introduction of new crop varieties more susceptible to insect pests, the elimination of natural enemies, the development of resistance to pesticides by pest populations, reduction in crop rotation, increase of monoculture of crops, use of aircraft application and the reduction in field sanitation (Asi *et al.*, 2013; Lacey, 2016). In response to concerns expressed by consumer groups, NGOs and others, the reduction of the use of synthetic pesticides has become a priority. Governments are enacting new legislation in order to regulate and reduce dependence on pesticides, including increasingly strict safety criteria for pesticide approvals (Isman, 2006; Lechenet *et al.*, 2014). This is leading to a significant reduction in the availability of chemically synthetised pesticide products (Steinmann *et al.*, 2010). Considering an increased global demand for food production, there is clearly a substantial need for more effective and sustainable systems of crop protection (Lechenet *et al.*, 2014). Most experts agreed that Integrated Pest Management (IPM) represent an important way to achieve sustainability, as it is based on the application of different crop protection technologies working together, in a synergistic and complementary way, with careful monitoring of pests and their natural enemies (Ehler, 2006). IPM methods do not exclude conventional chemical pesticides, but it also includes methods such as physical controls, cultural approaches (crop rotation), planting time and trap crops, biologically-based controls, plant breeding, or careful soil and water management. These methods provide additional pest control and could reduce the use of pesticides by 50% or more without reducing crop yields or cosmetic standards for some crops as flowers (Kim *et al.*, 2011). In Europe, for example, EU Directive 2009/128/EC (the Sustainable Use Directive on pesticides) places farmers under a

legal obligation to adopt IPM principles, and its main purpose is reducing the dependence on pesticides (Lechenet *et al.*, 2014).

1.2 Microbial control agents of arthropod pests of crops

Natural enemies have been used in crop pest management for centuries and in the 20th century the name biological control was used for first time (Orr & Lahiri, 2014). Therefore, biological control is defined as the utilization of introduced or resident living organisms to control the activity and population of plant pathogens (Pal & Gardener, 2006). There are different organisms used as biological control agents and include true predators, parasitoids, parasites, pathogens and microbial antagonists (Chandler *et al.*, 2010). Biocontrol agents (BCAs) have potential to play a key role in IPM because of their attractive properties including low impact on beneficial organisms, lack of residues in the environment, and low cost of development (Wright, 2014). Adoption of BCAs can lead to significant reductions in use of conventional chemical pesticides and cost savings for the grower (Asi *et al.*, 2013; Steinmann *et al.*, 2010). Biopesticides are based on biocontrol agents grouped in to three categories: microorganism (bacteria, fungi, oomycetes, viruses and protozoa), biochemicals (plant oils, compounds synthetized by other organisms) and semiochemicals (insect pheromones) (Chandler *et al.*, 2010). In the worldwide market, most of the available biopesticide products are based on microorganisms, such as bacteria, nematodes and fungi, the predominant mycoinsecticides are (a fungus based product to kill insects) South America, with 42.7 % of all products on the market, the USA representing 20.5%, Europe - Asia 12.3%, and Africa-Oceania less than 3% of the products (Faria & Wraight, 2007).

1.3 Entomopathogenic fungi

This project concerns the use of entomopathogenic fungi (EPF) as BCAs of agricultural pests. EPF are common microorganisms from the fungal kingdom, that infect insects or any other terrestrial arthropods, with over 700 species described from at least 90 genera. However only a few members of the Entomophthorales and Hyphomycetes have been well studied (Chandler, 2017; Khachatourians & Qazi,

2008; Roberts & Hajek, 1992). Fungi that infect insects are found in almost all taxonomic groups except the Basidiomycetes and Hyphomycetes (Roberts & Hajek, 1992). The two main fungal groups that are used for biological pest control are Hypocreales and Entomophthoromycota (Chandler, 2017). The majority of EPF species occur within the order Hypocreales of the class Sordariomycetes, phylum Ascomycota and include both anamorphic (haploid, asexually reproducing) and teleomorphic (diploid, sexual reproducing) forms. The anamorphic forms (blastospores) are usually used in industrial scale production of inundative microbial biopesticides, which is the use of a large amount of fungal biomass over a target pest population; whereas the teleomorphic form present sexual reproduction by production of ascospores and there are few individuals used for mass production (e.g *Cordyceps militaris*) (Chandler, 2017). The anamorph-teleomorph connection among these fungi is still not clear and that is the reason why different genera among anamorph-teleomorph strains that belong to the same lineage have been assigned to different genera. However, when this unsolved connection become more clear, the scientific names of these strains will be unified as appropriate (Kepler *et al.*, 2014). Most researches on EPF as BCAs has been done with species from well recognized genera of anamorphic fungi: *Beauveria*, *Metarhizium*, *Isaria* and *Lecanicillium, Hirsutella* and *Entomophthorales* (Asi *et al.*, 2013; Skinner *et al.*, 2014). These fungi cause infections in a range of arthropod hosts including orthopteran, homopteran, lepidopteran, coleopteran, dipteran and acarine pests (Khachatourians & Qazi, 2008). EPF infect their hosts using conidia (blastospores) which germinate on and penetrate the arthropod cuticle and then grow into the insect haemocoel (Roberts & Hajek, 1992). Host death occurs as a result of the production of specific metabolites by the fungus and other factors such as nutrient depletion and water loss, mechanical damage to cells and tissues, and conversion of host biomass to fungal cells, followed by the colonization of tissues and organs (Ravensberg, 2011b). The fungus infects its insect hosts by attaching to, and then penetrating the insect cuticle. This is done using aerial conidia which need sources of carbon and nitrogen to germinate, and which are obtained from the cuticle of the host insect. Penetration of the cuticle occurs as a result of enzymatic action, which enables the fungus to enter the haemocoel and after that, blastospores are produced to multiply within

the insect. Infection appears as dark brown spots over the insect and it can take 2 to 8 days to kill the insect, depending on the immune system of the host and environmental conditions. The insect continues eating and moving until its activity slows and a paralysis occurs immediately prior to death, which usually occurs once the fungus has used up most of the food supply within its host. Following host death, *B. bassiana* enters into a hyphal growth stage and sporulation occurs again to continue the life cycle (Griffin, 2007) (**Figure 1.1**). Wild type strains from EPF species are popular choices for development of commercial "biopesticides" for arthropod pest management (Ravensberg, 2011b). These "biopesticide" products consist of conidia formulated in an appropriate carrier that are applied to crops in a number of ways including as liquid suspension sprays, dusts, and granules. They are considered by regulatory authorities as presenting minimal risk to human and environmental health, and research has shown that they can be valuable components of IPM (Copping & Menn, 2000; Ravensberg, 2011b). The global market for biopesticides (which includes EPF and other microbial agents used for control of pests, plant pathogens and weeds) has grown substantially since the 1990s and it is expected to reach $3.2 billion by 2017 according with the Global Biopesticides Market-Trends and Forecasts (2012-2017) (Hassan, 2014). This trend is reflected in an increase in the number of biopesticide products registered worldwide, with currently more than 500 products available and with continued rapid growth expected in the near future (Hassan, 2014). In recent years, global agribusinesses, including Bayer, BASF, DuPont and Syngenta, have started to acquire small biopesticide companies in anticipation that the market for biopesticides will expand significantly (Ravensberg, 2011b). However, this market growth may not be achieved if biopesticides cannot be made to be more effective under field crop conditions.

Figure 1.1. *B. bassiana* on the body of an infected insect (Taken from Campbell, M).

1.4 Entomopathogenic fungi and their role in crop protection

The species of anamorphic hypocrealean EPF used as biopesticide are all comprised of a large number of genetic variants or strains. Individual strains differ in a range of phenotypic characteristics including virulence, host range, conidia production, enzyme production and response to environmental conditions (Ravensberg, 2011a). The efficacy of EPF biopesticides is normally dependent on having suitable environmental conditions, and can be adversely affected in particular by extreme temperatures, UV radiation and low humidity, all of which can inhibit fungal growth and conidial germination and restrict the use of these fungi as BCAs in the field (Aiuchi *et al.*, 2008a). Factors such as water availability (a_w) and temperature have a great influence on growth of EPFs like *Beauveria bassiana, Metarhizium anisopliae,* and *Paecilomyces farinosus* (Hallsworth & Magan, 1999). Enzyme production in EPFs and they role in pathogenicity is not fully understood, although several pieces of evidences support their importance. For instance, a study in *P. chlamydosporia* has proved that specific proteases, esterases, lipases and chitinases isolated from it, are active against nematodes eggs shell (Esteves *et al.*, 2009). For this reason, EPF have been used most successfully as biopesticides on protected (greenhouse) crops, where physical conditions are more amenable to fungal infection (Chandler *et al.*, 2010; Ravensberg, 2011b). However, there is an increasing

demand for using EPF biopesticides also in field crops in order to help fill the gap in crop protection products created by the withdrawal of conventional chemical pesticides (Steinmann *et al.*, 2010). At present, the strains used for commercial biopesticides are all wild types that have been selected on the basis of virulence and ease of mass production rather than on their ability to tolerate adverse environmental conditions (Hassan, 2014; Nunes *et al.*, 2013). However, some EPF strains have been identified which are better able to tolerate adverse environmental conditions than commercial EPF strains (Aiuchi *et al.*, 2008a). In some fungal species such as *Lecanicillium muscarium*, *Lecanicillium longisporum*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* water stress conditions (aw) were manipulated to obtain improved EPFs, and they found a more rapidly conidial germination on the surface of an insect host, and a fungal virulence increased with low relative humidity (Andersen *et al.*, 2006). This raises the question of whether commercial strains can be improved through a breeding program, by crossing them with others, non-commercial strains with complementary characteristics to produce an improved recombinant. The production of recombinant fungal strains is commonplace in other areas of microbial biotechnology, mainly in the pharmaceutical area (e.g. to improve antibiotic production), but it is not currently used in biopesticide science (Ravensberg, 2011b).

1.5 *Beauveria bassiana*: Taxonomic classification and its role as a biocontrol agent.

Beauveria bassiana s.l. is one of the most important entomopathogenic fungi worldwide, mainly because, as a species, it has a wide host range of insect pests primarily within the Coleoptera, Lepidoptera, Homoptera and Hemiptera (Faria & Wraight, 2007). It produces a range of biologically active secondary metabolites including non-peptide pigments and polyketides, non-ribosomally synthesized peptides and secreted metabolites involved in pathogenesis and virulence (including altering the feeding behaviour of the insect before death) and which also have potential in pharmaceutical and agricultural industries (Amnuaykanjanasin *et al.*, 2013; Xiao *et al.*, 2012). At least 58 biopesticides based on *B. bassiana* have been

developed worldwide since the 1960s, of which 45 are currently available as commercial products (Xiao *et al.*, 2012). The fungus is naturally widespread in the soil, but it has mainly been used as a biopesticide for the control of pests feeding on plant foliage. UV radiation, low humidity and extremes of temperature are the main factors that negatively affect the survival of *B. bassiana* in the host in the field, although the response to these factors varies widely depending on the origin of the strain and also its geographic origin (Fernandes *et al.*, 2007; Fernandes *et al.*, 2008). In general, strains that originate from the tropics have more tolerance to UV radiation that those from higher latitudes (Fernandes *et al.*, 2007), while strains from tropical countries also exhibit slower germination compared to those originating from countries that experience colder temperatures (Xiao *et al.*, 2012). Inactivation by UV radiation is a significant impairment to EPF biopesticide performance. Also, it has been proved that *B. bassiana* growth is highly affected by water stress (0.94 aw) and high temperatures (>35°C) (Borisade & Magan, 2014). The *B. bassiana* strain that is the active ingredient in the most widely used commercial biopesticide (Botanigard, produced by Laverlam Inc. USA) has a particularly poor UV tolerance and does not function well at high temperatures (Wraight & Ramos, 2015). However, it has other properties that make it a good biopesticide, in particular the ease with which it can be mass produced and its high virulence to lepidopteran pests. This raises the question of whether the environmental performance of this fungal strain could be improved through recombination with other strains. Persistency and efficacy of a formulation applied during the summer are affected particularly by UV-B, which appears to be directly related with a decrease in the survival of EPF conidia in field environments (Huang & Feng, 2009). In the field, high temperatures and sunlight damage the conidia. Some insect species are able to take advantage of this, and exhibit a "behavioral fever" in which they bask in sunlight, increasing their hemolymph temperature up to 47°C. High temperatures can have different effects depending upon humidity: it will change cellular and macromolecular structures under condition of high humidity, causing protein denaturation and membrane disorganization; on the other hand, if there is dry-heat exposure, DNA damage and generation of mutants will occur (Li & Lee, 2014).

The genus *Beauveria* consists of 13 phylogenetic species, all of which are entomopathogenic. *B. bassiana* is the most widely studied species within the genus because of its use as a biocontrol agent. The taxonomy of *Beauveria* has been updated recently using multi-locus nucleotide sequence data, which has produced the first reliable phylogeny of the genus. *B. bassiana* is now classified as: Kingdom Fungi, Phylum Ascomycota, Class Sordiaromycetes, Order Hypocreales, Family Cordycipitaceae, Genus *Beauveria* and Species *B. bassiana* (Rehner *et al.*, 2011). Because different species and clades within *Beauveria* have virtually identical morphologies, the application of molecular methods has been essential for their classification (Rehner & Buckley, 2005) (**Figure 1.2**). Recent studies have shown that *B. bassiana* is non-monophyletic and is comprised of two genetically distinct clades. It is likely that these clades will be redefined as separate species in the not too distant future. The first clade represents a globally distributed complex of *B. bassiana* strains and is likely to retain the name *B. bassiana*. The second clade is currently named as Clade C and has not yet been formally described taxonomically (Meyling *et al.*, 2009).

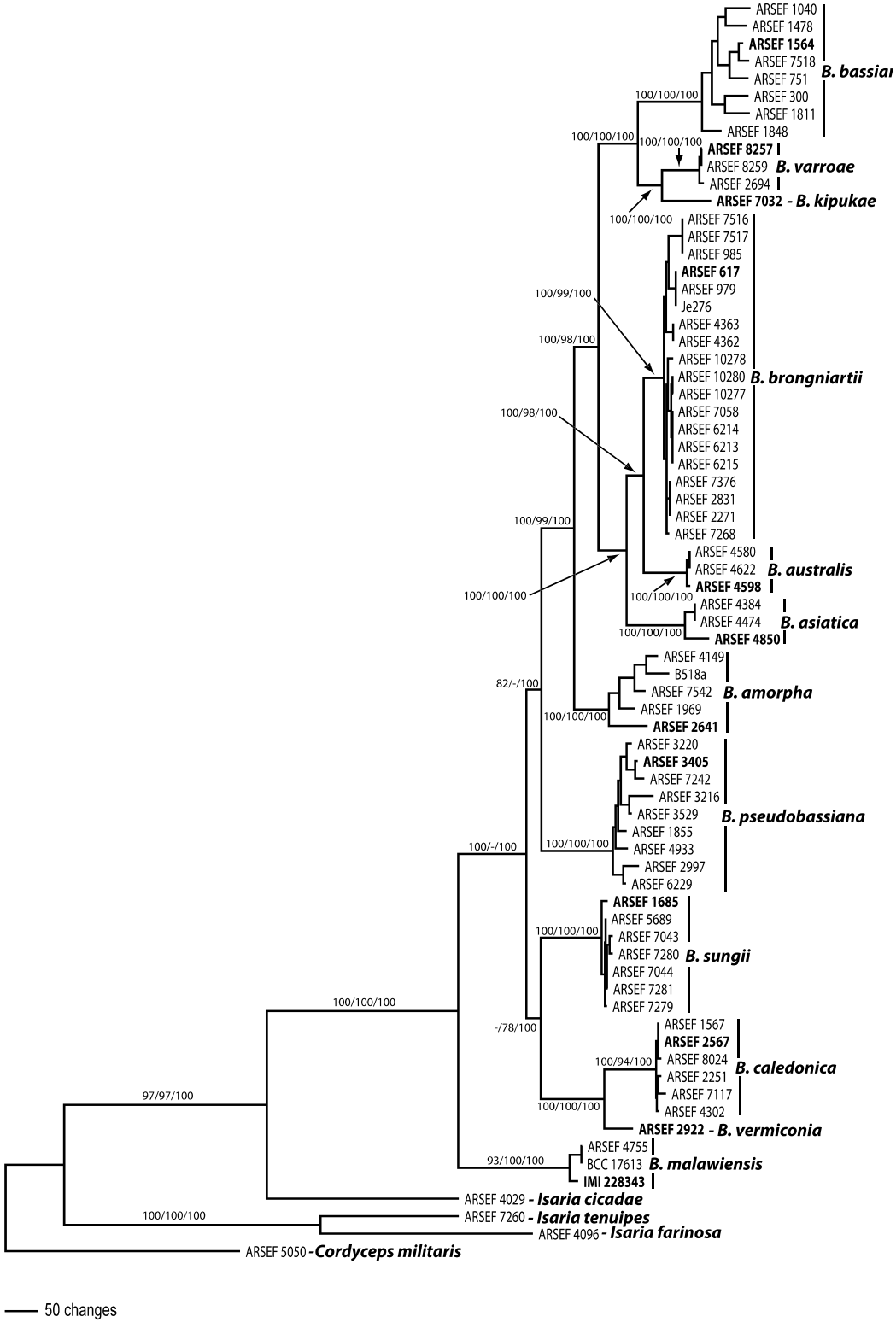


Figure 1.2. Phylogenetic tree shows relationship among different *Beauveria* species and clades (Rehner et al., 2011)

At least 58 biopesticides based on *B. bassiana* have been developed worldwide since the 1960s, of which 45 are currently available as commercial products (Xiao *et al.*, 2012). In order to have effective use of EPF within integrated pest management programs, it is necessary to select fungal pathogens tolerant of the temperature range and UV radiation found in the ecosystem in which they are used or produce recombinant strains through a strain improvement program. At the same time, developing new knowledge about the community structure of *B. bassiana*, its distribution, host range, reproductive biology and genetic variability between habitats could have a potential use in the production of some commercial strains of *B. bassiana* that can interact with the indigenous *Beauveria* community, and also the environmental effect could be predicted once the strains are in the field (Meyling *et al.*, 2009).

1.6 *Beauveria bassiana* and the need for strain improvement?

Because the anamorphic EPF do not have a sexual cycle, it is not possible to "breed" them using sexual crosses. However, it is possible to produce recombinants through the parasexual cycle, a non-sexual process for recombination in which the fusion of fungal hyphae (termed anastomosis) to form a heterokaryon is accompanied by fusion of nuclei to form an unstable diploid. This is followed by breakdown of the nucleus to produce haploid cells, which can result in the production of segregants as a result of mitotic crossing over and independent assortment of chromosomes during haploidization (which occurs through a series of short lived aneuploid states) (Kim *et al.*, 2011). Parasexual recombination to produce improved recombinant strains has been demonstrated recently with the genus *Lecanicillium* to develop hybrid strains possessing useful characteristics as BCAs. This was done through protoplast fusion of nitrate non-utilizing (nit) mutants with the selection of auxotrophic recombinants (Nunes *et al.*, 2013). However, it has not been investigated to any great extent in the fungal species that are used widely as biopesticides (*Beauveria and Metarhizium*)

An alternative strategy for strain improvement in EPF could be to induce sexual recombination (i.e. the teleomorph state) in anamorphic strains used for

biocontrol. The teleomorph-anamorph connections in the ascomycete EPF have recently become apparent, largely as a result of molecular phylogenies constructed from multilocus nucleotide sequencing (Nunes *et al.*, 2013). The teleomorph states of EPF are very slow growing and are not suitable for industrial scale mass production as biopesticides. However, induction of the ability to reproduce sexually could be an important mechanism for strain improvement in the anamorphic EPF. This has been done recently with a number of ascomycete fungi with industrial uses, including *Aspergillus* and *Penicillium* but has not been investigated in EPF (Böhm *et al.*, 2013).

1.7 Parasexual Recombination: How this technique works and its use for entomopathogenic fungi

The need for EPF with improved biocontrol characteristics raises the question of whether the phenotypes of commercial fungal strains can be improved through a breeding program, by crossing them with other, non-commercial strains with complementary characteristics to produce an improved recombinant. The production of recombinant fungal strains is commonplace in other areas of microbial biotechnology, mainly in the pharmaceutical area (e.g. to improve antibiotic production), but it is not currently used by the biopesticides industry because this area is still growing and the time for development of a product with the traits wanted last long than using the endemic wild type fungi (Ravensberg, 2011b). Because the anamorphic EPFs do not normally have a sexual cycle, it has not been possible so far to "breed" them using sexual crosses (but see Section 1.3 for a discussion of the possibilities of initiating sexual recombination in EPFs).

An alternative is to produce recombinants through the parasexual cycle, to form a heterokaryon and then by fusion of nuclei to form an unstable diploid and exchange of genetic material (Castrillo *et al.*, 2004) (**Figure 1.3**). The ability of two fungal strains to undergo hyphal anastomosis is determined by multiple incompatibility loci (*vic* or *het* loci) and requires identical alleles in the corresponding *vic* loci of the two potentially-anastomosing strains. Vegetative compatibility groups (VCGs) can be determined using complementation tests between nitrate non-utilizing (nit) auxotrophic mutants (Castrillo *et al.*, 2004). Hyphal fusion needs

heterokaryon compatibility, to have a cytoplasm with two distinct nuclei together. The frequency of hyphal fusion varies depending on the vegetative attraction of hyphae that is mediated by diffusible compounds. Once the hyphae make contact, the cytoplasm of the two fungi mix and this should result in the formation of the heterokaryon (Kimm, 2011). The same method can be used to generate recombinant phenotypes, which has a significant benefit in that *nit* phenotypes are produced as spontaneous mutants on a selective medium, rather than using a mutagenic agent that could introduce multiple mutations with detrimental effects on fungal fitness (Aiuchi *et al.*, 2008b). Parasexual recombination of *nit* mutants to produce improved recombinant strains has been demonstrated recently with the genus *Lecanicillium* to develop hybrid strains possessing useful characteristics as BCAs (Nunes *et al.*, 2013). However, it has not been investigated in the fungal species that are used widely as biopesticides (*Beauveria and Metarhizium*).

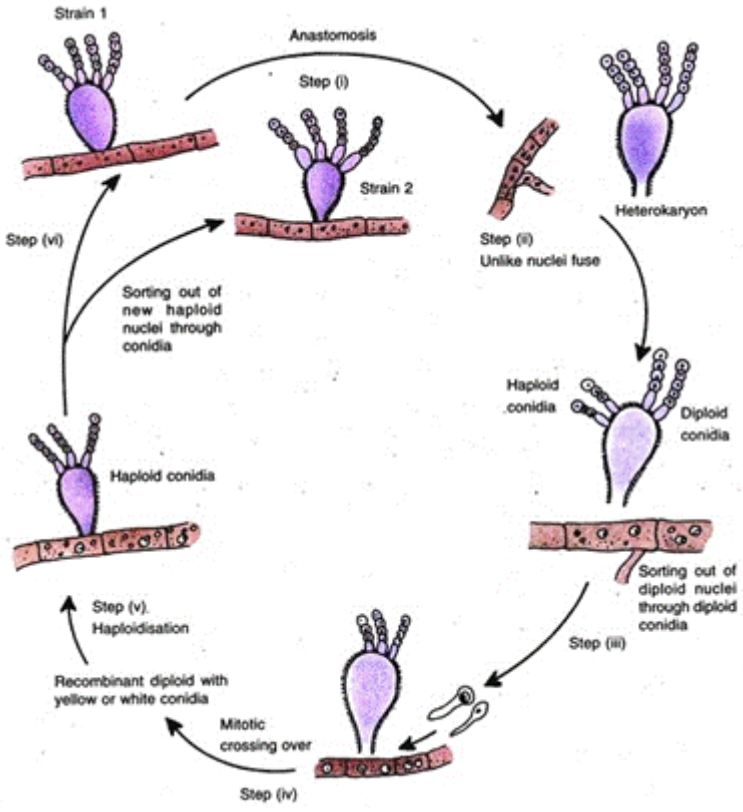


Figure 1.3. Fungi parasexual cycle (Esser & Kuenen, 2012)

1.8 Sexual Recombination: Highlights of its use as a new technique to obtain recombinant strains in asexual fungi.

An alternative strategy for strain improvement in EPF could be to induce sexual recombination (i.e. the teleomorph state) in anamorphic strains used for biocontrol. The teleomorph-anamorph connections in the ascomycete EPF have only recently become apparent, largely as a result of molecular phylogenies constructed from multilocus nucleotide sequencing (Nunes *et al.*, 2013). The teleomorph states of EPF are very slow growing and are not suitable for industrial scale mass production as biopesticides. However, induction of the ability to reproduce sexually could be an important mechanism for strain improvement in the anamorphic EPF. This has been done recently with a number of ascomycete fungi with industrial uses, including *Aspergillus* and *Penicillium* but has not been investigated in EPF (Böhm *et al.*, 2013). The development of a sexual recombination system for the anamorphic hypocrealean EPF could have many applications, including strain improvement, understanding the genetic basis of virulence, and in providing basic information on the anamorph-teleomorph connections in different taxonomic groups (Yokoyama *et al.*, 2004).

Sexual compatibility in the ascomycetous fungi is determined by a single locus called the mating type (*MAT1*) locus. This locus has two structurally unrelated allelic variants, *MAT1-1* and *MAT1-2*, and due to their high divergence are called idiomorphs rather than alleles (Bushley *et al.*, 2013). Fungi in the *Sordariomycetes* generally contain three genes in the *MAT1-1* idiomorph (termed *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*), and only one gene in the *MAT1-2* idiomorph (termed the *MAT1-2-1* gene) (**Figure 1.4**). Both idiomorphs are characterized by conserved genes in the proteins they encode:

- Heterothallic (self-sterile) fungal species present a system in which syngamy can occurs only between haploid cells with the opposite idiomorph or mating type (Bushley *et al.*, 2013).
- In contrast, homothallic (self-fertile) species contain both idiomorphs (*MAT1-1* and *MAT1-2*) in the same nucleus (Horn *et al.*, 2009). These can exist in the form of a single, physically combined MAT1-1 / MAT1-2 locus or as separate MAT1-1

and MAT1-2 loci on different locations in the nucleus, depending on the fungal species. Some species of homothallic fungi are able to determine which mating type allele is expressed (Billiard *et al.*, 2012).

- Pseudohomothallic, an intermediate mating system also exists in some fungal species, in which a stable, self-fertile heterokaryon can be formed when an individual (haploid) ascospore receives two nuclei of opposite mating types during meiosis, whereas other ascospores in the same ascus receive a single nucleus with a single mating type capable of outcrossing (Horn *et al.*, 2009).

It is thought that some groups of ascomycete fungi have undergone a pattern of repeated transitions between hetero- and homothallism throughout their evolutionary history. This evolutionary pattern is different for different fungal species and impacts on the molecular organization of the MAT1 locus, such that the precise way in which the locus is organized can be specific for individual homothallic species.

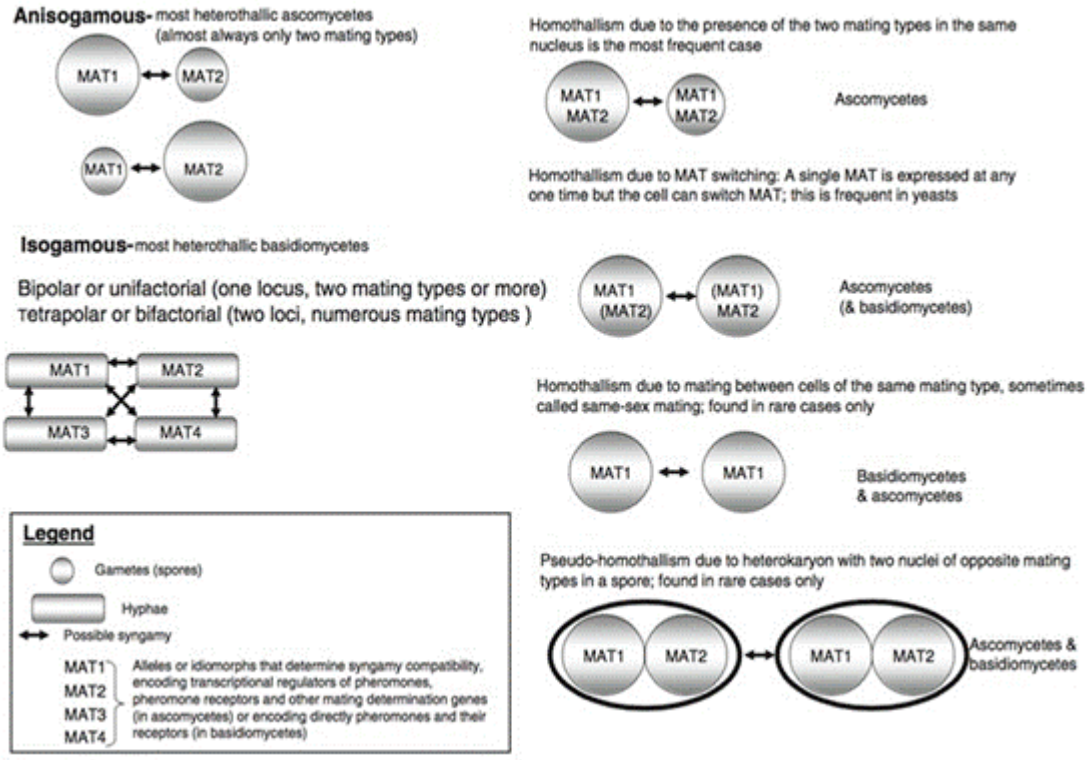


Figure 1.4. Syngamy modes in fungi (Billiard et al., 2011)

1.9 Aims and objectives

The aim of this project is to develop and investigate a system to improve strains of *Beauveria bassiana* through genetic recombination. The long-term aim is to produce recombinant strains that are more efficient as biocontrol agents and for this purpose the specific objectives were:

a) To select 50 fungal candidate strains of genus *Beauveria* with different geographic origins and insect hosts .

b) To perform phylogenetic characterization of the 50 strains of *Beauveria bassiana* to determine genetic relationships.

c) To assess phenotypically 50 strains of *Beauveria bassiana,* with respect to sporulation, virulence against *Plutella xylostella*, thermotolerance and tolerance to UV radiation to choose a group of strains for the following experiments.

d) To develop parasexual and sexual recombination between fungal strains selected previously with different phenotypes using different techniques applied for other fungal species before, with the ultimate aim of generating a strain improvement system for *Beauveria bassiana* and to evaluate recombinant strains if found.

2 Phenotype characterization of *Beauveria* fungal strains

2.1 Introduction

Despite the efficacy of chemically synthesized pesticides for crop protection, these products have been associated with negative effects on human health and the environment, due to their inherent recalcitrance, toxicity and unspecific mode of action that affect non-target animals altering more than one ecosystem (Horrigan *et al.*, 2002). The use of microorganisms in agriculture for crop protection (microbial pesticides) has become a promising alternative to replace the use of synthetic pesticides. Microbial pesticides are part of the bigger and diverse group of compounds called biopesticides, whose formal definition has not been established at the European level. However, according to The United States Environmental Protection Agency (US EPA), biopesticides are crop-protection agents derived from natural materials such as animals, plants, bacteria and certain minerals; although some researchers have suggested that only biopesticides based on living organisms and their bioactive compounds (metabolites produced directly from these organisms), should be addressed as such (Glare *et al.*, 2012; Villaverde *et al.*, 2014). Biopesticides represent a safer alternative to chemical pesticides, as these compounds tend to be less toxic, are easier to be degraded in nature, and usually present high specificity towards specific pests. Moreover, the use of biopesticides can lead to a diminished use of synthetic pesticides, lowering the content of residual chemicals on food and increasing the quality of products for consumers (Hassan & Gökçe, 2014). Microbial pesticides in liquid media (blastospores) represent the vast majority of commercially available biopesticide products on the market, and they can be based on bacteria, viruses or fungi; with fungal-based products aimed at killing insects (mycoinsecticides), the most common type of microbial pesticides produced (Glare *et al.*, 2012; Hassan & Gökçe, 2014). The global market for biopesticides based on microbial pesticides was worth US$ 113 million and US$ 289 million in 2009 and 2014, respectively (Glare *et al.*, 2012). While most microbial pesticides act by

ingestion, mycoinsecticides conidial infect by penetrating the insect cuticle. The infective stage can be either the asexual or sexual (ascospores) spores. Mycoinsecticides have been formulated as conidia or blastospores in oil, powders, water and in emulsifiable oils (Copping & Menn, 2000). Despite the benefits associated with the use of biopesticides, in practice their application on crop fields is limited due to several barriers, including problems related with the low quality or varying efficacy of their formulations; slower mode of action and higher production costs compared with synthetic pesticides; short shelf-life; and depending on each countries policies, biopesticides may face significant challenges for their development, production, registration and commercialization (Chandler *et al.*, 2011). Some traits that can been considered as advantages for biopesticides, can also be perceived as negative qualities depending on circumstances. For instance, the high specificity that some biopesticides show (e.g. the fungus *Lecanicillium lecanii*) could be a problem when a broad spectrum activity is required; or their rapid degradation in the environment, caused by sunlight, heat or humidity, have led to a higher dosage scheme, so the inoculum present in the biopesticides often need a high frequency of application (Aiuchi *et al.*, 2008a; Glare *et al.*, 2012).

The development of strains with novel or enhanced properties represents an effective way to overcome some of the problems that have restricted the commercialization of mycoinsecticides. The low virulence and sensitivity to abiotic stress can be listed as the main problem to be solved. Recombinant DNA and molecular techniques comprise powerful tools available currently for strain improvement of living-organisms (either plant, animal or microorganisms), and in the context of BCAs, they have made possible significant advances in insecticidal efficacy and stress tolerance, including resistance to UV light (Fang *et al.*, 2012). However, those studies rely on basic research that, in first instance, made possible the identification of target genes or regulatory networks. Breeding programs represent a valuable technique to solve complex biochemical mechanisms and identify genes involved in specific characteristics, as it allows the establishment of the relationship between changes in the phenotype of a given organism with changes in its genotype (genetic mutations). Furthermore, breeding programs could potentially result in improved strains that could be directly mass produced and commercialized using

fungal strains that have showed specific characteristics as biocontrol agents. This approach comprises three basic steps: firstly, a collection of organisms with useful genetic variation is needed; secondly, individuals of this population must be identified and characterized phenotypically to establish significative differences in specific traits; and thirdly, a selection of parent strains whose crossing could result in a progeny that outperform the original strains (Fang *et al.*, 2012; Moose & Mumm, 2008). In fungi, breeding programs have focused on reducing the pathogenicity of plant diseases, including fungi from the genus *Fusarium* (Puhalla, 1985); understanding changes produced by genetic mutations, such as the identification of chlorate resistance genes in *Aspergillus* (Cove, 1976); or improvement of desirable traits, for instance tolerance to temperature or increased virulence of *Lecanicillium lecanii* (Aiuchi *et al.*, 2008a). All of these studies were carried out based on the previous knowledge of strains with certain superior qualities, emphasizing the importance of prior phenotypic characterization.

In this study, the entomopathogenic fungus *B. bassiana* was selected as a model organism to develop a strain improvement system through a breeding program, and in this section, the phenotype characterization of isolates of *B. bassiana* is presented. The entomopathogenic fungus *B. bassiana* is a common microorganism found in soil, whose virulence against 100 species of insect has made it a strong candidate as a biocontrol agent (Roberts & Hajek, 1992). For phenotyping studies, three main characteristics of this fungus were assessed: thermotolerance, resistance to UV light and virulence, since an improvement of these qualities would result in a longer survival of this fungus in the environment and a faster mode of action. Biocontrol products based on *B. bassiana* usually are effective against arthropod pests in temperatures ranging from 8°C to 35°C, depending on the source of the fungus strain. Temperature tolerance in EPF could vary from 0 °C to 40 °C, however, optimum temperature is usually more restricted (Lacey *et al.*, 2001). Mathematical models can be used to describe fungal growth under different environmental conditions and regression models have been applied to estimate the optimum temperature for mycelial growth in EPF (Edelstein *et al.*, 2005; Terashima & Fujiie, 2007). The optimal temperature for *B. bassiana* could range from 18 to 30°C, however the viability and virulence of conidia is highly affected by sudden changes

in temperature during both summer and winter, which reduce the conidial ability for penetration (Fargues *et al.*, 1997; Inglis *et al.*, 1996). Little is known about thermotolerance mechanisms in entomopathogenic fungi (EPF), though a correlation between thermotolerance; enzymatic defence; expression of heat shock proteins that protect other proteins from denaturation; and changes in the lipid composition of the cell membrane has been reported (Lindquist & Kim, 1996; Noventa-Jordão *et al.*, 1999). UV-B radiation, it has been reported that sun light is responsible for a significant part of cellular damage and also has effect on viability and virulence (Nicholson *et al.*, 2000). In nature, the UV-B total energy per area irradiated (kJ/m^2) shows a large variation depending on the time of the day, season and geographic location. However, this variation of UV-B radiation could be predicted based on geographic latitude and altitude (Fernandes *et al.*, 2007; Piazena, 1996).

Few hours of exposure to sunlight in a tropical place is enough to inactivate conidia and delay the germination of the surviving conidia of most EPF, including *B. bassiana* (Fernandes et al., 2007). Studies in *Metarhizium, Hirsutella, Isaria, Lecanicillium, Beauveria* and *Paecilomyces*, have been performed by using different wave lengths, from a few minutes to several hours, to determine the tolerance of conidia to UV-B radiation. These studies found that tolerance to UV radiation varies and depends upon each strain, regardless of the fungal genus (Aiuchi *et al.*, 2008a; Huang & Feng, 2009; Rangel *et al.*, 2005). These findings emphasize the importance of microorganism collections, since novel and valuable properties could be found among strains that are already adapted to specific environmental conditions. For instance, the efficacy of a biopesticide formulated with a fungus endemic from a low UV-B irradiation zone, would have a lower efficacy if it is applied in a place with high UV-B irradiation (Braga *et al.*, 2001). Conversely, a strain with high tolerance to UV-B would have a better probability of persistence under high doses of sun exposure whereby such a strain can be considered as a good candidate for biopesticide formulations (Fargues *et al.*, 1996). Finally, understanding the mode of action of entomopathogenic fungi and the factors that limit their virulence and pathogenicity in the field is crucial for biopesticide production. It has been found that *Beauveria* produces an extracellular protease that is responsible for the hydrolysis of the insect cuticle to open the way to infect the insect (Bidochka & Khachatourians, 1990;

Fargues *et al.*, 1996). The infection process can be summarized as follows: (1) Conidial attachment to the insect cuticle and germination; (2) invasion and penetration within the insect cuticle; (3) reaching of the hemocoel and triggering of the host response to fungus attack; (4) overcoming of the insect immune system; and (5) fungal growth, external sporulation (Amnuaykanjanasin *et al.*, 2013). *B. bassiana* shows virulence towards a large range of insect hosts, and the degree of infection varies depending on each strain. In this study, Diamondback moth (DBM) was selected as the target host. The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), is an important and destructive pest of around 175 different plant species, and it is distributed widely around the world (Zalucki *et al.*, 2012). The cost to control this moth has been estimated as $2.3 billion dollars (Talekar & Shelton, 1993; Zalucki *et al.*, 2012). In Southeast Asia, this moth could causes crop losses > 90%, whereas in some parts of USA it represents up to 90% of defoliating lepidopterans of canola (Verkerk & Wright, 1996). Females of this moth can lay > 200 eggs on the upper leaf surface, then the first instar larvae hatch within one week and they feed on mesophyll tissue. The second, third and fourth instar larvae feed on the leaves, buds and flowers, causing substantial damage to the plant (Talekar & Shelton, 1993). One of the reasons for this problem is that the pest has developed insecticidal resistance; however, EPF have shown potential to control DBM (Grzywacz *et al.*, 2010). It has been reported that a third instar larvae population infected with an EPF (*Zoophtora radicans*) decreases larval feeding by 44%; while infected adult female moths infected with *B. bassiana* laid significantly less eggs and exhibited 100% mortality under controlled laboratory conditions (Furlong *et al.*, 2004; Sarfraz *et al.*, 2005). There are some commercially available products based on *B. bassiana*, such as Naturalis® (Biogard) or BotaniGard (BioWorks Inc.), whose strains have proven their efficacy to suppress the population of DBM under controlled conditions and in the field (Vandenberg *et al.*, 1998).

In this section, the phenotype characterization of 50 different strains of *B. bassiana* is described. The virulence to 2nd instar DBM larvae and the effect of six temperatures and 3 doses of UV-B radiation on germination and growth were examined. In addition, the strains were subjected to a molecular analysis to establish their phylogenetic relationship. Although, the strains within the *Beauveria* genus are

genetically related, their classification is still under examination mainly due to their morphological similarities and their anamorph-teleomorph connection with the genus *Cordyceps* (Rehner *et al.*, 2006; Sung *et al.*, 2007). There are different genetic markers available to perform phylogenetic analysis in *Beauveria*, including Internal transcribed spacer (ITS), Elongation factor or Bloc, whose sequences provide valuable information about how different *Beauveria* strains are grouped (Fernandes *et al.*, 2006; Rehner & Buckley, 2005). These phenotype characterization experiments were the basis for a selection of the best strains in terms of sporulation, tolerance to high temperatures, tolerance to high UV-B radiation and virulence against the DBM; to be used subsequent for complementation and protoplast fusion in further experiments (**section 3.3**).

2.2 Materials and Methods

2.2.1 Fungal selection and preparation of conidial suspension

Fungal selection

For storage, an aliquot of a conidial suspension with a concentration of 10^7 conidia/mL was pipetted onto cryotolerant plastic beads, which were stored at -80 ^oC, and working cultures store on slopes at 5^oC (Chandler, 1994).

For this research, every six months two beads per isolate were removed from deep freeze and spread on Sabouraud dextrose agar (SDA) (Oxoid, UK) contained in a 30 mL Universal tube, known as a slope, using a sterile plastic loop (Fisher Scientific). Slope cultures were grown for 14 days at 20^oC in darkness and then kept at 5^oC. Fungal cultures for experiments were grown from slope cultures when required. A sterile loop spreader was used to transfer fungal material from the slope culture to SDA contained within a 9 cm Petri dish (Merck). The sterile slope was streaked evenly across the SDA plate. Cultures were grown in the dark at 20 ^oC for 10-14 days (in a Sanyo Gallenkamp cooled incubator) before conidia were harvested for experiments.

A total of 50 fungal isolates were used in this study, of which 42 were obtained from the Warwick Crop Centre collection (Wellesbourne), six isolates from the USDA-ARSEF collection and two from commercial products (**Table 2.1**). Strains

from the Wellesbourne collection were selected based on their geographical location source to ensure a pool of strains with a diverse range of phenotypes; whereas USDA-ARSEF strains were selected based on previously reported characteristics in the literature that might have been useful for the aim of this study (e.g. high virulence against *Plutella xylostella* (1758-15), thermotolerance (252), or resistance to UV light (2882)) (Fernandes *et al.*, 2007; Huang & Feng, 2009; Wraight *et al.*, 2010); two commercial strains, Botanigard and Naturalis, were included in this study.

Preparation of conidial suspension

Fungal strains were grown on SDA petri plates at 22 ^{o}C in darkness. After 14 days, 10 mL of sterile 0.05% Triton X-100 (BDH) was added to the colony and the conidia harvested by gentle agitation using a 'L' shaped spreader (Fisher Scientific, UK). Suspensions were filtered through sterile milk filter paper (19cm diameter) (Goat Nutrition Ltd, Kent, UK) to remove impurities (hyphal and agar debris) and the conidia suspension collected.

The conidial suspension concentration was determined by using an improved Neubauer haemacytometer (Merck, UK). Two channels of the haemacytometer were filled with diluted suspension (1/10) and examined under a light microscope (X 40). All suspensions on 0.05% Triton X-100 (BDH) were adjusted to 1 x 10^{7} /mL and were grown in SDA plates at 22 ^{o}C for 13 days in darkness.

Table 2.1. Collection of fungal strains used in this study for phenotyping characterization.

N°*	Isolate **	Country	Host	N°	Isolate	Country	Host
1	478-00	Canada	Diptera	**26**	919-05	UK	Lepidoptera
2	1757-15	Canada	Lepidoptera	**27**	920-05	UK	Lepidoptera
3	432-99[a]	USA (Naturalis)	Lepidoptera	**28**	969-05	UK	Lepidoptera
4	433-99 [b]	USA (Botanigard)	Lepidoptera	**29**	986-05	UK	Lepidoptera
5	434-99	USA	Hymenoptera	**30**	990-05	UK	Lepidoptera
6	476-00	USA	Diptera	**31**	997-05	UK	Lepidoptera
7	495-00	USA	Diptera	**32**	1094-05	UK	Lepidoptera
8	1756-13	USA	Coleoptera	**33**	1334-05	UK	Lepidoptera
9	1758-15	USA	Lepidoptera	**34**	1750-11	UK	Lepidoptera
10	1759-15	USA	Lepidoptera	**35**	480-00	France	Diptera
11	252[c]	USA	Coleoptera	**36**	486-00	France	Diptera
12	2861 [c]	USA	Homoptera	**37**	491-00	Denmark	Diptera
13	2864 [c]	USA	Homoptera	**38**	61-82	Italy	Lepidoptera
14	2880 [c]	USA	Homoptera	**39**	1754-13	Turkey	Lepidoptera
15	2882 [c]	USA	Homoptera	**40**	520-01	Kenya	Lepidoptera
16	2883 [c]	USA	Homoptera	**41**	521-01	Kenya	Lepidoptera
17	299-86	Colombia	Coleoptera	**42**	525-01	Kenya	Lepidoptera
18	251-85	Brazil	Lepidoptera	**43**	247-85	China	Hemipteran
19	252-85	Brazil	Coleoptera	**44**	1335-05	China	Diptera
20	488-00	Brazil	Diptera	**45**	231-85	China	Hemipteran
21	455-99	UK	Lepidoptera	**46**	233-85	China	Hemipteran
22	465-99	UK	Lepidoptera	**47**	133-82	Vietnam	coleoptera
23	493-00	UK	Diptera	**48**	274-86	Thailand	Lepidoptera
24	805-05	UK	Lepidoptera	**49**	315-87	Phillipines	Lepidoptera
25	910-05	UK	Lepidoptera	**50**	1333-05	Australia	Lepidoptera

* Isolate number to be use in the experiments

** Isolate number in the Warwick Crop Centre culture collection (isolate number from culture collection of origin.

a. Isolate forms the active ingredient in the proprietary mycopesticide 'Naturalis' (Troy Biosciences Inc., 113 South 47th Avenue, Phoenix, AZ 850433, USA).

b. Isolate forms the active ingredient in the proprietary mycopesticide 'Botanigard' (BioWorks, Inc. 100 Rawson Road, Suite 205 Victor, NY 14564, USA)

c. Isolate from the ARSEF collection and supplied by Dr Richard A. Humber, The USDA-ARS Biological Integrated Pest Management Research Unit, 538 Tower Road, Ithica, USA

2.2.2 Phylogenetic analysis of candidate strains of *Beauveria*

Mycelium preparation

Sterilized 250 mL flasks containing 100 of mL Sabouraud dextrose broth (SDB) were inoculated with 100 µL of fungal conidia suspension (10^7conidia/ml) and incubated at 22 ^oC in darkness in an orbital shaker at 150rpm. After 14 days, growth fungal biomass was filtered through sterile milk filter paper (19cm diameter) (Goat Nutrition Ltd, Kent, UK) and washed several times with sterile distilled water. The filter paper containing wet mycelium was squeezed gently to remove any excess water and removed from the filter paper and placed into a conical flask, which was frozen at –20^oC for 24 hours, prior to freeze-drying for 24 hours. After freeze-drying, the tubes were stored at –20°C until required.

DNA Extraction

Frozen mycelium (25mg) was transferred into labelled grinding tubes containing 0.5 g of zirconia ceramic beads (0.1mm) and 10 acid washed glass beads (2.5-3.5 mm). The tubes were ground for 20 seconds in a fast prep 24 machine and the procedure was repeated twice to assure homogeneity. Then, 300 µL of extraction solution (ABI Prepman Ultra Sample Preparation Reagent from ThermoFisher Scientific) was added to milled cells and the mixture was manually shaken. The tubes were placed in a heat block at 108°C for five minutes, gently mixed, and returned to the heat block for a further five minutes. Tubes were centrifuged for five minutes at maximum speed (14000 rpm) twice, rotating the tubes 180 degrees each time. The supernatant (150 µL) was transferred into a clean tube and diluted 10 times in sterile water. After quantifying DNA using a NanoDrop (LabTech), both stock and diluted lysate were stored at -20 °C.

Polymerase Chain Reaction (PCR)

Elongation factor 1α, β-tubulin, DNAlyase, and Internal transcribed spacer (ITS) sequences were used as molecular markers for phylogenetic studies. All primers were ordered from Sigma-Aldrich. The detailed list of molecular markers and primers used for PCR are listed in **Table 2.2**.

Table 2.2. Molecular markers and primers used for amplification

Molecular marker	Primer name		Primer sequence	Reference
elongation factor 1α	GP1F	Forward	AGG ACA AGA CTC ACA TCA AC	(Prince & Chandler, 2006) unpublished
	613R	Reverse	CTT GAG CTT GTC AAG AAC C	
β-tubulin	β-tubulin F2	Forward	CAA CTG GGC TAA GGG TCA TT	(Prince & Chandler, 2006) unpublished
	β-tubulin R3	Reverse	GGG AGC AAA GCC GAC C	
DNAlyase	DNAlyase F	Forward	ACA TTT CAG GCC ATG TTT GAC	(Prince & Chandler, 2006) unpublished
	DNAlyase R	Reverse	GCT ATG AGG TTT CGT ATC CG	
ITS	ITS1	Forward	TCC GTA GGT GAA CCT GCG G	(White *et al.*, 1990)
	ITS4	Reverse	TCC TCC GCT TAT TGA TAT GC	

All PCR reactions were performed in a volume of 25 µL. The set-up reaction consisted of a master mix solution (12.5 µL) (REDTaq Readymix, Sigma-Aldrich, USA), 10 µM of forward and reverse primers (1 µL each), genomic DNA (1 µL) and nuclease free water (9.5 µL). PCRs were run on a Gene Amp PCR System 9700 (Applied Biosystems, USA). PCR conditions for elongation factor 1-α, β-tubulin, DNA Lyase, and Internal transcribed spacer (ITS) were as follows: initial denaturation at 94°C for 3 minutes; 35 amplification cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes (White *et al.*, 1990). The PCR products were separated on a 1.2% (w/v) agarose gel (Sigma-Aldrich, USA) (with 2µl per 100ml of GelRed) at 90V for 90 minutes. PCR products were cleaned up by a QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol and sequenced by GATC Biotech Company (Coventry, UK) using the forward primer (5 µM) for each molecular marker.

Following sequencing, data was aligned, edited, saved as consensus sequences and an individual phylogenetic tree was obtained for the four markers using the software CLC Workbench (Qiagen, https://www.qiagenbioinformatics.com/). To generate the phylogenetic trees, the best fitting model was determined, the maximum likelihood method was evaluated and models with the lowest Bayesian information criterion (BIC) scores were chosen as the best fitted. The bootstrap consensus tree was tested using 1000 replicates

(Kumar *et al.*, 2016). The final tree was a consensus of the four individual trees generated before. The sequences obtained for these genes (ITS, E-factor, DNA lyase and B-tubulin) were integrated into one tree, using the same software, to have a more precise relation among the *Beauveria* strains.

As the 50 strains selected presented phenotypic differences among them, three genetically confirmed strains from the genus *Beauveria*: *B. bassiana* (ARSEF 1564), and *B. pseudobassiana* (ARSEF 3405)(Rehner *et al.*, 2011) were included in the phylogenetic analysis to confirm the presence of one or more species within the group studied. In the phylogenetic tree mating type idiomorphs (MAT genes) are included as information but is discussed later in **Chapter 4**.

2.2.3 Comparison of conidial production on candidate strains of *Beauveria*

A conidia suspension (1 x 10^7 conidia/ml) was prepared for each one of the 50 fungal strains of the collection used in this study, divided in five groups of ten strains, by the method described in **Section 2.2.1**. and 100 µL was spread on to Sabouraud dextrose agar (SDA) plates using a sterile L Shaped spreader and incubated at 22 ^oC in darkness. After 13 days, the conidia were harvested as described previously and their concentration was determined using an improved Neubauer haemacytometer (Merck). The experiment was repeated on three occasions.

2.2.4 Effect of temperature on fungal colony extension rate and conidial germination

Effect of temperature on fungal growth

Conidial suspensions from each strain (**section 2.2.1**) divided into five groups of ten strains, were adjusted to a concentration of 1 x 10^7 conidia/mL. 200 µL of the adjusted conidia suspension was spread over a SDA plate using a sterile L shaped spreader, and incubated at 25^oC in darkness for 48 hours. A 10 mm cork borer was used to obtain a plug of inoculum, which was then inverted over the centre of a new

SDA plate marked with an X/Y axis to measure colony extension rate (Davidson *et al.*, 2003).

Two SDA plates per each strain were incubated at six different temperatures (10ºC, 15ºC, 20ºC, 25ºC, 30ºC and 33ºC) in a Sanyo Gallenkamp incubator. The diameter of the fungal colonies was measured each week, for four weeks. The experiment was repeated on three occasions.

Effect of temperature on germination of conidia

Conidial suspensions were adjusted to a concentration of 1 x 10^7 conidia/mL using the procedure described in **Section 2.2.1** and divided into five groups of 10 strains. An aliquot of 20 µL was pipetted in three previously marked circles on SDA Petri dishes (35 x 10 mm, Fisher, UK). Plates were sealed in plastic bags and incubated (Sanyo Gallenkamp) in the dark at 6 different temperatures (10ºC, 15ºC, 20ºC, 25ºC, 30ºC and 33ºC). Germination was stopped after 24 hours of inoculation by pipetting a drop of lactophenol methylene blue (Sigma-Aldrich, UK) over each marked circle. Plates were sealed and stored at 4 ºC before examination under a light microscope (magnification x200) (Olympus BH-2, Japan). Incidence of germination was recorded for approximately 100 conidia per circle. Germination was defined as the point when an emerging germ tube was equal to, or larger than, the length of the conidia. The experiment was repeated on three occasions.

2.2.5 Effect of UV-B radiation on germination of conidia

Conidial suspensions were prepared as described in **Section 2.2.1**. with the addition of benomyl (Sigma-Aldrich, USA) (0.002 w/v) in the SDA medium to inhibit germ tube growth, allowing a clearer observation under the microscope (Fernandes *et al.*, 2007; Fernandes *et al.*, 2008).

Three strains (11, 4, 12) were initially selected and evaluated to develop the exposure time for future experiments. These strains were selected as they had been previously reported to be thermotolerant (11) (Fernandes *et al.*, 2008), resistant to UV-B radiation (12) (Huang & Feng, 2009), and the commercially available strain 4 was used as a control. A 20 µL aliquot (1 x 10^7 conidia/mL) was pipetted on to

Sabouraud dextrose agar (SDA) with benomyl (0.002w/v) plates (35 x 10 mm, Fisher). The plates were placed in a Sanyo 2279 cabinet, equipped with 2 fluorescent bulbs (T12 40/12) with 25 cm of distance between the plates and the bulbs. The radiance emitted inside the cabinet was 1100 mW/m^2 (5.94 kJ/m^2) and was measured using a 919P-003-10 High Sensitivity Thermopile Sensor (Newpot Corporation). This dose of radiation is equivalent to the highest UV-B radiation month in United States and middle Europe-Asia; or the lowest UV-B radiation month in Central America, or middle region in South America (Fernandes *et al.*, 2007) (**Appendix 1**). Plates were covered with Pyrex glass dishes (35 x 23 cm) to block any radiation lower than 290 nm (Lepre *et al.*, 1998), and the plates exposed to 1100 mW/m^2 for 30, 60, 90, 120, 150 and 180 minutes while the cabinet was maintained at 25 ^oC. This was done to determine the exposure time that produced the largest variation between strains in germination when compared with a negative control. Once the exposure time was selected (90 minutes), the 50 strains were irradiated at 1100 mW/m^2 following the same methodology mentioned above. The experiment was repeated on three occasions. As a negative control, plates of the strains 11, 4 and 12 were covered with aluminium foil to avoid radiation, in each replicate.

After UV-B light exposure, plates were incubated at 25 °C for 48 hours and germination stopped using a drop of lactophenol cotton blue. Plates were sealed and stored at 4 °C, until examination under a light microscope (400 X) to evaluate the effects of UV light. A minimum of 300 conidia per plate were evaluated and compared with the control. Germination was defined as conidia having a length of germ tube equal to or larger than 50 % of the diameter of the conidia.

Effect on time to germination was also evaluated on the same three strains used for calibrating time of exposure (11, 4, 12). Samples irradiated at 1100 mW/m^2 were compared with non-irradiated samples (controls) by assessing germination every 12 h up to 48 h (Fernandes *et al.*, 2007).

2.2.6 Conidial virulence of fungal strains against *Plutella xylostella* (diamondback moth)

Plant raising

Cauliflower (*Brassica oleracea*) seed (Skywalker F1 organically certified film coated seed, (Elsoms Seeds Ltd, Lincoln, UK)), were sown on damp vermiculite contained within a Perspex tray (18 x 12 x 4 cm). After seven days, individual plants were transferred to plastic plant pots (7 x 7 x 8 cm, Desch Plantpak) containing soil (F2+S, Levington seed and modular compost). Plants were then transferred to a controlled environment room (20 °C) (16:8 LD) and watered *ad libitum*.

Plutella xylostella Culture

The *Plutella xylostella* (diamondback moth, DBM) culture used in this study was obtained from a previously existing stock of DBM (Collected 18/12/1995, Wellesbourne, Warwickshire). The DBM was cultured on the cauliflower plants in a controlled environment room (20 °C). Two semi-mature cauliflower plants (two to three weeks old) were placed into a cage with adult moths (45 x 45 x 45 cm, BD44545 – Bugdorm) for 48 hours, to allow oviposition to take place. The plants with eggs were then removed and placed in a clean larval development cage. After egg hatching and up until pupation two new plants were added to the larval development cage every 48 hours to feed the DBM. Once the adults emerged after 14 days, they were transferred to an adult cage. A constant supply of fresh adults was needed, so two larval development cages were set up weekly to ensure a constant supply of adults.

Laboratory bioassays

All bioassays were done with fixed age populations of DBM larvae. For this purpose, one cauliflower plant of three to four weeks old was placed into the adult cage for 24 h, so that adults could oviposit on the leaves. The following day, this plant was refrigerated for 24hr whilst another cauliflower plant was placed into the adult cage. The plants were then removed from the adult cage and the refrigerator to be placed in a fresh larvae cage. Mature cauliflower plants were added to the cage to feed the developing larvae as required (16:8 DL).

Using a fine paintbrush, early second instar larvae (distinguished from other second instars by their smaller size and darker skin) were transferred to a 9 cm Petri dish lined with filter paper (12 larvae per dish). The Petri dishes were placed in the fridge at 5^{o}C for no longer than 1-2 hours before spraying. A Potter tower sprayer (Potter, 1952) was used to apply four mL conidial suspension (1 x 10^{7}conidia/ml) with a pressure of 0.345 Bar, to groups of 12 larvae on damp filter paper within a 9cm Petri dish. Control larvae were sprayed with four mL of Triton X-100.

A 18x18 mm cover slip was placed on the Petri dish with larvae, before been placed in the spray table of the Potter tower and secured below the spraying nozzle using a retractable metal arm. The conidial suspension (1x10^{7}conidia/m) was vortexed and 4ml of the conidia suspension was transferred to the spray tube and the spray initiated. Control larvae were sprayed with four mL of Triton x-100. To avoid cross-contamination, between each experimental spray, 70% alcohol and Triton X-100 were sprayed each run through the spraying equipment separately.

The Petri dish lid containing the larvae was then removed from the spray table. A leaf disc of mature cauliflower plants (3 cm diameter) was added to the Petri dish to feed the larvae and the dish sealed with parafilm and incubated for 24hr at 20^{o}C (16:8 LD) (Sanyo, MLR-351). After 24 hours, larvae were transferred to a new Petri dish with two holes in the lid (2cm diameter), covered with perforated plastic to ensure aeration. Leaf discs were changed daily to ensure fresh food, for the duration of the experiment (Wraight *et al.*, 2010). This experiment was repeated twice.

Survival data

Larvae mortality was assessed every 24 hours for seven days. Mortality on day one was considered to be due to handling and was removed from the analysis. Any dead larvae were removed and incubated on damp filter paper within Petri dishes (22 $\pm$ 1°C, darkness) for seven days, and inspected for the presence of mycelium on cadavers.

2.2.7 General statistics

Data from all the experiments was entered using Microsoft Excel Software, to perform specific statistical analysis. For the assessment of the 50 isolates of *Beauveria* a randomized block design was used for five blocks of 10 strains, each one analysed using one-way analyses of variance (ANOVA) to find significant differences. Duncan and Tukey tests were used to compare means and identified significantly different groups.

For colony extension rate, the diameter of the mycelial colony was calculated by taking the mean of the two diameter measurements. This figure was then halved to get the radius of the mycelial colony and the radial extension rate calculated by plotting mycelial colony radius against time for each EPF strain. A linear regression model was used to obtain the radial rate (cm/day). To describe the relationship between temperature, growth and conidial germination the various dependent variables recorded (germination proportion and colony extension rate of candidate isolates), the Lactin-1 non-linear model was used as reported for the EPF. Like *M. anisopliae* (Klass *et al.*, 2007). The model was fitted to data in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package Minpack.lm (version 1.2-0). The Lactin-1 model proposed by Lactin et al. (1995) had the equation:

$$y = e(pT)-e(pTmax-(Tmax-T/\Delta)$$

The Lactin-1 model is effective in describing the temperature range and has been used to describe the thermal development profile of DBM in the past (Roy *et al.*, 2002). Parameters T and y are temperature and the dependent variable, respectively. Tmax is the upper developmental threshold. Parameter p a is constant which defines the rate at the optimum temperature. Parameter Δ is the number of degrees above T at which temperature inhibition becomes the overriding influence. T0 is not estimated in this model as the curve does not intercept the horizontal axis. Topt was calculated by subtracting Δ from Tmax (Roy *et al.*, 2002).

Kaplan–Meier estimator in SPSS (Version 24, 2016) was used for analysis of larval mortality in virulence experiments (Busvine, 1967).

2.3 Results

2.3.1 Phylogenetic analysis of candidate strains of *Beauveria*

ITS, β-tubulin, elongation factor 1-α, and DNA Lyase regions were amplified from chromosomal DNA of the 50 fungal strains of the genus *Beauveria*. The lengths of the PCR products were according to the literature (Meyling *et al.*, 2009; Rehner & Buckley, 2005). The sizes of PRC products were as follows: ≈550 bp for ITS, ≈600 bp for β-tubulin, ≈650 for elongation factor 1-α, and ≈550 bp for DNA Lyase. The root of the phylogenetic tree for the 50 isolates of *Beauveria* was inferred from an initial parsimony analysis that included an isolate of *Cordyceps militaris.* This fungal species was previously determined to be closely related to but distinct from *Beauveria* in an 18S SSU rDNA phylogeny (Sung *et al.*, 2001).

A phylogenetic tree was generated in the software CLC Workbench (Qiagen, https://www.qiagenbioinformatics.com/) for the 4 genetic markers, and it was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter mode. This model had the lowest Bayesian information criterion (BIC) scores, the bootstrap consensus tree was tested using 1000 replicates (**Figure 2.1**). The resulting tree showed two main groups or branches, suggesting the presence of more than one genetic clade of *Beauveria,* whose identity could be associated with the common species of *Beauveria*: *bassiana* and *pseudobassiana (Rehner et al., 2011)*. To confirm this assumption, an additional set of three different species that had previously been confirmed belonging to the species *B. bassiana* (ARSEF 1564) *and B. pseudobassiana* (ARSEF 3405). There were used for verification of the phylogenetic analysis in addition to the strains obtained from the ARSEF collection that were confirmed *B. bassiana* (Rehner *et al.*, 2011). DNA of these strains was isolated and used for PCR amplification of the 4 genetic markers used in this study, and their sequences were used to feed into the phylogenetic three analysis. Results showed that strains in the fungal collection used in this study can be separated into two genetic different groups. The first two branches comprise 39 strains belonging to the species *B. bassiana*; while the third branch comprises eight strains belonging to the species *B. pseudobassiana* (**Figure 2.1**). It was not possible to visualize any correlation between insect host and the position of the isolates within the phylogenetic tree; however, a

correlation was found between the geographical origin of some strains and their position in the tree, as a strain from a given location tended to close in the tree with strains sourced from nearby locations. For instance, *B. bassiana* strains 40, 41 and 42 from Kenya are positioned in the same branch one followed after the other. Besides, same pattern was observed in all the strains obtained from the ARSEF collection. Five out of six strains (Lepidoptera host) where in the same branch one after other except for strain 11 that only differed in insect host (Coleopteran). In Figure 5 was included information about presence of mating types genes because all genes were evaluated at the same time. Procedures and results about mating types genes are described in **section 4.3.1**.

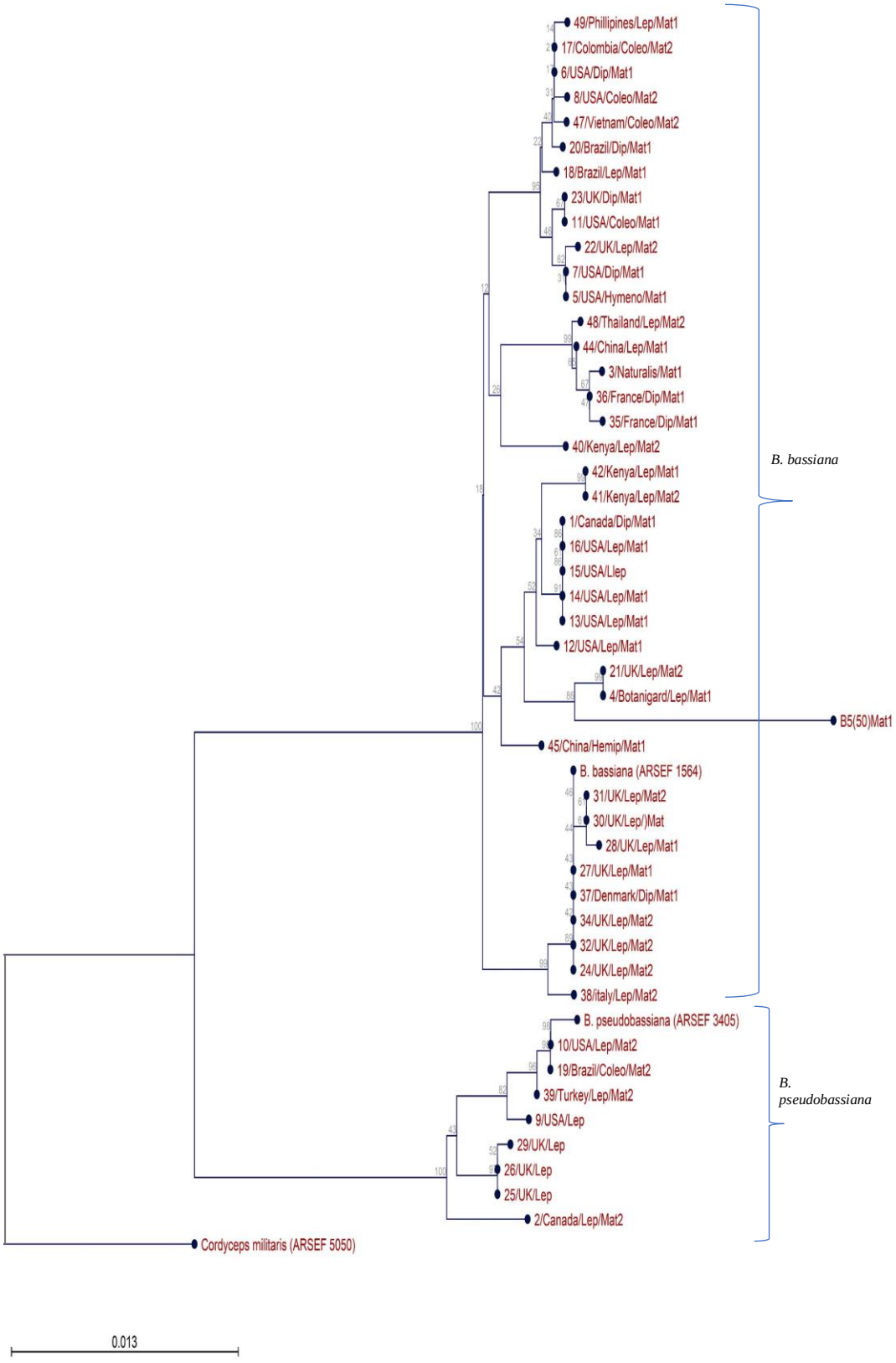


Figure 2.1. Phylogenetic Tree generated in CLC Workbench (Qiagen, https://www.qiagenbioinformatics.com/), by the Maximum Likelihood method based on the Kimura 2-parameter mode. Genetic markers used: ITS, Elongation factor, β-tubulin and DNA Lyase. Strains used to confirm species identity: *B. bassiana* (ARSEF 1564) and *B. pseudobiassiana* (ARSEF 3405).

2.3.2 Comparison of conidial production by candidate strains of *Beauveria*

Data obtained in this experiment was transformed to log 10 before statistical analysis. Mean conidia concentration of each strain of the fungal collection, was evaluated in a one-way analysis of variance (ANOVA, $p > 0.05$). After 13 days of growth at 22 °C, the number of spores produced by these strains showed differences among them (see **Appendix 2**). *B. bassiana* strains 34 (UK), 48 (Thailand) and 35 (France), produced significantly more conidia ($p > 0.05$), reaching concentrations > 1 x 10^9 conidia/mL. Conversely, *B. bassiana* strains 18 (Brazil), 2 (USA) and 41 (Kenya), produced significantly fewer conidia ($p > 0.05$) with conidial concentrations < 2 x 10^8 cfu/mL. The other 44 strains produced similar numbers of conidia (Duncan test, $p > 0.05$) (see **Appendix 2**). No correlation was found between the geographical source of a given strain and its conidial production level, under the conditions tested.

Table 2.3 One way ANOVA for Fungal Sporulation in 50 strains of Beauveria and Duncan (p>0,05)

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	21.74	49	0.44	5.15	<0.0001
Strains	21.74	49	0.44	5.15	<0.0001
Error	12.91	150	0.09		
Total	34.65	199			

Test:Duncan Alpha:=0.05

Error: 0.0861 df: 150

Strains	Means	n	S.E.								
18	7.32	4	0.15	A							
2	7.68	4	0.15	A	B						
41	7.84	4	0.15		B						
38	8.32	4	0.15			C					
39	8.34	4	0.15			C	D				
12	8.39	4	0.15			C	D	E			
44	8.39	4	0.15			C	D	E			
46	8.39	4	0.15			C	D	E			
43	8.41	4	0.15			C	D	E			
40	8.42	4	0.15			C	D	E			
50	8.43	4	0.15			C	D	E	F		
8	8.48	4	0.15			C	D	E	F	G	
26	8.52	4	0.15			C	D	E	F	G	H
25	8.53	4	0.15			C	D	E	F	G	H
30	8.55	4	0.15			C	D	E	F	G	H	I	
16	8.57	4	0.15			C	D	E	F	G	H	I	
29	8.57	4	0.15			C	D	E	F	G	H	I	
37	8.58	4	0.15			C	D	E	F	G	H	I	
4	8.61	4	0.15			C	D	E	F	G	H	I	J
10	8.63	4	0.15			C	D	E	F	G	H	I	J
3	8.63	4	0.15			C	D	E	F	G	H	I	J
11	8.64	4	0.15			C	D	E	F	G	H	I	J
19	8.65	4	0.15			C	D	E	F	G	H	I	J
32	8.67	4	0.15			C	D	E	F	G	H	I	J
22	8.67	4	0.15			C	D	E	F	G	H	I	J
6	8.70	4	0.15			C	D	E	F	G	H	I	J
23	8.71	4	0.15			C	D	E	F	G	H	I	J
1	8.71	4	0.15			C	D	E	F	G	H	I	J
27	8.72	4	0.15			C	D	E	F	G	H	I	J
28	8.73	4	0.15			C	D	E	F	G	H	I	J
15	8.73	4	0.15			C	D	E	F	G	H	I	J
45	8.76	4	0.15			C	D	E	F	G	H	I	J
13	8.76	4	0.15			C	D	E	F	G	H	I	J
24	8.80	4	0.15			C	D	E	F	G	H	I	J
20	8.82	4	0.15			C	D	E	F	G	H	I	J
33	8.82	4	0.15			C	D	E	F	G	H	I	J
42	8.82	4	0.15			C	D	E	F	G	H	I	J
47	8.85	4	0.15			C	D	E	F	G	H	I	J
5	8.85	4	0.15				D	E	F	G	H	I	J
31	8.86	4	0.15				D	E	F	G	H	I	J
7	8.86	4	0.15				D	E	F	G	H	I	J
14	8.87	4	0.15				D	E	F	G	H	I	J
21	8.88	4	0.15					E	F	G	H	I	J
49	8.95	4	0.15						F	G	H	I	J
17	8.98	4	0.15							G	H	I	J
9	9.02	4	0.15								H	I	J
36	9.03	4	0.15								H	I	J
34	9.03	4	0.15								H	I	J
48	9.06	4	0.15									I	J
35	9.11	4	0.15										J

2.3.3 Effect of temperature on fungal growth

All 50 strains grew at all the temperatures examined in the present study. Radial extension was taken as the mean of two diameters. Mean diameters were plotted over time for each strain, replicate and temperatures (10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 33 °C). Colony extension rates were analysed using ANOVA and significant results were analysed with Tukey HSD ($p > 0.05$) (see **Appendix 3**).

The variation in growth presented a bell-shaped distribution for each strain. Nevertheless, the individual strain behaviour was different with some strains showing a high thermotolerance such as 17 (Colombia) while other strains were thermosensitive, for instance, strain 14 from the USA showed good growth at 20 ^{o}C and 25 ^{o}C, but at 10 ^{o}C and 15 ^{o}C, the growth ratio of the colony was significantly affected (**Figure 2.2**). When the whole range of temperatures assayed were considered, strain 43 showed the greatest variation in fungal growth, which would suggest a sensitivity of this strain to temperature. Conversely, strain 38 showed the lowest variation, suggesting that this strain would be thermostable (**Figure 2.2**). Some strains displayed some degree of thermotolerance, as they were able to grown at 30 °C, including strains: 23 (UK), 20 (USA), 17 (Colombia), 41 (Kenya), 20 (Brazil), 45 (China), 8 (USA), 43 (China), 49 (Phillipines), 50 (Australia) (see **Appendix 4**).

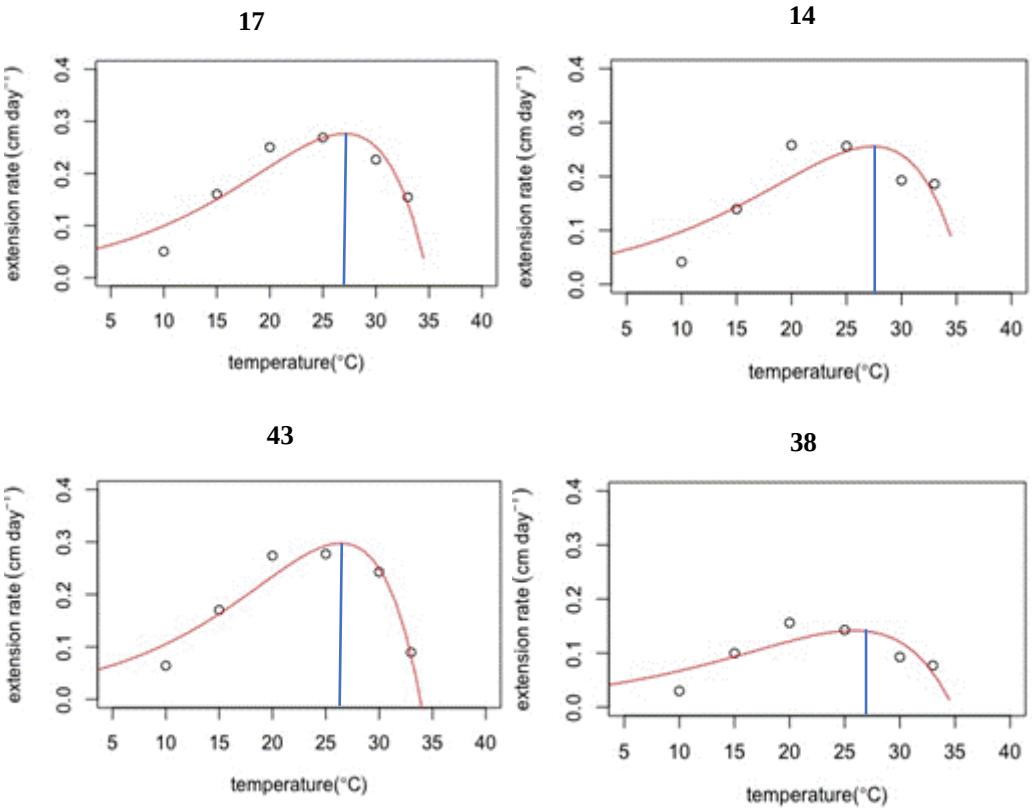


Figure 2.2 Lactin-1 non-linear model fitted to mean colony extension rate (cm/day) plotted against temperatures for four *B. bassiana* strains.

Lactin-1 was considered the best fitting model for this study after the Akaike information criterion (AIC) values were compared with other three models (Logan, Polynomial and Briere). AIC estimates the relative quality of each model for a given set of data and for the present study has been used to evaluate the performance of colony extension rates in other EPF (Klass *et al.*, 2007) (**Appendix 5**). The variation within the data is explained by r^2 values, where the closer data points are to the statistical regression line, the closer to 1 the value of r^2 is. This model displayed r^2 values between 0.54 and 0.97 among the 50 strains of *Beauveria*. On the other hand, AIC values reflects the accuracy between models, where the lowest AIC is considered to be the most appropriate in explaining the data set. AIC values for the present study were between -13.43 and -33.79. Optimum temperature (Topt), was similar, for most of the *Beauveria* strains and displayed values between 25.08 °C and 28.12 °C; while Maximum temperature (Tmax) displayed values between 33.34 °C and 37.32 °C for the 50 strains of *Beauveria*.

Table 2.4 Fitted parameters of Lactin-1 model fitted to colony extension rates (cm/day) at six temperatures for 50 isolates of Beauveria.

Strain	Tmax	Topt
33	35.56	26.57
29	35.02	26.50
20	35.03	27.32
26	34.36	26.29
34	34.15	26.90
24	33.65	25.10
22	37.32	27.83
47	34.75	25.74
2	33.98	25.94
12	34.39	25.95
42	34.16	26.98
38	34.82	25.89
18	34.65	26.83
14	35.74	27.45
1	35.47	27.29
11	34.26	26.37
10	36.26	27.44
32	33.42	25.50
28	34.03	26.37
7	33.72	27.00
9	33.52	26.40
3	35.74	26.06
50	33.70	26.63
46	33.58	26.11
21	34.63	26.92
37	33.47	25.46
45	33.43	26.99
4	33.76	26.74
30	34.83	27.10
40	33.39	25.54
13	34.12	26.62
31	33.91	26.23
19	33.34	25.08
41	33.48	26.94
15	34.16	26.65
48	34.28	27.32
17	34.90	27.11
5	34.37	26.60
6	34.19	26.59

Table 2.4 Continued, fitted parameters of Lactin-1 model fitted to colony extension rates (cm/day) at six temperatures for 50 isolates of Beauveria.

Strain	Tmax	Topt
27	34.14	26.68
35	34.17	27.29
23	35.04	26.78
35	35.53	28.12
16	35.20	27.15
25	35.38	26.53
49	34.26	26.79
44	34.15	26.30
43	33.88	26.46
8	35.37	27.93
39	33.63	26.31

As an illustration, the colony area of different strains on the whole range of assayed temperatures can be seen in **Figure 2.3**. These results provide valuable information for further experiments, as those strains that showed increased growth capability could be considered as good candidates for biopesticide formulations or strain improvement programs to be used not only in green houses but also in the field, due to their improved ability for growing on the whole range of temperatures.

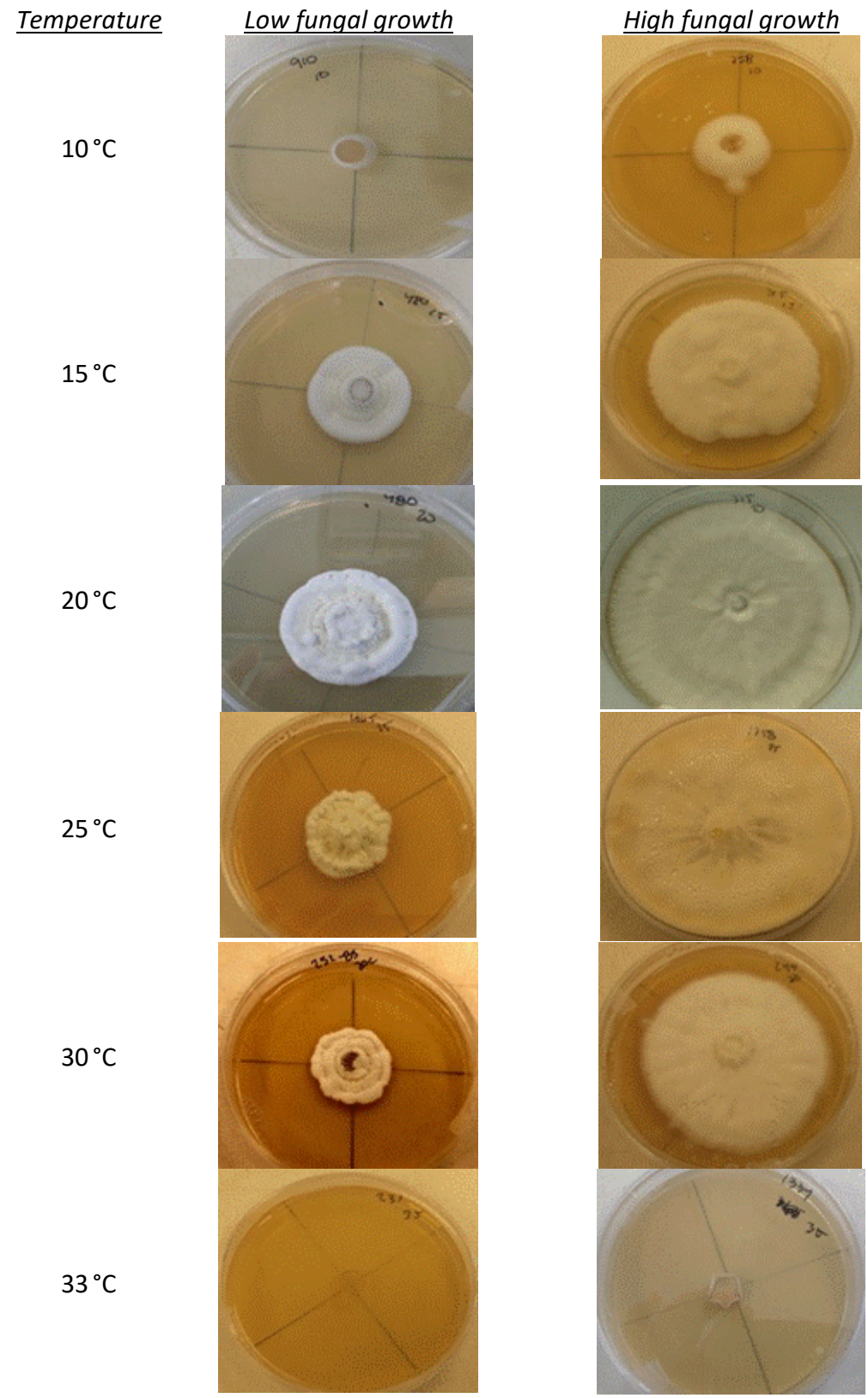


Figure 2.3. Examples of fungal colony of different strains of *Beauveria* over the whole range of assayed temperatures, after four weeks incubation.

2.3.4 Effect of Temperature on germination

The relationship between temperature and percentage germination of populations of the candidate strains was evaluated after 24 h incubation. For germination data, percentage germination of EPF conidia were calculated from numbers of germinated and ungerminated conidia after 24 h incubation at one of six temperatures. Results showed differences of incubation statistically significant by an ANOVA and Tukey (p>0,05) analysis (see **Appendix 6**). Germination proved to be dependent on temperature, showing a behavior that resembles the effect of temperature on growth experiments, as the bell-shaped distribution was observed for germination (**Figure 2.4**). Germination was highly affected also at 10 ^{o}C and 33 ^{o}C, where this germination was completely inhibited in most of the cases. Strains 4 displayed the highest percentage of germinated conidia in all the temperatures, except for 10 ^{o}C where this was completely inhibited.

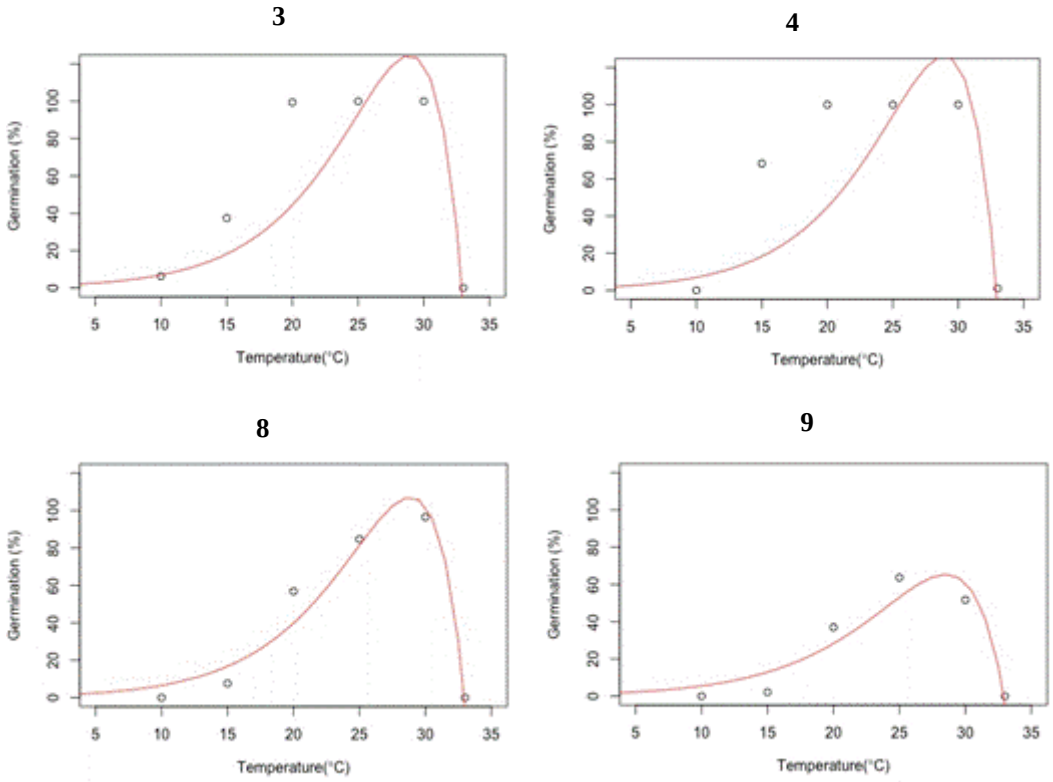


Figure 2.4. Percentage germination for four strains used in this study, after 24 hours incubation at different temperatures.

Lactin-1 model was fitted to the relationship between temperature and percentage germination (**Appendix 7**). Thermal optima and maxima were relatively similar among the 50 isolates within the range of 29.08 °C to 29.48 °C and 32.9 ^{o}C to 33 ^{o}C respectively (**Table 2.5**).

Table 2.5 Fitted parameters of Lactin-1 model fitted to percentage of germination of conidia at six temperatures for 50 isolates of Beauveria.

Strain	Tmax	Topt
33	32.9361	29.4573
29	32.9334	29.4236
20	32.9913	29.18329
26	32.9278	29.4273
34	32.9446	29.3753
24	32.9353	29.4387
22	32.9498	29.422
47	32.9626	29.4075
2	32.935	29.4377
12	32.9312	29.4569
42	32.9381	29.4306
38	32.9409	29.4065
18	32.9415	29.4424
14	32.9401	29.4564
1	32.9515	29.4246
11	32.9536	29.2906
10	32.9354	29.3609
32	32.9386	29.4262
28	32.9486	29.3756
7	32.9729	29.3443
9	32.9357	29.0833
3	32.9303	29.4579
50	32.9373	29.4382
46	32.9329	29.451
21	32.9281	29.4667
37	32.9325	29.4381
45	32.9415	29.4341
4	32.937	29.4766
30	32.9291	29.425

Table 2.5 Continued, fitted parameters of Lactin-1 model fitted to percentage of germination of conidia at six temperatures for 50 isolates of *Beauveria*.

Strain	Tmax	Topt
40	32.9315	29.4375
20	32.9346	29.4474
31	32.9265	29.4321
19	32.9361	29.4257
41	32.9367	29.3114
15	32.9325	29.452
48	32.9504	29.4232
17	32.9359	29.4524
5	32.9379	29.4401
6	32.949	29.4266
27	32.9346	29.4254
35	32.9441	29.4343
23	32.9491	29.4416
36	32.9525	29.4052
16	32.9314	29.4567
25	32.8676	29.2118
49	32.9341	29.4401
44	32.9255	29.3179
43	32.9296	29.4577
8	32.9758	29.3927
44	32.9542	29.4035

2.3.5 Effect of UV radiation on candidate strains of *Beauveria*

Dose selection and effect on time to germination

Three strains (4, 11, 12) were assessed to set up the working conditions for this experiment. The effect of UV-B on germination was first seen after 60 minutes exposure. Isolates 4 and 12 responded similarly with 76.6% and 96% of germination respectively. Isolate 11 was more susceptible with 45% conidial germination. Germination decreased as the time of exposure increased for all the isolates examined (**Figure 2.5**). As a working condition for further experiments on exposure time of 90 minutes was selected as allowed the evaluation of significant differences in germination among the assayed strains, without complete inhibition of germination.

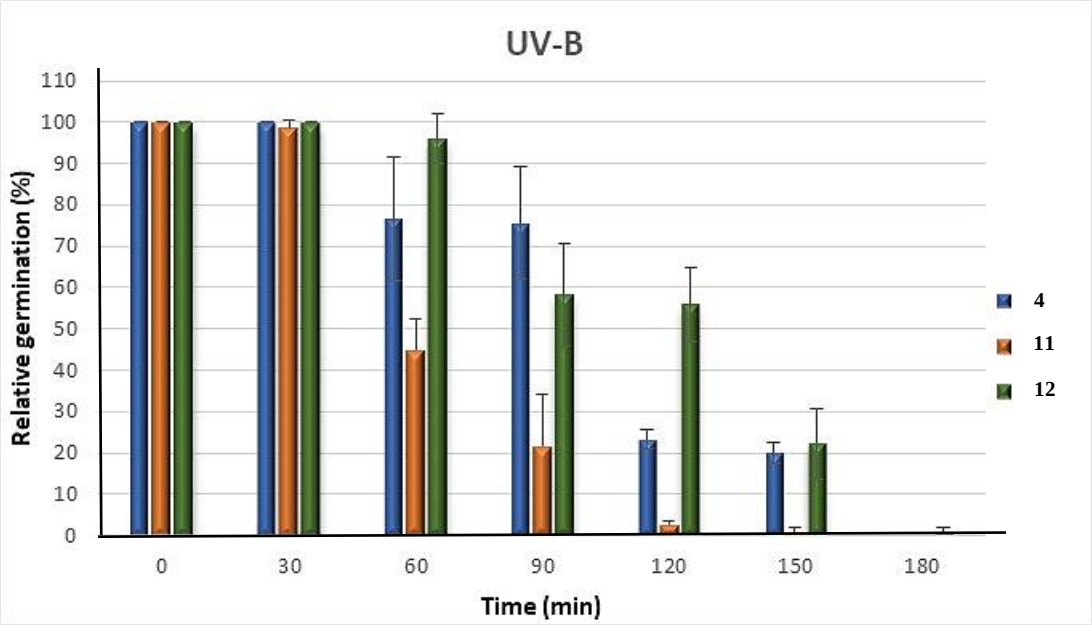


Figure 2.5. Mean of germination of *Beauveria* strains after exposure at 5.94 kJ/m2 of irradiance, for different periods of time was statistical different.

After 12 h, very little germination was observed in both controls and the UV-B treated strains (1% to 16.9%). After 24 h, all control conidia exhibited 100% germination whereas in the UV-B treated conidia, germination varied from 11.2% to 36.1%. For further experiments, conidia were allowed to germinate for 48 h before being assessed (**Figure 2.6**).

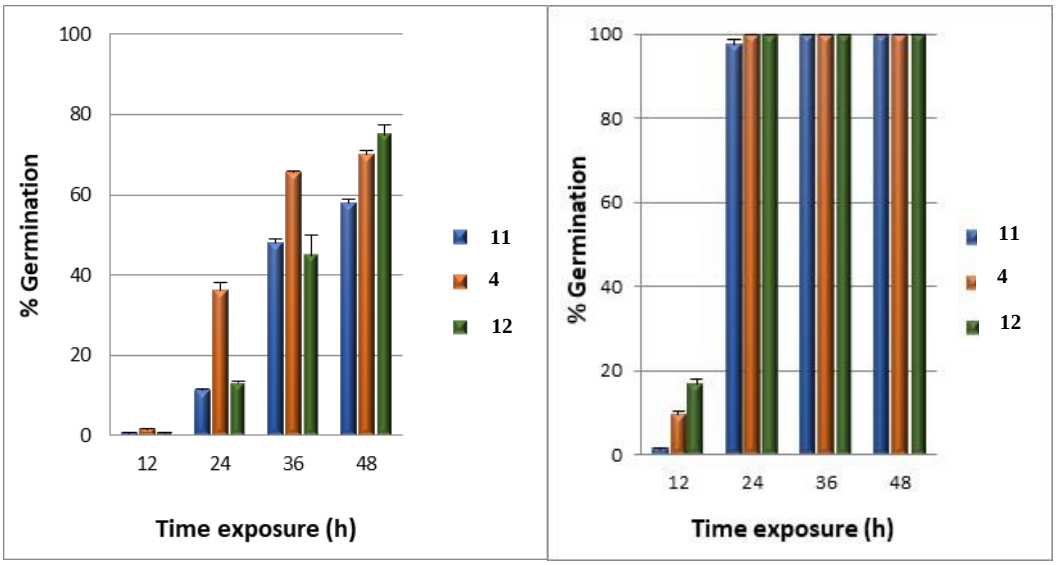


Figure 2.6. Effect on time to germination (germination recovery) evaluated every 12 h, for 48 h, after exposure to UV-B light. Left: irradiated strains 11, 4, 12. Right: same strains, non-irradiated (controls).

Tolerance to UV-B irradiation

The conidia exhibited significant differences in germination in response to UV-B exposure among the 50 strains of *Beauveria* (ANOVA and Tukey, $p>0.05$) (see **Appendix 8**). The strains were categorized into three groups, according to their relative percentage germination. Low tolerance for 10 isolates with a range of germination between 0% and 29.7%, medium tolerance for 18 isolates displaying a range of germination between 30.6% and 58.2% and high tolerance for 22 isolates displaying germination between 60.9% and 88.1% (**Table 2.6**). No relationship was found between the geographical origin of strains and their germination response to UV-B radiation. For instance, two different strains from warm climate, Kenya: 40 and 41 were classified as high and low tolerant to UV-B, respectively; and strains 18 and 20 from Brazil were classified as low and medium tolerant, respectively. Conversely, strains from the UK were among the most tolerant to UV-B light, i.e., strains 26 and 23, showing over 80% germination after irradiation exposure. Strain 29 did not germinate at all.

Table 2.6 Sensitivity to UV-B radiation. Mean percentage germination of 50 strains of Beauveria after 90 minutes exposure to UV-B radiation at 1100 mW/m^2 (5.94 kJ/m^2). Low tolerance (<30%), medium tolerance (30%-60%) and high tolerance (>60%)

Beauveria Strain	Mean % Germination	STERROR	Classification
29	0,0	0,0	LOW
43	4,7	2,2	LOW
41	13,5	1,7	LOW
27	14,9	3,0	LOW
46	18,7	13,4	LOW
47	19,0	1,8	LOW
2	23,9	11,3	LOW
11	25,3	8,8	LOW
7	25,6	5,7	LOW
32	28,3	9,0	LOW
38	29,3	12,1	LOW
25	29,7	11,7	LOW
39	30,6	13,4	LOW
18	31,4	5,7	LOW
42	31,4	8,7	MEDIUM
8	32,8	8,2	MEDIUM
36	34,3	3,3	MEDIUM
9	35,4	15,9	MEDIUM
20	36,7	13,4	MEDIUM
34	38,2	22,1	MEDIUM
22	40,1	22,9	MEDIUM
6	43,5	24,1	MEDIUM
37	45,4	15,3	MEDIUM
10	48,6	20,2	MEDIUM
19	49,6	20,2	MEDIUM
1	55,1	0,5	MEDIUM
3	56,1	9,8	MEDIUM
17	57,5	18,3	MEDIUM
48	58,2	3,4	MEDIUM
30	60,9	10,6	HIGH
24	64,7	7,6	HIGH
4	65,5	27,0	HIGH
5	65,6	2,7	HIGH
15	69,0	9,8	HIGH

Table 2.7 Continued. Sensitivity to UV-B radiation. Mean percentage germination of 50 strains of *Beauveria* after 90 minutes exposure to UV-B radiation at 1100 mW/m^2 (5.94 kJ/m^2). Low tolerance (<30%), medium tolerance (30%-60%) and high tolerance (>60%).

Beauveria Strain	Mean % Germination	STERROR	Classification
40	72,4	4,3	HIGH
44	73,9	6,8	HIGH
50	75,6	3,8	HIGH
28	76,5	8,8	HIGH
21	79,0	5,9	HIGH
31	79,9	12,2	HIGH
13	80,6	0,8	HIGH
33	80,8	9,3	HIGH
12	80,8	5,9	HIGH
45	81,1	3,5	HIGH
16	82,1	9,9	HIGH
23	83,9	7,1	HIGH
35	85,0	15,0	HIGH
14	85,5	9,1	HIGH
49	86,6	0,8	HIGH
26	88,1	7,5	HIGH

2.3.6 Virulence of fungal strains against *Plutella xylostella* (DBM)

Mortality from seven days after inoculation was analyzed to compare virulence of the 50 strains of *Beauveria* against DBM. The time of infection assessed for *Beauveria* strains towards second instar larvae of DBM was seven days. After this time, 9 strains showed a low incidence of mortality (<50%); 15 strains killed > 50% of larvae but < 80% of them; and the remaining 26 strains caused the mortality of > 80% of larvae, proving to be highly virulent (**Figure 2.7**). Control mortality values ranged from 10% to 16.02%, so data was not corrected. Strains exhibited a wide range of virulence to DBM ranging from 17.7% (strain 6) to 100% (strain 29) and mortality varied significantly among the 50 isolates (ANOVA and Tukey, p>0,05) (see

Appendix 9). Moreover, the cadavers of DBM exhibited sporulation on the cuticule surface, supporting successful infection (**Figure 2.8**).

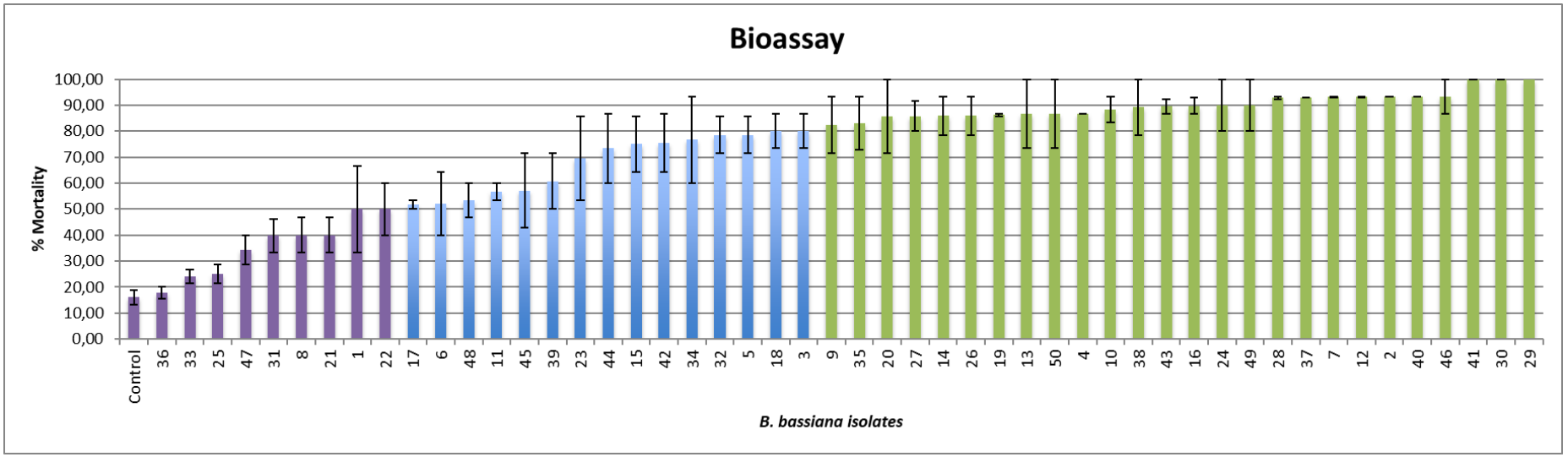


Figure 2.7. Percentage mortality of second instar larvae of DBM after seven days infection incubated at 22°C (16:8 LD). DBM larvae were sprayed with a conidial suspension (10^7conidia/ml) of each of the 50 strains of *Beauveria*. In purple are highlighted strains with low virulence (< 50% mortality), in blue are highlighted strains with medium mortality (50-80% mortality) and in green are highlighted strains with high virulence (> 80% mortality). Error bars are standard error of the mean, n= 3.

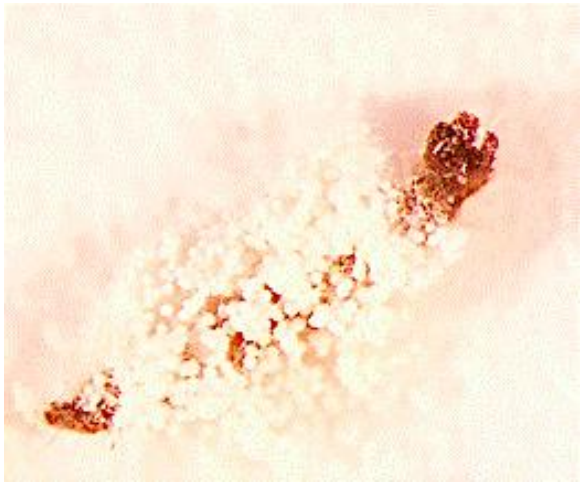


Figure 2.8. *Putella xylostella* larvae cadaver after 7 days *Beauveria* infection, showing sporulation on its surface.

Most of mortality occurred between days 4 and 5 (**Figure 2.9**). However, a few strains, including strain 35 (France) and strain 37 (Denmark), needed until day 7 to kill ~30% of the larvae.

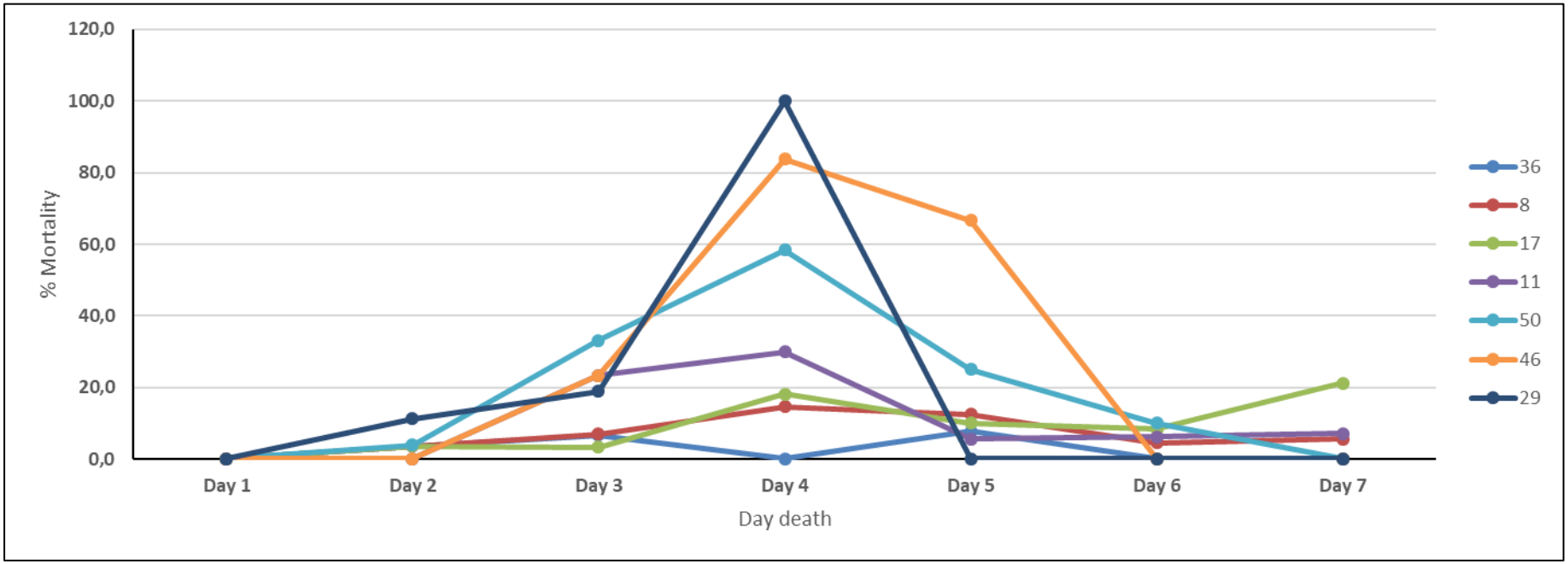


Figure 2.9. Survival Analysis made for DBM after seven days of application of *Beauveria* strains incubated at 22°C (16:8 LD). Ten strains of *Beauveria* have been selected to high light the bell shape behaviour from the low virulent to the most virulent strains.

Interestingly, larvae treated with strain 29 (UK) was the fastest to kill compared with larvae infected with the other 49 strains. Two days after inoculation and on the third day, they had ceased to feed, resulting in 100% mortality after four days.

2.4 Discussion

2.4.1 Phylogenetic analysis of candidate strains of *Beauveria*

At the beginning of this study, 50 putative *B. bassiana* s.l. strains were selected based on their morphological characteristics. The strains were chosen to represent a range of geographical locations and insect hosts. Results from a phylogenetic analysis of the strains using nucleotide sequence data generated from four genes revealed four main branches, suggesting the presence of two distinct genetic clades within the *Beauveria* collection. While *Beauveria* shows little morphological variation within the genus, there is evidence showing high genetic diversity (Ghikas *et al.*, 2010; Meyling *et al.*, 2009; Rehner *et al.*, 2011). The most recent updated of *Beauveria* classification, based on four nucleotide sequence data identified six different clades corresponding to existing morphological species, but also identified six additional genetic clades, and proposed that the genus be divided into 13 different phylogenetic species (Rehner et al., 2011). By incorporating sequence data for three recognized phylogenetic species of *Beauveria* (*B. bassiana, B. brongniartii and B. pseudobassiana*) into the current analysis, it was possible to assign the clades to recognized phylogenetic species. The larger clade (provisionally entitled clade A) was mapped to *B. bassiana*; and the small branch (clade B) was mapped to *B. pseudobassiana*. Clade A (*B. bassiana* s.s) is a large group, including geographically widespread genotypic subgroups within the species as well as undetermined lineages that are present in both natural and agricultural habitats (St Leger *et al.*, 1992); which can explain the presence of multiple phylogenetic species within this big clade (Ghikas *et al.*, 2010; Rehner *et al.*, 2006). No evidence of correlations between geographic location and host insect was found it. Both, *B. bassiana* and *B. pseudobassiana* exhibited a wide range of origins within the isolates. The isolates belonging to *B. pseudobassiana* had as the host, lepidopteran insects, except for 19 which prefered coleopteran insects. On the other hand, it has been

reported that *B. pseudobassiana* is not immediately related to *B. bassiana*, yet it has the same conidial shape, wider range of habitats-host and size which are undistinguishable from *B. bassiana* conidia, unless a phylogenetic analysis is performed (Rehner *et al.*, 2011). No evidence of correlations between geographical location and host insect were found. Virulence of *B. pseudobassiana* against coleopteran pests (e.g. *Ips sexdentatus* and *I. typographus*) has been reported showing good potential for biopesticide activity (Kocacevik *et al.*, 2016; Álvarez-Baz *et al.*, 2015).

2.4.2 Effect of temperature on fungal growth and conidial germination

Fungal growth

After evaluating the growth of 50 strains of *Beauveria* at six different temperatures, and modelling the growth-temperature interaction, it was possible to identify the temperatures for maximum and optimum growth. It was clear that the strains showed a different pattern of growth depending on temperature conditions. It has been reported that thermotolerance is a trait often related to the country of origin of a given strain (Braga *et al.*, 2001; Fernandes *et al.*, 2007; Rangel *et al.*, 2005); however, the results found in this study did not find such a correlation. Eight out of 50 isolates came from low latitudes (**Table 2.1**) and just two of them (50 (Australia) and 17 (Colombia)) stood out as having a high temperature tolerance. However, at low temperatures strains belonging to high latitudes (e.g. from Europe) presented the highest growth rate originating previously for *B. bassiana* (Fernandes *et al.*, 2008; Vidal *et al.*, 1997). Therefore, although country of origin provides some useful information about possible climate conditions or level of light irradiation within the strains native range, it is probably more important to have specific details of the habitat from which the strain was isolated (i.e., its endemic conditions) and even the specific point of collection. For instance, strains from the same country will perform differently depending on whether they have been collected in a forest or in agricultural land (Tumuhaise *et al.*, 2018). Differences could emerge even in small areas, as highly complex and diverse micro-environments can be found within the same ecosystem, such as a tropical rain forest, where environmental conditions (UV

light irradiation, temperature, water availability, soil structure and nutrients) can vary significantly even over relatively small distances (Cabanillas & Jones, 2009; Fargues *et al.*, 1997). These facts could explain the differences observed for strains sourced from the same country and also corroborate the findings in other *Beauveria* studies, where isolates collected from forested, agricultural habitats and the Canadian Arctic concluded that a population structure within Hypocreales insect-pathogenic fungi is controlled by habitat selection and not by insect host selection (Bidochka *et al.*, 2002). Optimum temperatures for growth in *B. bassiana* is generally around 25 °C (Ying & Feng, 2011). Some models, such as Polynomial, Briere and Lactin, have been done in order to understand interaction fungus-host in other EPF (Inglis *et al.*, 1996; James *et al.*, 1998). However, few studies have been done on *Beauveria* colony extension growth (Davidson *et al.*, 2003) and most of the published studies have not used statistical regression models to identify the cardinal temperatures for growth. Results regarding temperature showed that all *Beauveria* strains tested were sensitive to temperatures higher than 30 °C, with growth being almost completely inhibited at 33 °C. Conversely, all isolates grew at 10 °C, indicating cold tolerance, which has been reported before in *Beauveria*, especially in *B. bassiana* (Fernandes *et al.*, 2008; James *et al.*, 1998). For optimum temperature in the present study the values range between 25 ^{o}C and 28 ^{o}C, with all strains displayed a bell-shaped distribution which depended on the strain could be steep or broad. Then, was no relationship between optimum or maximum temperatures and place of origin or insect host. Other studies in *B. bassiana* have found levels of tolerance for some strains as high as 35 to 37 °C, making them interesting candidates for applications in greenhouses, where temperatures > 35 °C can be reached (Fargues *et al.*, 1997); whereas the 50 strains assayed in this study could represent good candidates for applications in temperate zones, due to their sensitivity to temperatures above 30 °C.

Conidial germination

Conidial germination was evaluated after 24 h incubation, and was highly affected at temperatures > 30 °C. No germination was visible for 46 strains at 33 °C while the other 4 strains had a low level of germination (>1 %) under these

conditions. Germination optima was higher when compared to colony growth optima and were relatively similar among the 50 isolates within the range of 29.1 ^{o}C to 29.5 ^{o}C, whereas maximum temperature was lower than colony growth maxima with values of 32.9 ^{o}C to 33 ^{o}C. This results are in line with reports for *M. anisopliae* and *B. bassiana* isolates in a previous study on colony growth (Smits *et al.*, 2003) and there was no correlation between germination and place of origin or host. It has been found that the damage produced by high temperatures, delayed the process of germination in *B. bassiana* and *M. anisopliae* and this effect has been explained as a recovery time needed for the cell to repair itself before germination begins (Fernandes *et al.*, 2008). Despite these results, some isolates did show high thermotolerance, such as strain 433-94 (*B. bassiana*), whose germination surpassed 60 % at 15 °C, while it reached 100 % germination at 20 °C-30 °C. This strain along with *B. bassiana* strains 21, 14 and 33 exhibited some limited germination (< 1%) at 33 °C (see **Appendix 6**). The ability to recover from damage after high temperature exposure represents a big advantage, especially for strains that could be used for formulations of biocontrol products, considering that their low efficacy under high temperatures represents one of their most important problems (Bugeme *et al.*, 2008; Devi *et al.*, 2005; Fernandes *et al.*, 2007; Fernandes *et al.*, 2008; James *et al.*, 1998).

2.4.3 Effect of UV radiation on candidate fungal strains

There was a high variability in UV-B tolerance among the 50 strains of *Beauveria*. Following the exposure to UV-B, the germination percentage for different strains varied from 0% to 90%, which has also been reported for other *B. bassiana* strains (Li & Lee, 2014). As expected, the delay in germination was observed after 90 minutes irradiation at 5.94 kJ/m^2. This dose was equivalent to the highest UV-B radiation month in United States and middle Europe-Asia; or the lowest UV-B radiation month in Central America, or middle regions in South America (Fernandes *et al.*, 2007). This result is attributed to cell damage produced by UV-B exposure, so that before germination begins, surviving conidia require time to repair the cell (Nicholson *et al.*, 2000). In another study, it was found that the geographical latitude of origin in *M. anisopliae* is a factor that plays a role in tolerance to UV-B light, since

the amount of radiation is inversely proportional to the latitude (Braga *et al.*, 2001). Therefore, strains belonging to countries with lower latitudes would be more tolerant to UV-B radiation than strains belonging to countries with higher latitudes. Nevertheless, in the current study no relationship between UV-B tolerance and strain origin was found, with some exceptions, such as *B. pseudobassiana* strain 29 (UK, higher latitude), which was completely inhibited at these conditions. No correlation was found among colony extension rate, germination and UV-B tolerance. Moreover, some *B. bassiana* strains (43 (China, Hemipteran) and 8 (USA, Coleoptera)) that had good growth and germination at high temperatures were highly susceptible at UV-B radiation. Few studies have been conducted in *B. bassiana* to expand the current knowledge on this regard. Some studies have indicated that there is no relationship between tolerance to UV-B radiation and geographical origin of the strains (Braga *et al.*, 2001); while other studies have suggested an inverse correlation between latitude of origin and tolerance to UV-B radiation (Fernandes *et al.*, 2007; Li & Lee, 2014). There are many environmental aspects, including host, niche, season of collection, daily variation in sun inclination or humidity, that should be taken into account to explain the behaviour of fungi under specific conditions, even though they belong to the same family or place of origin (Bidochka *et al.*, 2002). Thus, screening programmes should be implemented in the research focused on EPF because latitude of origin did not influence tolerance to UV-B. In fact, the environment (i.e., in the field) or the media (i.e., in vitro) where the fungal conidia growth is an important factor to consider at the time of UV-B tolerance evaluation, because it has been shown that a change in the nutritional factors in EPF´s will affect the physiology and biochemistry of the conidia (Magan, 2001). For instance, a study done on *M. anisopliae* found that conidia from infected dead larvae were more susceptible to UV-B radiation than conidia developed on potato dextrose agar with yeast (PDAY) or rice due to laboratory conditions are less harmful than the environmental conditions (Rangel *et al.*, 2004).

2.4.4 Virulence of candidate fungal strains against *Plutella xylostella*

The high virulence of *B. bassiana* towards arthropod insects is one of the most important reasons to include this fungus as a valuable potential microbial control agent (Vandenberg *et al.*, 1998; Wraight *et al.*, 2010). As expected, virulence against *Plutella xylostella* (diamondback moth, DBM) among the 50 isolates varied, revealing strains with high virulence and a fast mode of action (infection in a short time), whereas there were other strains that needed more time to produce mortality. A critical step was found during this experiment, in that the precise age of the larvae used for virulence bioassays needed to be carefully controlled in order to obtain reproducible results. To reduce variation among repetitions, only recently moulted second instar larvae were used, which were identified by a darkened cuticle (Harcourt, 1957). Later instar larvae were less susceptible to the action of the EPFs than early instar larvae, since moulting to the next stage of the larvae acts as a defence mechanism became fungal-infected old epidermis (skin) is replaced by an un-infected new one, shortening the time for spores to penetrate through to the haemocoel, thus preventing infection (Vandenberg *et al.*, 1998).

Regarding the unusual infection of *B. pseudobassiana* strain 29 (early feeding inhibition), this effect has been reported in adult maize weevils (*Sitophilus zeamais*) sprayed with *B. bassiana*, where 3 days after infection, these insects did not respond to external stimuli, did not have coordinated movements and their feeding decreased (Adane *et al.*, 1996). In another study, a feeding decrease by 76.2 % was found on fourth-instar Colorado potato beetles, 3 days after infection by *B. bassiana* (Fargues *et al.*, 1994).

Mortality by *B. bassiana* of other insect species has been shown, such as migratory grasshoppers, aphids, beetles, moths, and in ticks, producing up to 80 % mortality (Bidochka & Khachatourians, 1990; Castrillo *et al.*, 2011; Fang *et al.*, 2005; Hussein *et al.*, 2012; Kaaya & Hassan, 2000). Sporulation was observed in most of the larval cadavers, which indicates that the fungi overcome the defence of the larvae during the penetration of the cuticle to achieve proliferation inside the host.

No correlation was found among virulence and host, place of origin or thermotolerance. Nevertheless, correlation was found between virulence and

germination. The five most virulent strains, 29 (*B. pseudobassiana*), 30 (*B. bassiana)*, 40 (*B. bassiana*), 41 (*B. bassiana*) and 46 (*B. bassiana*), displayed a percentage germination between 95% and 100% in a range of temperatures between 20 °C and 30 °C. This is in line with reports for *M. anisopliae* and *Paecilomyces fumosoroseus* where faster germination was correlated with higher virulence (Altre *et al.*, 1999; Rangel *et al.*, 2004; Samuels *et al.*, 1989).

The results found in this section of the current research will be used as a basis for the following experiments. Values from tolerance to high temperatures, susceptibility to UV-B radiation and virulence were evaluated to select a specific number of strains considering the time involved on each experiment, as result, seven strains for parasexual recombination and nine strains for sexual recombination.

3 Parasexual Recombination of candidate fungal strains

3.1 Introduction

The parasexual cycle is a natural mechanism present in non-sexual organisms that allows the transfer of genetic material without the generation of sexual structures (Pontecorvo, 1956). In asexual fungi the process involved in this cycle is yet to be fully elucidated, albeit it is known that two fungi fuse their hyphae exchanging cytoplasmic material, conserving the haploid nuclei of both parents (Leslie, 1993). The newly formed cell with more than one nucleus is named as a heterokaryon, and it can be generated artificially in the laboratory through protoplast fusion or induction of hyphal fusion between auxotrophic mutant strains (Adams *et al.*, 1987). It has been reported that in some filamentous fungi (i.e. *Rhizoctonia solanii, B. bassiana*), the two nuclei in the heterokaryon can lead to the formation of an unstable diploid cell that can lose chromosomes in some hypha stages, resulting in a recombinant haploid showing different characteristic compared with the parental strains, such as aggressiveness and host range, however this phenomenon is uncommon (Ogoshi, 1987; Paccola-Meirelles & Azevedo, 1991).

The fusion between the hyphae from two parental fungi and the heterokaryon stability depends on how vegetatively compatible the strains are (Castrillo *et al.*, 2004). Vegetative compatibility works differently for each group of strains, but in general terms this process occurs when a particular set of loci (such as *vic* and *het*) are identical between the two strains in the process, prompting hypha fusion to form a stable heterokaryon; whereas when one of the loci is different the strains are unable to fuse and the two fungal strains are grouped as vegetative incompatibles, suggesting that incompatible strains would be genetically different from each other (Couteaudier & Viaud, 1997; Leslie, 1993). Therefore, a new phylogenetic characterization can be performed by taking into account the vegetative compatibility groups (VCGs), where VCGs represent a subdivision within the same fungal species and whose members can fuse hyphae (anastomosis) to form heterokaryons and exchange genetic information (Couteaudier & Viaud, 1997). A considerable number of VCGs indicate a significant genetic diversity and this could suggest the possibility of a sexual cycle in some fungi before having an asexual cycle. In asexual fungi, the exchange of genetic information would be limited to strains belonging to the same VCGs and they could share characteristics like selection, mutation, migration and drift because they would be considered as clones of a single parental strain (Hartl & Clark, 1998). For instance, Couteaudier and Viaud (1997) found that some strains of *B. bassiana* share the same genotype despite the fact that they came from different countries, indicating a clonal population (Couteaudier & Viaud, 1997). As a consequence, not only strains but also VCGs could be lost by genetic drift and the population would be less diverse (Leslie, 1993).

To force a parasexual recombination under laboratory conditions, auxotrophic mutants are needed to obtain prototrophic heterokaryons after fusion, either by protoplast fusion or hyphal anastomosis. The main advantage of auxotrophic mutant utilization is to allow selection of prototrophic heterokaryons, as they can grow under conditions that the auxotrophic mutants cannot, confirming the recombination and enabling selection at the same time (Leslie, 1993). The auxotrophic nitrate non utilizing (nit) mutants can be used with this aim, as they present advantages compared with other auxotrophic mutants generated by the action of chemical or physical mutagens. They do not require an exogenous inductor

that prompts the mutation, enabling mutants to be obtained in a shorter time, using less steps and a less modified genome (Puhalla, 1985). The generation nit mutants is based on the capacity of fungi to synthesize nitrate to nitrite and nitrite to ammonium by the enzyme nitrate reductase, in the nitrate reduction pathway (Cove, 1976). The nitrate reductase pathway is also responsible for the transformation of chlorate into chlorite, the latter being toxic to fungi. When fungal strains are grown in the presence of chlorate, this is transformed into chlorite, which in turns inhibits fungal growth; however, a spontaneous mutation can cause the generation of some strains which lack the ability to transform chlorate into chlorite (and nitrate into nitrite), resulting in auxotrophic mutants resistant to chlorate (Korolev & Katan, 1997). The mutants are chlorate resistant, and since chlorate and nitrate are analogues, because they share the nitrate reduction pathway, the resulting mutants are nitrate resistant as well and cannot utilize nitrate as a nitrogen source, i.e., they become auxotrophic for nitrate (**Figure 3.1**) (Puhalla, 1985). The auxotrophic mutants that grow in chlorate media have aerial mycelium. However, once the mutants are plated on media with nitrate, the growth of the mycelium is non-aerial , which is used as a probe of the mutation (Aiuchi *et al.*, 2004). The behaviour of each fungus is different, and even fungi from the same family can show a preference for different media, albeit one of the media that allows the generation of more mutants is water agar chlorate (WAC) (Korolev & Katan, 1997). A paramount factor to keep in mind is the purity of mutants. It has been reported that cultures of *Veriticillium dahliae* in the presence of chlorate showed suppression of the aerial mycelium. However radial growth was unaffected and the margins of the fungal colony growth mixed or resistant sectors, suggesting that a mixture of nit mutants and wild type strains had occurred (Daayf *et al.*, 1995). To purify nit mutant cultures, several transfers in WAC media is required. In addition, it has been found that the number of purification steps can be reduced by increasing the chlorate concentration (2%-5%) (Korolev & Katan, 1997).

Figure 3.1. Fungal growth on minimal media (MM) supplemented with nitrate. Left: growth of a prototrophic (wild type) strain. Right: growth of an auxotrophic (nit mutant) strain, incapable of using nitrate as a nitrogen source, resulting in non-aerial mycelial growth.

It is still not clear exactly where the mutations occur, nevertheless it is known that the mutations are present in some loci responsible for encoding enzymes involved in transformation of some nitrogen sources like nitrate, nitrite and ammonium (Aiuchi *et al.*, 2008b). To identify mutants, a media with four different nitrogen sources (nitrate, nitrite, hypoxanthine and ammonium) is required (Bayman & Cotty, 1991). Depending on what part of the nitrate reductase activity is affected the mutants can be classified into 3 different groups (**Table 3.1**) (Correll *et al.*, 1987).

1) Nit 1, when the mutation is at the nitrate reductase structural locus and mutants cannot utilize nitrate as nitrogen source, thus these mutants show non-aerial growth on MM supplemented with nitrate;

2) Nit 3, when the mutation is in the nitrate assimilation pathway-specific regulatory locus and mutants cannot utilize nitrite as a nitrogen source, thus these mutants show non-aerial growth on MM supplemented with nitrite;

3) Nit M, when the mutation is in at least 5 loci that affect the assembly of molybdenum cofactor which is necessary for nitrate reductase activity and mutants cannot utilize hypoxanthine as a nitrogen source. Thus, these mutants show non-aerial growth on MM supplemented with hypoxanthine.

Nit 1 and nit 3 do not complement to form a prototrophic strain, whereas nit M is complementary with both mutants becoming the most reliable tester in

vegetative compatibility and also helping to avoid false-negative complementation (Correll *et al.*, 1987). When dense and aerial growth is formed between the contact zone of two auxotrophic mutants, a recombination event has occurred and an heterokaryon has been formed (Leslie, 1993) (**Figure 3.2**).

Table 3.1. Classification of nit mutants based on growth on different nitrogen sources. "+" means a dense aerial growth is formed; "-" means non-aerial growth (Correll *et al.*, 1987).

	Nitrate	Nitrite	Ammonium	Hypoxanthine
Wild type	+	+	+	+
Nit 1	-	+	+	+
Nit 3	-	-	+	+
Nit M	-	+	+	-

Once mutants are identified, a search for self-compatible strains is performed by crossing nit 1 or nit 3 mutants with nit M mutants, from a single strain. The formation of heterokaryon indicates that the strain in question is self-compatible (Campbell *et al.*, 1992). Only self-compatible strains will be used for crossing experiments, to avoid the formation of an self-incompatible heterokaryon (HSI) as these kinds of heterokaryons commonly revert to the wild type (Correll *et al.*, 1989). Self-compatibility can be tested through hyphal or protoplast fusion. The hyphal fusion approach requires a crossing event between auxotrophic mutants on minimal media, with nitrate as nitrogen source in different combinations (Bayman & Cotty, 1991). In the protoplast fusion approach, the auxotrophic mutants are subject to enzymatic treatment to degrade the cell wall and then, under certain conditions of temperature and buffers, protoplasts are centrifuged to prompt a fusion of cells and hence form a hybrid (Zhang *et al.*, 2016). The heterokaryon obtained by protoplast fusion is different from the heterokaryon obtained by hyphal fusion (hyphal anastomosis). If a given heterokaryon is generated by hyphal fusion from two vegetative incompatible strains, once a diploid cell is formed their incompatible nuclei will lead to cell death (Molnar *et al.*, 1990); whereas an heterokaryon generated by protoplast fusion does not show this behaviour and the incompatibility is overcome (Leslie, 1993).

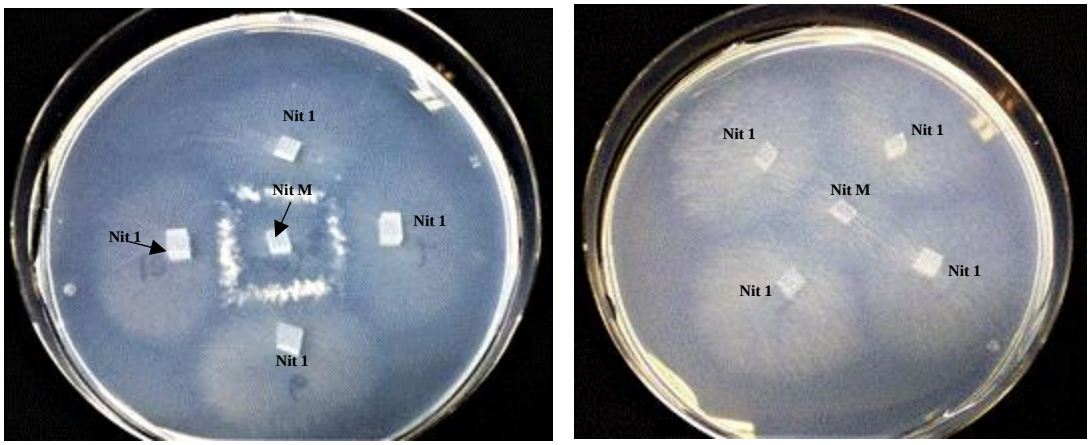


Figure 3.2. Pairing between nit mutants on minimal media with nitrate. Left: Vegetative compatibility among Nit M (centre) and all Nit 1 (sides). Right: Vegetative incompatibility among Nit M (centre) and all Nit 1 (sides).

Parasexual recombination represents a valuable technique for strain improvement in asexual fungi. However, most of the studies have involved plant pathogenic fungi with the main focus to diminish pathogenicity rather than increase the virulence of strains. The genus *Beauveria* has high genetic diversity (St Leger *et al.*, 1992) and there has been several attempts to use recombination techniques to improve strains with valuable traits for biocontrol (e.g. virulence, thermotolerance) through these techniques (Gadau & Lingg, 1992). For instance, there are studies of recombination between strains from the same species (*B. bassiana* x *B. bassiana*) (Castrillo *et al.*, 2004; Kim *et al.*, 2011) and strains from different species (*B. bassiana* x *B. sulfurescens*) (Couteaudier *et al.*, 1996), that have achieved strain improvement on thermal tolerance characteristics and increase in virulence against *O. nubilalis*.

Complementation trials were assessed to determine the different vegetative compatibility groups (VCGs) for all 50 strains used in this study, to provide additional information that could be used in further experiments. In this study, seven strains of the genus *Beauveria* were selected based on their phenotypic characteristics, to perform parasexual recombination assays. With this aim, non-utilizing mutants (nit) mutants were used to identify complementation events (Glass & Kuldau, 1992). Hyphal fusion (hyphal anastomosis) and protoplast fusion were performed in the seven selected strains to find a method to obtain recombinant strains with improved characteristics.

3.2 Materials and Methods

3.2.1 Fungal selection for parasexual recombination:

A total of seven strains were selected to perform strain improvement assays through parasexual recombination (**Table 3.2**). The strains were selected based on an analysis of the results obtained in the phenotypic experiments (see **Section 2.3**), considering their ability to growth at high temperatures (strain 49), tolerance to UV-B radiation (strain 23) and generation of mortality (virulence) against DBM (strain 29). In addition, the position of the strains in the phylogenetic tree (**Figure 2.1**) was also used to select *B. bassiana* strains 40 and 41; which belong to the same place of origin (Kenya) and contain opposite mating types. In strains 29 and 17, was not possible to find mating type genes, besides 29 is from a different species (*B. pseudobassiana*), hence was a good candidate for parasexual recombination. Mating type genes, their amplification and utilization are discussed in **Chapter 4**.

Table 3.2. Strains selected for parasexual recombination.

Strain	Beauveria	Origin	Host	M. Type	Temp	UV-B	Virulence
23	*bassiana*	UK	Diptera	MAT 1	High	High[a]	Medium
29	*Pseudo bassiana*	UK	Lepidoptera	N.F*	Medium	Low	High[b]
32	*bassiana*	UK	Lepidoptera	MAT 2	Medium	Low	Medium
49[c]	*bassiana*	Phillipines	Lepidoptera	MAT 1	High	High	High
11	*bassiana*	USA	Lepidoptera	MAT 1	Medium	Low	Low
42	*bassiana*	Kenya	Lepidoptera	MAT 1	Medium	Medium	Medium
41	*bassiana*	Kenya	Lepidoptera	MAT 2	High	Low	High[d]

*N.F: Not found, a: Tolerance to UV-B potential (>80% germination), b: High virulent (100% mortality), c: high performance in all the evaluation, d: High colony growth at 25 °C and 30 °C. Results in section 2.3

3.2.2 Generation of Nitrate non-utilizing mutants (nit)

Potassium chlorate concentration in media is crucial to generate nit mutants and depending on the organism, this concentration varies. It has been reported a high tolerance to chlorate for *Beauveria*, reaching values as high as 4% to 6% chlorate, in contrast with other fungi (*Lecanicillium, Verticillium*) that use 2% of chlorate to obtain nit mutants (Aiuchi *et al.*, 2008b; Castrillo *et al.*, 2004). To

standardize the percentage of chlorate to be used in the water agar a preliminary experiment to optimise the chlorate concentration of the media was performed with six strains and three chlorate concentration (4-6%). The smallest number of reversions among the mutants were found on 6% of water agar chlorate (WAC) media and this concentration was used in all further experiments.

Conidia suspension from the seven strains selected for parasexual recombination were prepared as described in **section 2.2.1** and adjusted to 10^3 conidia/mL. To generate nit mutants, 50 µL of conidia suspension were spread (L Shaped spreader (Fisher Scientific, UK)) onto 10 plates of Water Chlorate Agar (WAC) 6% per isolate, incubated in darkness at 25 °C and examined after four days of incubation (Castrillo et al., 2004). Colonies were selected and a plug (3 mm^3) was transferred for three additional subcultures onto WAC 6% media (Korolev & Katan, 1997). Sectors of chlorate-resistant colonies were further transferred to a minimal medium (MM) supplemented with nitrate (Aiuchi *et al.*, 2008b) and incubated for 10 days at 25 °C in darkness. Thirty mutants per isolate distinguished by thin expansive colonies with non-aerial mycelia were identified as nit mutants as described by (Puhalla, 1985) and stored at 4°C.

The nit mutants were classified according to the method of (Correll *et al.*, 1987)(**Table 3.1**). Minimal media supplemented with four different nitrogen sources (nitrate, nitrite, ammonium and hypoxanthine) were required to classify the nit mutants because the growth on each media will show what kind of mutation was produced (Aiuchi *et al.*, 2008b). Mycelial plugs of each mutant were taken from 14 days old nit mutants grown on MM with nitrate and placed in the centre of a 9 cm Petri dish containing each of the four media mentioned before. The plates were incubated in darkness at 25 °C. After 10-14 days, colony morphology was evaluated and completed as shown in **Table 3.1**. Ammonium media was the positive control due to mutants showing aerial growth in both nitrate and ammonium media (Correll *et al.*, 1987). Classification for each mutant was generated on two occasions. The preparation of all media used in this experiment are shown in **Appendix 10**.

3.2.3 Vegetative Compatibility and Hyphal anastomosis

Vegetative compatibility was evaluated by complementation tests with the 50 strains of *Beauveria* (**Table 2.1**). For each strain, 10 mutants (one Nit M and nine Nit 1 or Nit3) were tested for self-compatibility by transferring a mycelial plug ($3mm^3$), as described above, from the Nit M mutant culture in the centre of an 9 cm Petri dish containing MM. Four mycelial plugs from Nit 1 or 3 mutants from the same strain were placed 1 cm apart from the centre plug of Nit M (**Figure 3.2**). The MM plates with the plugs were incubated in darkness at 25 °C for 15-20 days. Self-complementation was visible by dense aerial prototrophic growth in the contact zone between the two mutants, while the lack of prototrophic growth indicated vegetative incompatibility and no heterokaryon formation (Couteaudier & Viaud, 1997). VCGs among the different strains of *Beauveria* were determined by the same procedure described above, albeit a Nit M mutant from one strain was paired with a one genetically different Nit 1 or 3 mutants from a different strain. Prototrophic growth was transferred to a new plate of MM to purify the growth and single colonies were picked and grown for molecular analysis using the same four genes used in **Section 2.2.2**.

3.2.4 Protoplast fusion

A total of 8 different combinations of nit mutants were subject to generate recombinant strains through protoplast fusion (**Table 3.3**). For instance, *B. pseudobassiana* wild type 29 was the most virulent strain against DBM. However, its germination was completely restricted under UV-B conditions used in the experiments, thus this strain were crossed with a strain which had a high tolerance to UV-B such as *B. bassiana* strain 49 (**Table 3.3**).

Conidial suspensions were prepared as described in **Section 2.2.1**, and adjusted to 1 x 10^7conidia/mL from Nit M mutants and Nit 1 mutants from each strain. An aliquot (100 µL) was used to inoculate a 250 mL flask containing 100 mL of sterile SD broth. Each flask was incubated in a shaker at 28 °C for 2-7days, at 1000 rpm. Mycelium from each flask was harvested on a sterile milk filter paper (19cm diameter) (Goat Nutrition Ltd, Kent, UK), washed with RO water and 40 mg/mL of

wet mycelium was resuspended in 8 mL of Buffer (1M $MgSO_4$) + Lysing enzymes (0.03 g/mL) (L1412-5G (sigma Aldrich lysing enzymes)) and incubated in a shaker for 5h at 28 °C, 100 cycles/min. After this time, the solution was filtered through sterile milk filter paper (19cm diameter) (Goat Nutrition Ltd, Kent, UK) and centrifuged at 4000 rpm for 10 minutes before being resuspended in 2 mL of Sorbitol solution (1M Sorbitol, 50mM CaCl2, 10nmTris-Hcl 7.4). The solution was centrifuged twice at 4000 rpm for 10 minutes and washed with sorbitol solution after each centrifugation step. The concentration of protoplasts was adjusted to 1 x 10^5 protoplast/mL for each mutant by using an improved Neubauer haemacytometer (Merck, UK). A mixture was made by adding 100 µL of protoplast suspension from the Nit1 and Nit M mutants. Then, the mixture was treated with 1.25 mL of 30% PEG 6000 (50 mM CaCl2, 10 mM tris HCl 7.5) and incubated at 25 °C for 45 minutes. After this time, the solution was centrifuged at 4000 rpm for 5 minutes and resuspended in 2 mL of 1 M sorbitol solution. 50 µL of suspension was added to a 9 cm Petri dish containing MM and incubated until any prototrophic colonies became visible. A plug (3 mm^3) of prototrophic colonies was individually subcultured on MM media supplemented with Sodium nitrate (**Annex 10**) for purification and selection of stable hybrids. After 4 generations, five stable colonies were selected per hybrid to perform the subsequent experiments.

Table 3.3. Combinations of nit mutants for protoplast fusion incubated at 25°C. Parental phenotype shows the characteristics that were combined between the nit mutant parents.

Nit M x Nit 1 or 3	Code	Parental phenotype
49 - 29	S	High tolerance UV-B, high virulence – Highly sensitive to UV-B, high virulence
49 - 32	T	High tolerance UV-B, high virulence – Low tolerance to UV-B, medium virulence
42 - 41	U	Medium sporulation, medium tolerance UV-B – Low tolerance to UV-B, low virulence
32 - 49	V	Low tolerance to UV-B, medium virulence – High tolerance UV-B, high virulence
11 - 49	W	Low tolerance to UV-B, low virulence – High tolerance UV-B, high virulence
42 - 29	X	Medium sporulation, medium tolerance UV-B – highly sensitive to UV-B, high virulence
23 - 29	Y	High tolerance UV-B, medium virulence – highly sensitive to UV-B, high virulence
32 - 11	Z	Low tolerance to UV-B, medium virulence – Low tolerance to UV-B, low virulence

3.2.5 Characterization of recombinant strains

Five prototrophic single spore colonies from three different combinations (S (49 x 29), U (42 x 41) and X (42 x 29)) were selected to perform phenotype characterization, according to the same methodology described in **Section 2.2**. Hybrid strains were examined for effects of temperature on fungal growth, UV radiation on conidial germination and virulence to DBM. Colony morphology was assessed to find differences between hybrids and wild type parents by molecular analysis. For the molecular analysis of the hybrids DNA extraction of each hybrid was performed. Then, extracted DNA was used as a template for PCR reactions. The methodology for DNA extraction, PCR reactions and four molecular markers for phylogenetic analysis described in **Section 2.2.2** was used. Elongation factor 1α, DNA lyase and Internal Transcribed Spacer (ITS), were detailed primers can be seen in **Table 2.2**. Alignments and generation of phylogenetic trees were obtained by CLC Workbench software (Qiagen, https://www.qiagenbioinformatics.com/).

3.3 Results

3.3.1 Generation of Nitrate non-utilizing mutants (nit)

After five days, the number of resistant colonies was not significantly affected by the concentration of chlorate regardless of fungal strains confirming that *Beauveria* has a relatively high tolerance to this compound (**Figure 3.3**). However, there was a difference in the stability of the mutants generated among the three tested concentrations of chlorate with less colony reversion to the wild type observed for mutants grown on WAC at 6 % chlorate concentration. Therefore, this concentration was selected for the generation of mutants.

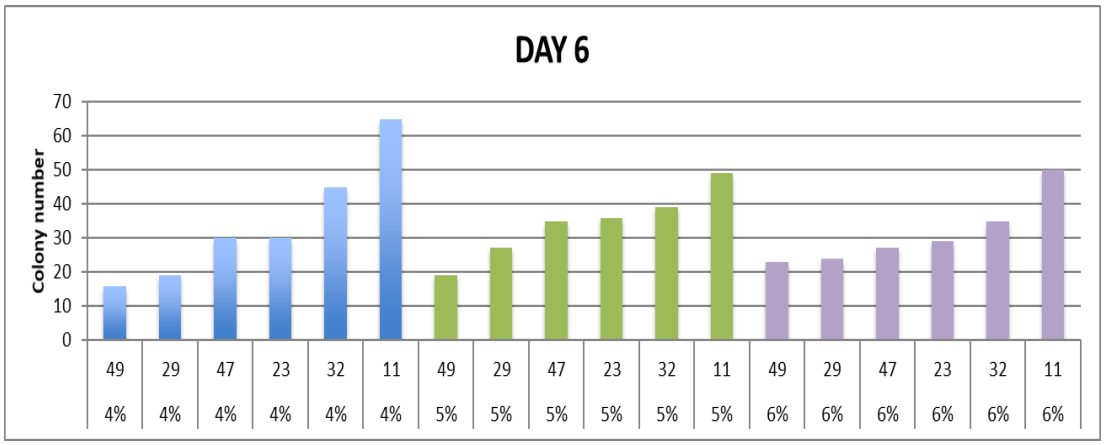


Figure 3.3. Growth of chlorate resistant colonies from six different strains of *Beauveria*, on 3 different concentrations of chlorate media (4%, 5% and 6%).

Colonies were first observed after four to seven days depending on the fungal strain. Thirty resistant colonies for each strain (1500 in total) were picked and transferred twice to fresh WAC 6% plates to recover pure mutants. Growth of the 1500 colonies on MM with sodium nitrate confirmed the generation of 664 nit mutants (non-aerial growth) (**Table 3.4**). Four *B. bassiana* strains (43, 12, 8, 46) did not generate any nit mutants, despite generating colonies resistant to chlorate. It is possible that these strains required a different chlorate concentration than the ones tested here, for instance, other fungal species such as *Aspergillus* or *Lecanicillum* produce nit mutant at 2% of chlorate concentration. Phenotypic characterization confirmed that of 664 strains generated 55.72%, were nit 1 mutants (unable to use nitrate as nitrogen source), 5.57% were nit 3 mutants (unable to use nitrite as

nitrogen source) and 13.25% were nit M mutants (unable to use hypoxanthine as a nitrogen source) (**Figure 3.4**). Of the 46 that produced nit mutants, only 11 strains generated “nit 3” mutants and 31 strains generated “nit M” mutants, but all of the 46 strains generated “nit 1” mutants, hence this was the prevalent type of nit mutant among most of the strains. Only strains 28 and 33 produced more “nit 3” mutants (77.78 %) or “nit M” mutants (90.91 %) respectively, than “nit 1” mutants (**Table 3.4**).

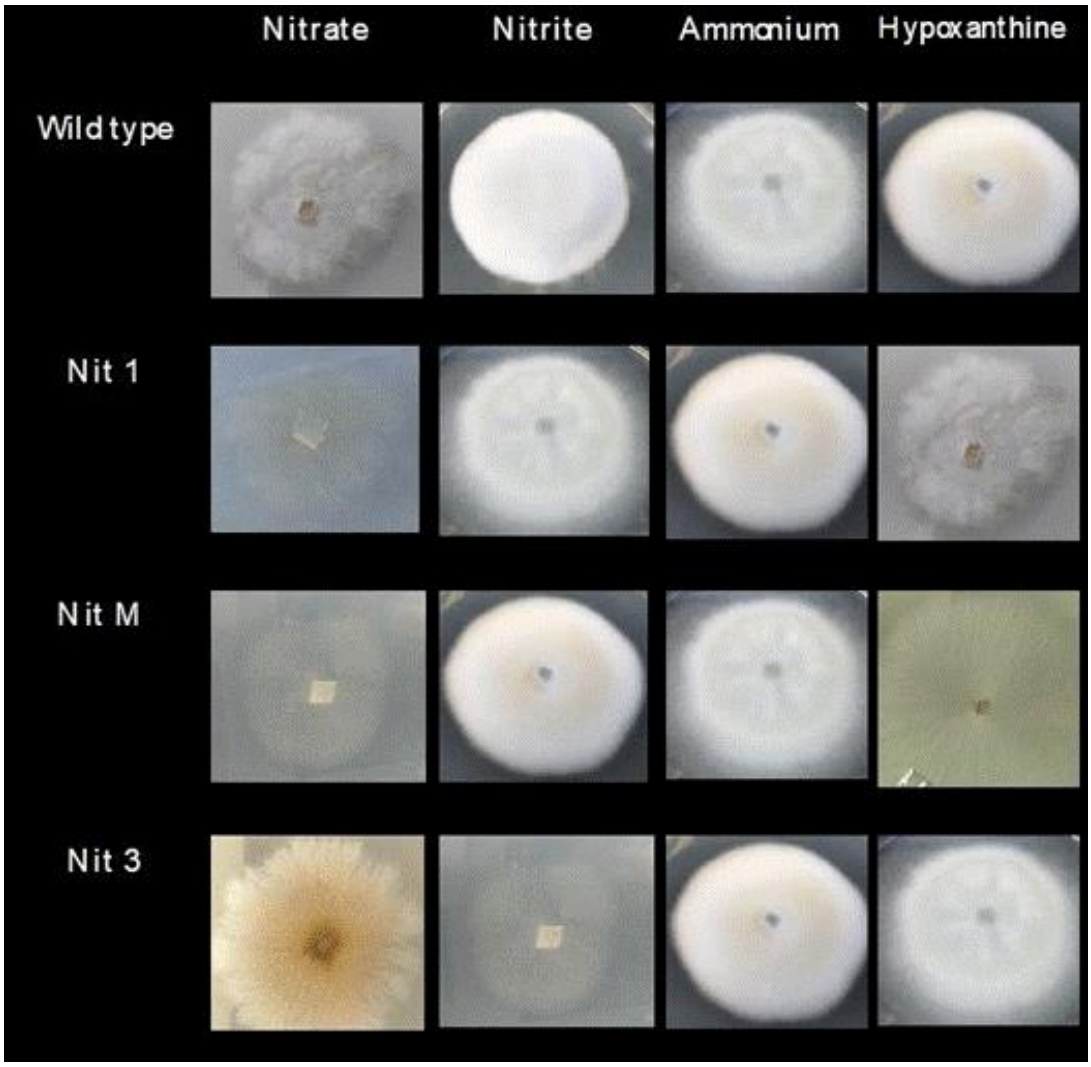


Figure 3.4. Phenotypic characterization of nit mutants from *B. bassiana* and *pseudobassiana* strains by growth on MM supplemented with four different nitrogen sources.

Table 3.4. Frequency of nit mutants and phenotypes ("nit-type mutants") obtained from strains of *B. bassiana* and *B. pseudobassiana* on Water agar chlorate (WAC) and minimal media (MM) supplemented with different nitrogen sources. Highlighted in green the seven strains used for parasexual recombination (continues on next page).

Species	Strain	Resistant colonies	% Nit mutants	% Nit1	% Nit3	% NitM
B. bassiana	**5**	30	73	14	0	0
	48	30	27	63	25	0
	6	30	30	44	0	11
	20	30	20	83	0	17
	17	30	53	25	13	13
	27	30	37	73	18	9
	21	30	30	67	11	11
	3	30	80	33	0	0
	45	30	83	32	0	0
	47	30	27	25	13	0
	4	30	53	19	0	0
	38	30	73	45	9	14
	41	**30**	**40**	**33**	**0**	**25**
	37	30	63	32	11	0
	35	30	47	14	0	0
	7	30	40	58	0	8
	1	30	63	63	0	26
	42	**30**	**30**	**67**	**0**	**11**
	30	30	23	57	0	0
	24	30	50	67	0	13
	32	**30**	**23**	**71**	**0**	**0**
	23	**30**	**30**	**89**	**0**	**0**
	11	**30**	**30**	**78**	**0**	**22**
	31	30	33	80	0	20
	49	**30**	**57**	**82**	**0**	**12**
	34	30	70	48	38	10
	36	30	50	73	0	20
	50	30	43	77	8	15
	40	30	50	87	0	13
	28	30	60	6	78	17
	14	30	60	72	0	28
	22	30	30	89	0	11
	16	30	63	58	0	42
	33	30	73	9	0	91

Table 3.4. Continued. Frequency of nit mutants and phenotypes ("nit-type mutants") obtained from strains of *B. bassiana* and *B. pseudobassiana* on Water agar chlorate (WAC) and minimal media (MM) supplemented with different nitrogen sources. Highlighted in green the seven strains used for parasexual recombination.

Species	Strain	Resistant colonies	% Nit mutants	% Nit1	% Nit3	% NitM
B. bassiana	**44**	30	60	83	0	17
	15	30	50	80	0	20
	20	30	67	90	10	0
	18	30	50	93	0	0
	43	30	0	0	0	0
	12	30	0	0	0	0
	8	30	0	0	0	0
	46	30	0	0	0	0
B. Pseudobassiana	**29**	**30**	**40**	**92**	**0**	**0**
	26	30	30	78	0	22
	910-05	30	27	88	0	13
	2	30	30	67	0	33
	44	30	43	85	0	8
	10	30	47	93	0	7
	9	30	70	76	0	10
	19	30	60	22	0	0
	TOTAL	**1380**	**47**	**56**	**6**	**13**

3.3.2 Vegetative Compatibility and Hyphal anastomosis

Of the 46 strains that generated nit mutants, 28 strains produced self-compatible mutants. Three *B. bassiana* strains (22, 40 and 33) were self-incompatibles, therefore their "nit 1" mutants were discarded from further experiments. However, their "nit M" mutants were used for compatibility experiments with other strains. Additionally, *B. bassiana* strains: 20, 18, 5, 48, 3, 45, 47, 4, 37, 35, 30, 32, 23 and *B. pseudobassiana* strains 29, 19 did not generate any "nit M" mutants and self-compatibility could not be tested for these strains (**Table 3.4**).

Since the remaining 28 strains were self-compatibles, their corresponding "nit 1", "nit 3" and "nit M" were crossed in all possible combinations to test compatibility among the other mutants (**Table 3.5**).

Table 3.5. Nit mutants used for the crosses in all possible combinations (Nit M x Nit 1; Nit M x Nit 3) to determine VCG's among the 46 strains of *Beauveria*.

Nit M	Nit 1	Nit 3
50 Nit M	9 Nit 1	38 Nit 3
33 Nit M	32 Nit 1	28 Nit 3
44 Nit M	50 Nit 1	34 Nit 3
34 Nit M	44 Nit 1	
44 Nit M	44 Nit 1	
2 Nit M	10 Nit 1	
9 Nit M	45 Nit 1	
10 Nit M	18 Nit 1	
11 Nit M	48 Nit 1	
14 Nit M	20 Nit 1	
15 Nit M	14 Nit 1	
16 Nit M	15 Nit 1	
17 Nit M	16 Nit 1	
49 Nit M	17 Nit 1	
21 Nit M	49 Nit 1	
22 Nit M	4 Nit 1	
6 Nit M	5 Nit 1	
1 Nit M	1 Nit 1	
36 Nit M	36 Nit 1	
20 Nit M	37 Nit 1	
7 Nit M	23 Nit 1	
40 Nit M	7 Nit 1	
41 Nit M	41 Nit 1	
42 Nit M	42 Nit 1	
38 Nit M	24 Nit 1	
24 Nit M	910-05 Nit 1	
910-05 Nit M	26 Nit 1	
26 Nit M	27 Nit 1	
27 Nit M	29 Nit 1	
28 Nit M	30 Nit 1	
31 Nit M	31 Nit 1	

After two to three weeks of incubation, depending on the strain, anastomosis (hyphal fusion) formation was visualized through prototrophic growth among the complementary mutants. The 46 strains used in these pairings resulted in 19 vegetatively compatible strains, which were identified through dense prototrophic growth between the hyphae; whereas 27 strains were vegetatively incompatible and no anastomosis in the contact zone were found amongst these strains (**Figure 3.5**). In summary, 35 vegetative compatibility groups (VCG's) were identified, the first group is composed of strains 17, 49, 21, 42, 24, 27, 29, 30, 31, 32, 11, and 23; the second group is composed by strains 45 and 28; the third group is composed by strains 47, 36 and 44; the fourth group is composed by strains 6 and 41; while the remaining 28 groups are comprised by individual strains which were not compatible with any other strain (**Table 3.6**). No correlation was found between country of origin and insect host within the VCG's. A wide range of geographical regions within the individuals VCG's was observed (**Figure 3.6**). For instance, in VCG 1, which is the largest group, there were strains from Kenya, UK, Colombia, Philippines and the USA.

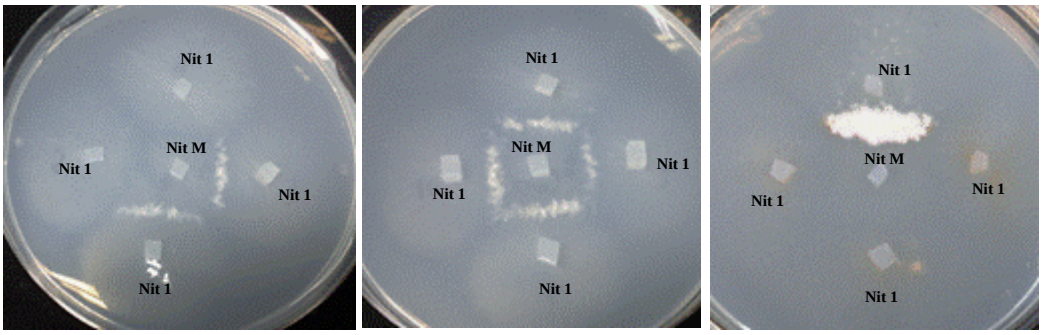


Figure 3.5 Growth amongst the different combinations of mutants to determine VCG's. Prototrophic growth means the same VCG's, no prototrophic growth means different VCG's.

Table 3.6. Vegetative compatibility groups amongst 50 strains of *B. bassiana* and *B. pseudobassiana* (Continues the next page).

Strain	VCG	Origin	Host	*Beauveria* specie
42	1	Kenya	Lepidoptera	*B. bassiana*
21	1	UK	Lepidoptera	*B. bassiana*
30	1	UK	Lepidoptera	*B. bassiana*
31	1	UK	Lepidoptera	*B. bassiana*
24	1	UK	Lepidoptera	*B. bassiana*
27	1	UK	Lepidoptera	*B. bassiana*
32	1	UK	Lepidoptera	*B. bassiana*
17	1	Colombia	Coleoptera	*B. bassiana*
49	1	Phillipines	Lepidoptera	*B. bassiana*
11	1	USA	Coleoptera	*B. bassiana*
23	1	UK	Diptera	*B. bassiana*
29	1	UK	Lepidoptera	*B. pseudobassiana*
44	2	Beijin. China	Diptera	*B. bassiana*
36	2	France	Diptera	*B. bassiana*
47	2	Vietnam	Coleoptera	*B. bassiana*
41	3	Kenya	Lepidoptera	*B. bassiana*
6	3	USA. New York	Diptera	*B. bassiana*
45	4	Hunan. China (1985)	Hemipteran	*B. bassiana*
28	4	UK	Lepidoptera	*B. bassiana*
20	5	Parma, Idaho.	Homoptera	*B. bassiana*
1	6	Canada	Diptera	*B. bassiana*
14	7	USA, Idaho.	Homoptera	*B. bassiana*
15	8	USA, Idaho.	Homoptera	*B. bassiana*
16	9	USA, Idaho.	Homoptera	*B. bassiana*
12	10	USA, Idaho.	Homoptera	*B. bassiana*
50	11	Australia	Lepidoptera	*B. bassiana*
4	12	USA	Lepidoptera	*B. bassiana*
40	13	Kenya	Lepidoptera	*B. bassiana*
48	14	Thailand	Lepidoptera	*B. bassiana*
3	15	USA	Lepidoptera	*B. bassiana*
35	16	France	Diptera	*B. bassiana*

Table 3.6. Continued. Vegetative compatibility groups among 50 strains of *B. bassiana* and *B. pseudobassiana*.

Strain	VCG	Origin	Host	*Beauveria* specie
38	17	Bologna	Lepidoptera	*B. bassiana*
34	18	USA	Lepidoptera	*B. bassiana*
37	19	Denmark	Diptera	*B. bassiana*
20	20	Brazil, Alagoas	Diptera	*B. bassiana*
18	21	CNPAF. Brazil (1982)	Lepidoptera	*B. bassiana*
5	22	USA. Florida	Hymenoptera	*B. bassiana*
7	23	New York	Diptera	*B. bassiana*
22	24	UK	Lepidoptera	*B. bassiana*
8	32	USA. Tennessee	Coleoptera	*B. bassiana*
46	33	China	Hemipteran	*B. bassiana*
43	34	China	Hemipteran	*B. bassiana*
33	35	UK	Lepidoptera	*B. bassiana*
2	25	Canada	Lepidoptera	*B. pseudobassiana*
26	26	UK	Lepidoptera	*B. pseudobassiana*
25	27	UK	Lepidoptera	*B. pseudobassiana*
9	28	USA	Lepidoptera	*B. pseudobassiana*
44	29	Turkey	Lepidoptera	*B. pseudobassiana*
10	30	USA	Lepidoptera	*B. pseudobassiana*
19	31	Goiana. Brazil (1982)	Coleoptera	*B. pseudobassiana*

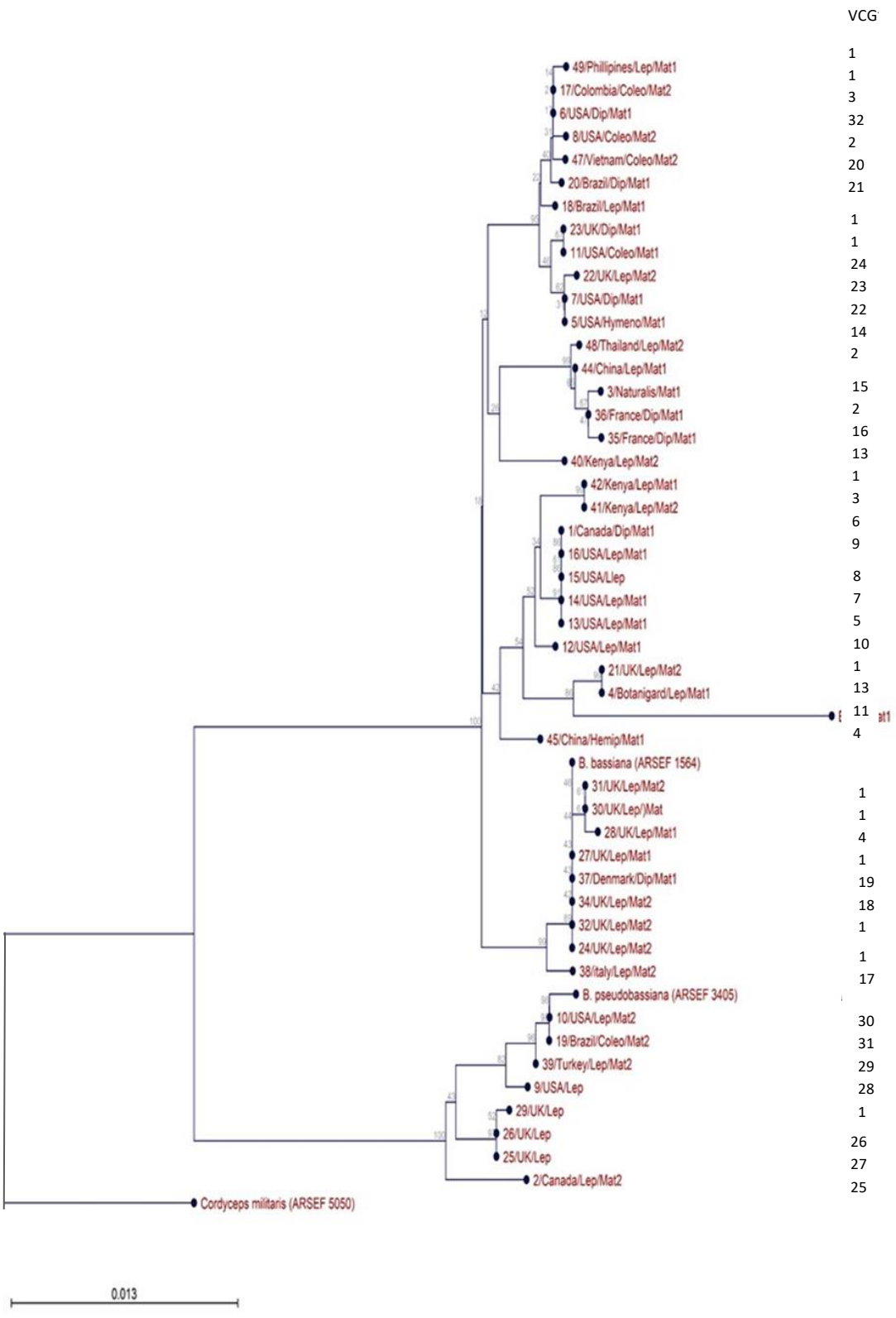


Figure 3.6. Phylogenetic tree generated in CLC Workbench (Qiagen, https://www.qiagenbioinformatics.com/) with the 50 strains of Beauveria. VCG's indicated in the righthand column for each isolate.

3.3.3 Protoplast fusion

Since no “nit M” mutants were generated in three (*B. bassiana* strains: 32, 23 and *B. pseudobassiana* 29) out of seven of the strains selected for parasexual recombination through anastomosis (hyphae fusion), a protoplast fusion approach was performed to avoid incompatible heterokaryons (**Figure 3.7**, **A, B, C and D**).

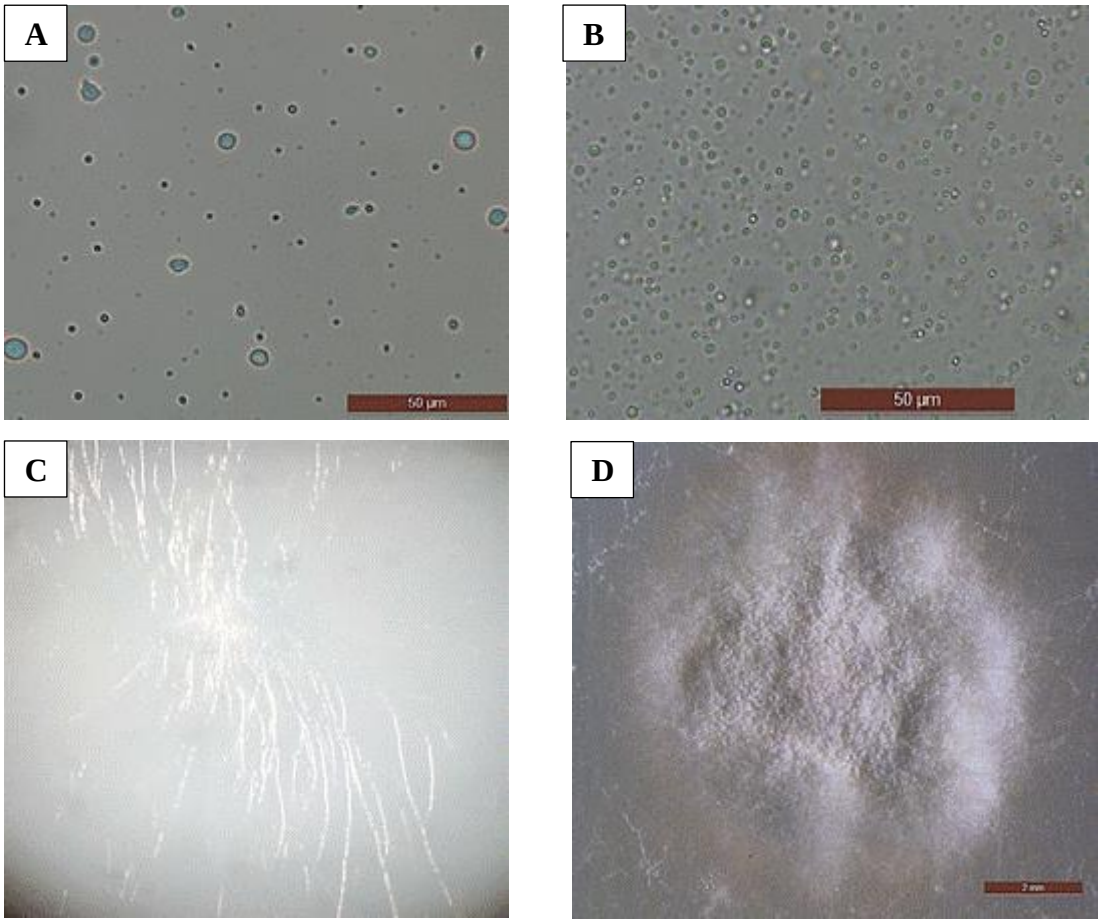


Figure 3.7. (A) Protoplasts (40 x 0.8 magnification), before fusion. (B) Fused protoplasts (40 x 0.8 magnification). (C) Auxotrophic colony (nit mutant colony) on MM with nitrate. (D) Prototrophic colony (presumable recombinant) on MM with nitrate.

All combinations tested showed prototrophic colonies. However, only three combinations (named as S (49 (*B. bassiana*) x 29 (*B. pseudobassiana*), U (42 (*B. bassiana*) x 41 (*B. bassiana*)and X (42 (*B. bassiana*) x 29 (*B. pseudobassiana*)) (**Table 3.3**) produced consistently high number of stable prototrophic colonies and these were selected for characterization by phylogenetic and genetic experiments, to confirm the occurrence of a recombination event. All wild types parents produced

white colonies without sectors which generally were not clearly distinguishable from their hybrids. Nevertheless, a few hybrids from the three combinations differed from their corresponding parent's morphology, showed halos around the colony and dense or thin mycelium (**Figure 3.8**).

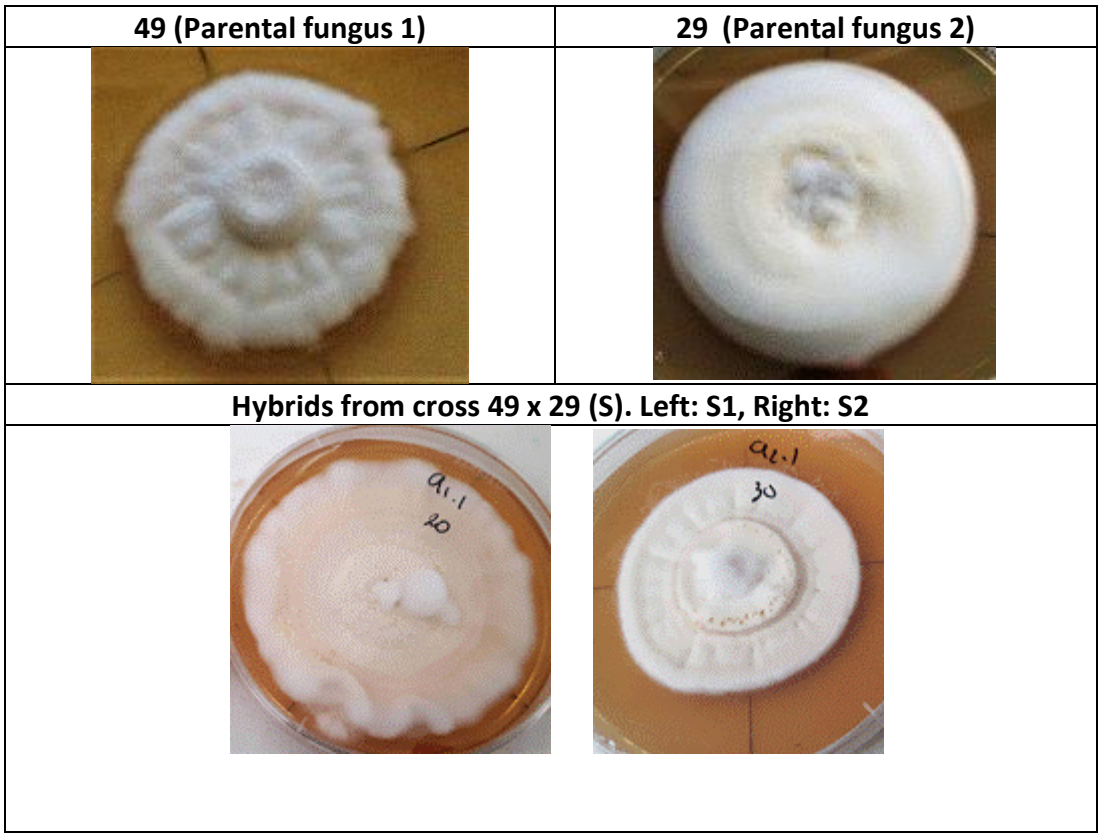
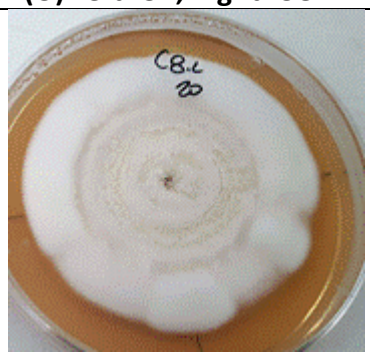


Figure 3.8. Comparison in morphology between parent wild types strains from the three combinations S (49 x 29), U (42 x 41) and X (42 x 29) and hybrid strains after protoplast fusion (Continues on the next page).

42 (Parental fungus 1)	41 (Parental fungus 2)
	
Hybrids from cross 42 x 41 (U) Left: U4, Right: U8	
	

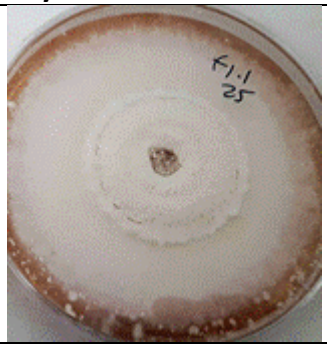
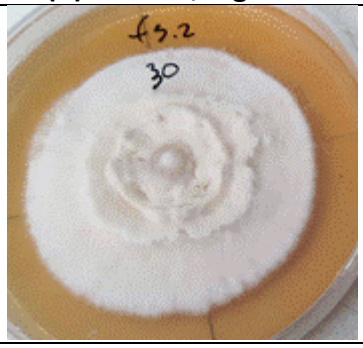
42 (Parental fungus 1)	29 (Parental fungus 2)
	
Hybrids from cross 42 x 29 (X) Left: X1, Right: X3	
	

Figure 3.8. Continued. Comparison in morphology between parent wild types strains from the three combinations S (49 x 29), U (42 x 41) and X (42 x 29) and hybrid strains after protoplast fusion.

3.3.4 Characterization of recombinant strains

Comparison of conidial production between hybrid strains and wild type parents

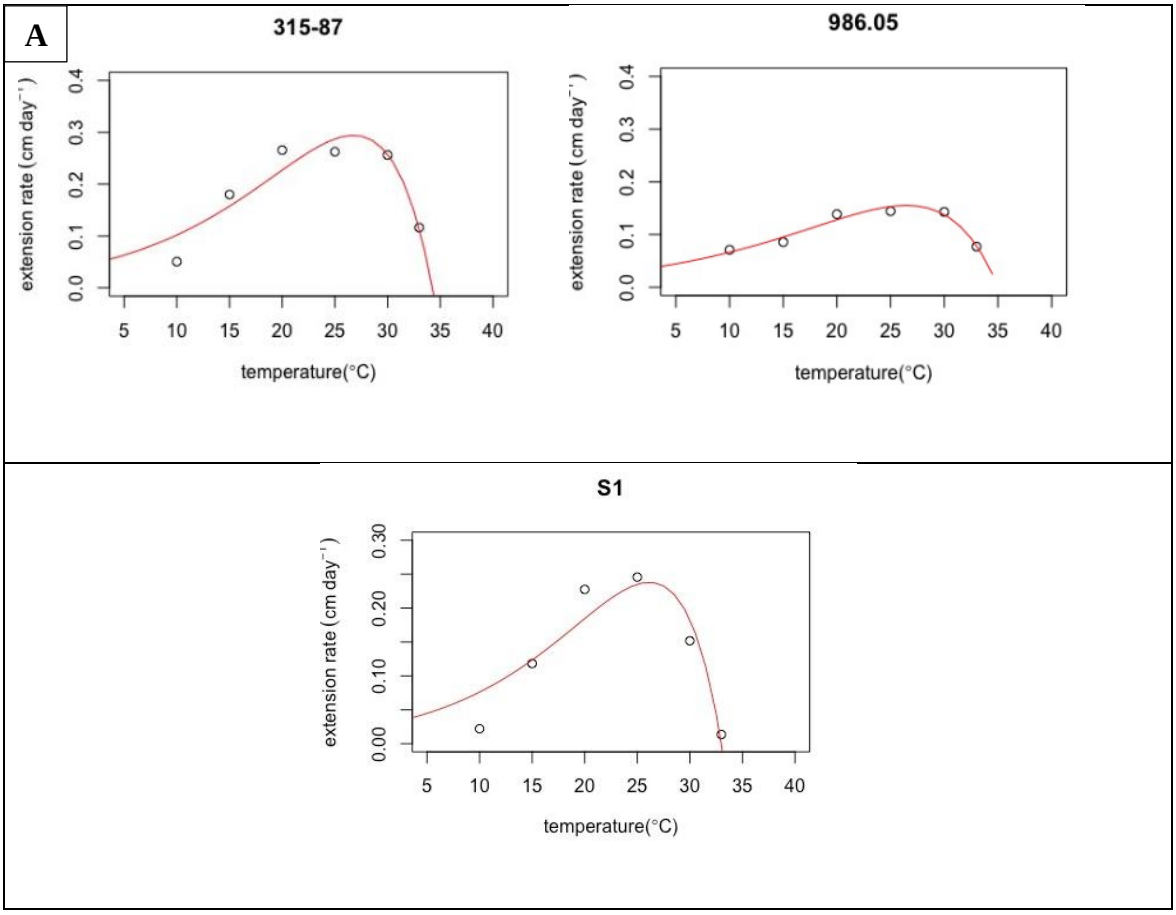
Data obtained in this experiment was transformed to log 10 before statistical analysis. Mean conidial concentration of each strain of the fungal collection, was evaluated in a one-way analysis of variance (ANOVA, $p > 0.05$). Conidial yield of 15 hybrids was not significantly different compared with their corresponding parental wild types strains (**Table 3.7**). Of the three combinations examined, only strains in combination "S" (49 x 29 produced more spores after 14 days incubation at 22 °C, and hybrids S1 (9.5 x 10^8 conidia/mL), S5 (9.35 x 10^8 conidia/mL) were the only hybrids that slightly surpassed the conidial production of their wild type parents (8.96 x 10^8 conidia/mL and 4.65 x 10^8 conidia/mL) (**Table 3.7**). In contrast, hybrids from combination "U" (42 x 41) generally showed the lowest conidia production (<2 x 10^8) and their performance was similar to one of the parental strains (1.79 x 10^8 conidia/mL) (**Table 3.7**). Hybrids from combination "X" (42 x 29) gave the highest variability in conidial yield between the five hybrids selected and were generally lower (between 1.20 x 10^8 conidia/mL and 5.6 x 10^8 conidia/mL) than their corresponding wild type parents (**Table 3.7**).

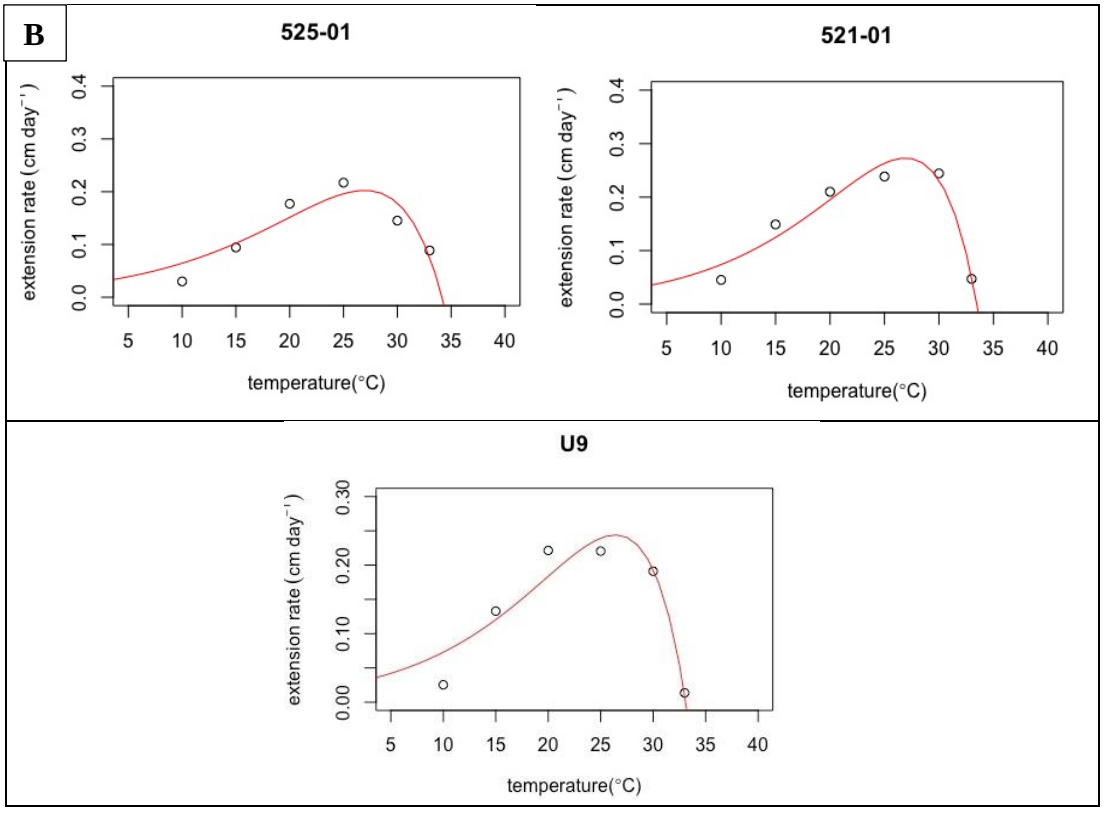
Table 3.7 Conidial production (log 10/mL) of different hybrid strains and their wild type parents after 14 days incubation at 25°C. Combination S (49 x 29), combination U (42 x 41) and; combination X (42 x 29).

Hybrid	Sporulation	Parental strains	
	(Log 10)	315-87	986-05
S1	8,98	8,95	8,67
S2	8,86	8,95	8,67
S5	8,97	8,95	8,67
S6	8,91	8,95	8,67
S8	8,87	8,95	8,67
		525-01	521-01
U4	8,21	8,91	8,25
U6	8,16	8,91	8,25
U7	8,18	8,91	8,25
U8	8,26	8,91	8,25
U9	8,24	8,91	8,25
		525-01	986-05
X1	8,35	8,91	8,67
X2	8,75	8,91	8,67
X3	8,08	8,91	8,67
X8	8,19	8,91	8,67
X9	8,54	8,91	8,67

Effect of temperature on colony extension between hybrid strains and wild type parents

Radial growth in the hybrid strains at different temperatures followed the same growth profile as seen in the wild type parents (see **Appendix 11**). However, in all cases the response at each temperature varied from their parental wild type, increasing or decreasing their tolerance (**Table 3.8**). The hybrids generally exhibited greater tolerance than the lower tolerant parent, peak growth still fell between 20 to 30°C and very little growth was observed at 33 °C. Only combination "X" (42 (*B. bassiana*) x 29 (*B. pseudobassiana*)) showed an improvement in their growth at certain temperatures except for hybrid "X9" which had a similar performance than parental wild type strains when compared with their parental wild type (**Figure 3.9, C**). As an illustration, colony growth of three hybrids (S1, U9 and X2) from the three combinations are shown in **Figure 3.9**, the remaining curves are shown in **Appendix 13** and **Table 3.9**.


A
315-87
986.05
S1
extension rate (cm day⁻¹)
temperature(°C)


B
525-01
521-01
U9
extension rate (cm day⁻¹)
temperature(°C)

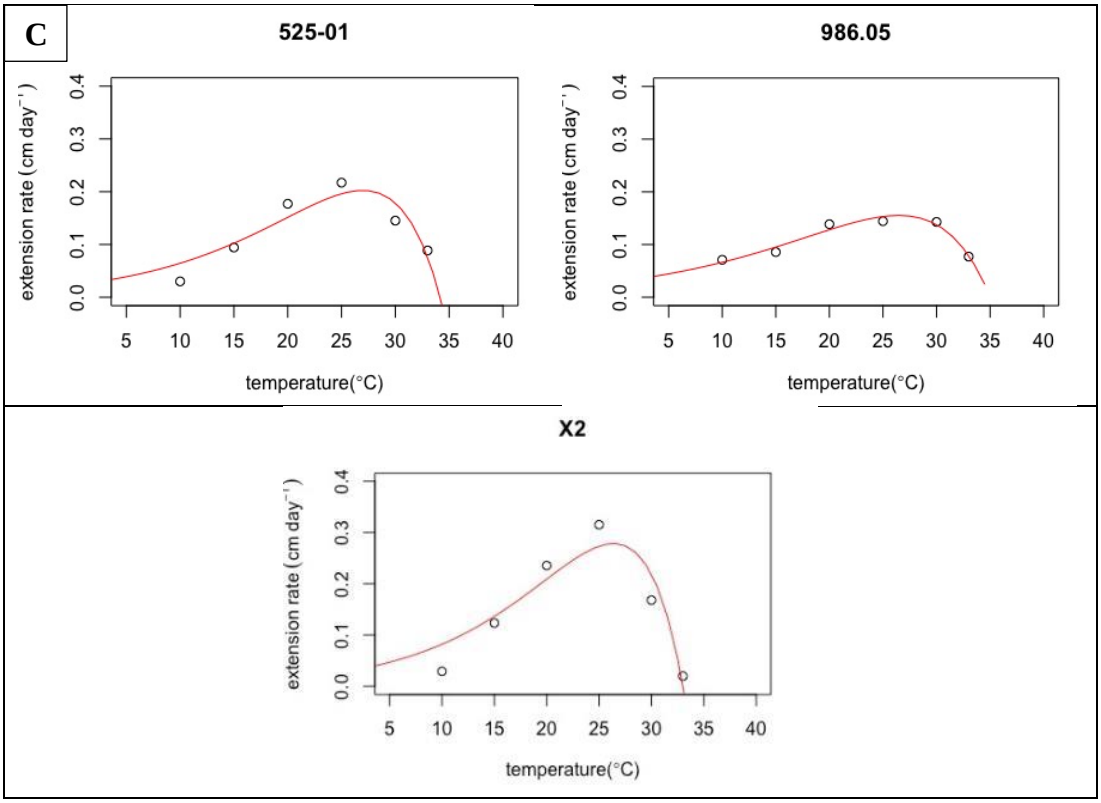


Figure 3.9. Lactin-1 models for comparing colony growth profile between three hybrid strains at six different temperatures and their corresponding wild type parental strains(A) Combination S (49 x 29). (B) Combination U (42 x 41) and; (C) combination X (42 x 29).

Table 3.8. Mycelial growth rates of 15 *Beauveria* hybrids and their respective parental strains, after four weeks incubation at six different temperatures. Combinations: S (49 x 29), U (42 x 41) and X (42 x 29) (Continues on next page).

Hybrid	Temperature (°C)	Rate (cm/day)	**49**	**29**
S1	10	0.02	0.05	0.07
	15	0.12	0.18	0.09
	20	0.23	0.27	0.14
	25	0.24	0.26	0.14
	30	0.15	0.26	0.14
	33	0.01	0.12	0.08
S2	10	0.02	0.05	0.071
	15	0.11	0.18	0.09
	20	0.21	0.27	0.14
	25	0.1914	0.2624	0.1443
	30	0.1507	0.2562	0.1429
	33	0.0182	0.1162	0.0771
S5	10	0.0232	0.0505	0.071
	15	0.1207	0.18	0.0857
	20	0.2146	0.2657	0.1386
	25	0.2536	0.2624	0.1443
	30	0.1639	0.2562	0.1429
	33	0.0132	0.1162	0.0771
S6	10	0.0232	0.0505	0.071
	15	0.1368	0.18	0.0857
	20	0.2104	0.2657	0.1386
	25	0.2254	0.2624	0.1443
	30	0.1486	0.2562	0.1429
	33	0.0193	0.1162	0.0771
S8	10	0.0236	0.0505	0.071
	15	0.1304	0.18	0.0857
	20	0.2014	0.2657	0.1386
	25	0.26	0.2624	0.1443
	30	0.1421	0.2562	0.1429
	33	0.0164	0.1162	0.0771

Table 3.8. Continued. Mycelial growth rates of 15 hybrids and their respective parental strains. Combinations: S (49 x 29), U (42 x 41) and X (42 x 29).

Hybrid	Temperature (°C)	Rate (cm/day)	**42**	**41**
U4	10	0.0221	0.03	0.0452
	15	0.1196	0.0943	0.149
	20	0.2214	0.1771	0.21
	25	0.2286	0.2171	0.2386
	30	0.1911	0.1452	0.2443
	33	0.0125	0.0886	0.0471
U6	10	0.0271	0.03	0.0452
	15	0.1314	0.0943	0.149
	20	0.2325	0.1771	0.21
	25	0.2125	0.2171	0.2386
	30	0.1754	0.1452	0.2443
	33	0.0139	0.0886	0.0471
U7	10	0.0236	0.03	0.0452
	15	0.1211	0.0943	0.149
	20	0.23	0.1771	0.21
	25	0.2221	0.2171	0.2386
	30	0.1757	0.1452	0.2443
	33	0.0171	0.0886	0.0471
U8	10	0.0257	0.03	0.0452
	15	0.1232	0.0943	0.149
	20	0.2246	0.1771	0.21
	25	0.2271	0.2171	0.2386
	30	0.2	0.1452	0.2443
	33	0.0168	0.0886	0.0471
U9	10	0.0254	0.03	0.0452
	15	0.1329	0.0943	0.149
	20	0.2214	0.1771	0.21
	25	0.2204	0.2171	0.2386
	30	0.1911	0.1452	0.2443
	33	0.0136	0.0886	0.0471

Table 3.8. Continued. Mycelial growth rates of 15 hybrids and their respective parental strains. Combinations: S (49 x 29), U (42 x 41) and X (42 x 29).

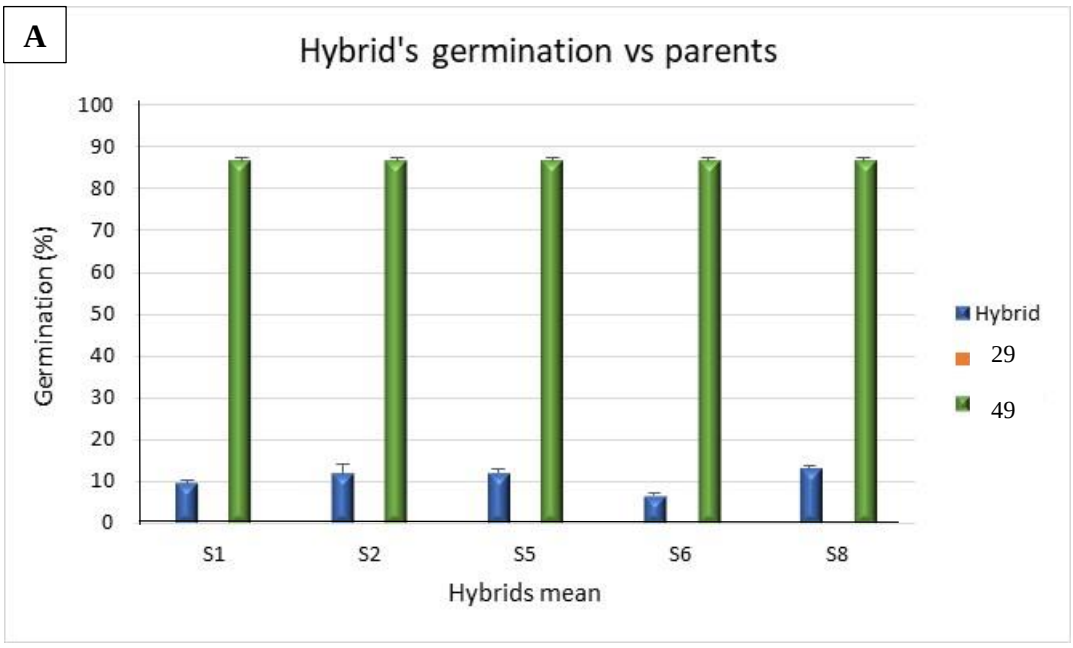
Hybrid	Temperature (°C)	Rate (cm/day)	**42**	**29**
X1	10	0.0289	0.03	0.071
	15	0.1386	0.0943	0.0857
	20	0.2261	0.1771	0.1386
	25	0.2611	0.2171	0.1443
	30	0.1871	0.1452	0.1429
	33	0.0214	0.0886	0.0771
X2	10	0.0293	0.03	0.071
	15	0.1236	0.0943	0.0857
	20	0.2357	0.1771	0.1386
	25	0.3154	0.2171	0.1443
	30	0.1682	0.1452	0.1429
	33	0.02	0.0886	0.0771
X3	10	0.0275	0.03	0.071
	15	0.1432	0.0943	0.0857
	20	0.2171	0.1771	0.1386
	25	0.2571	0.2171	0.1443
	30	0.1818	0.1452	0.1429
	33	0.0168	0.0886	0.0771
X8	10	0.0268	0.03	0.071
	15	0.125	0.0943	0.0857
	20	0.2071	0.1771	0.1386
	25	0.2896	0.2171	0.1443
	30	0.1646	0.1452	0.1429
	33	0.0246	0.0886	0.0771
X9	10	0.0379	0.03	0.071
	15	0.1418	0.0943	0.0857
	20	0.2264	0.1771	0.1386
	25	0.2161	0.2171	0.1443
	30	0.1611	0.1452	0.1429
	33	0.0196	0.0886	0.0771

Lactin-1 model were fitted for the relationship between temperature and colony extension rates for the 15 hybrids (see **Appendix 12** and **Appendix 13**). Thermal optima were relatively similar between hybrids and wild type parents. However, for the maximum temperatures there was a slight increase in hybrid strains for all combinations when compared with the parental wild types. Hybrid Topt

ranged from 25.78 °C to 26.62 °C and 26.5 °C to 26.98 °C for parental strains. For Tmax, the values were between 33.01 °C and 33. 16 °C, whereas for parental strains was around 32.94 °C. This model displayed r^2 values of between 0.86 and 0.92 among the 15 hybrid strains. AIC values were between -14.5 and -19.2. (see **Appendix 12**).

Effect of UV-B radiation between hybrid strains and wild type parents

The germination of 15 hybrid strains were assessed by UV-B tolerance following the same methodology used in **Section 2.2**. All 15 hybrid strains were highly susceptible to UV-B radiation with no hybrid exhibiting more than 45% germination after 90 minutes at 5.94 kJ/m UV-B radiation (**Figure 3.10**). Hybrids from the combination "S (49 x 29)" were the most susceptible to UV-B radiation with no more than 15% germination (**Figure 3.10, A**). Strains from combination "U (42 x 41)" did not show a better tolerance compared with wild types, reaching a germination percentage from 20% to 30% (**Figure 3.10, B**). Of all the hybrids listed in combination "X (42 x 29)" only hybrid "X1" exceeded the parental tolerance to UV-B radiation, reaching a germination > 40%, nevertheless all hybrids had more UV-B tolerance than the parent 29 strain (**Figure 3.10, C**).



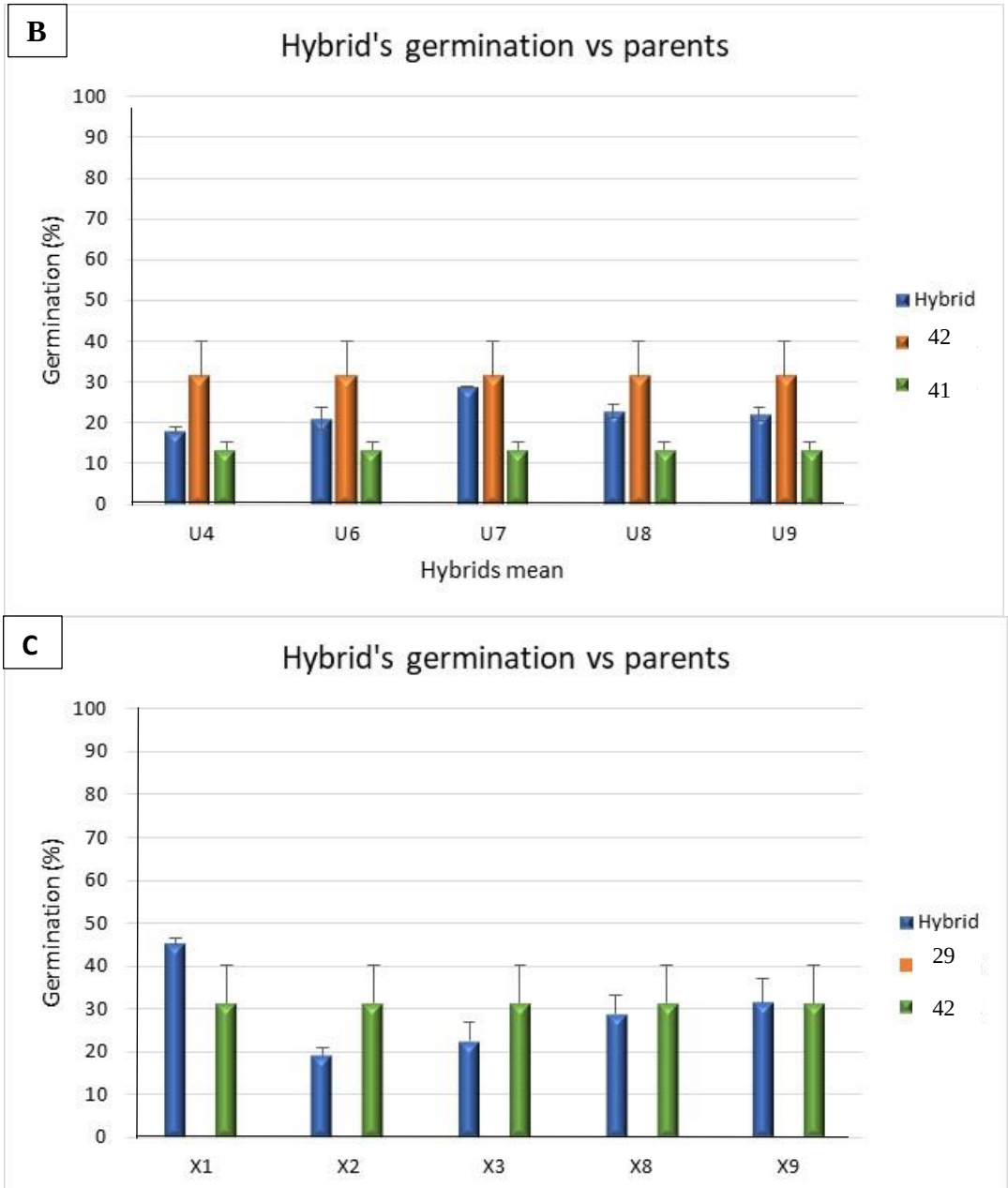


Figure 3.10. Percentage germination of different hybrid strains and their wild type parents, after exposure to UV-B radiation. (A) Combination S (49 x 29). (B) Combination U (42 x 41) and; (C) Combination X (42 x 29). Strain 29 is not visible in graphs (A) and (C) due to germination was completed restricted after UV-B radiation.

Virulence of hybrid strains *against Plutella xylostella* (DBM)

Pathogenic activity was evaluated by bioassays against lepidopteran pest DBM under the conditions previously described in **Section 2.2.6**. Virulence to 2nd instar DBM larvae of the wild type parents displayed mortality values of 75.9% (42 (*B. bassiana*)), 88% (49 (*B. bassiana*) and 100% (41 (*B. bassiana*) and 29 (*B. pseudobassiana*). Only four hybrid strains, S1 (49 x 29), U8 (42 x 41), X1 (42 x 29), X2,

were as virulent to DBM as their parental strains with 100% of mortality (**Figure 3.11**). There was no evidence of a reduction in feeding in any of the hybrids, regardless of wild type parent 29 strain, which reduced larvae feeding in day 2 previously observed in virulence assays (**Section 2.3.6**). Despite, there was no evidence of feeding reduction, three hybrids (S1, X1 and X2) exhibited similar high speed of kill, as the parental strain 29, with all of the larvae having died four days post inoculation.

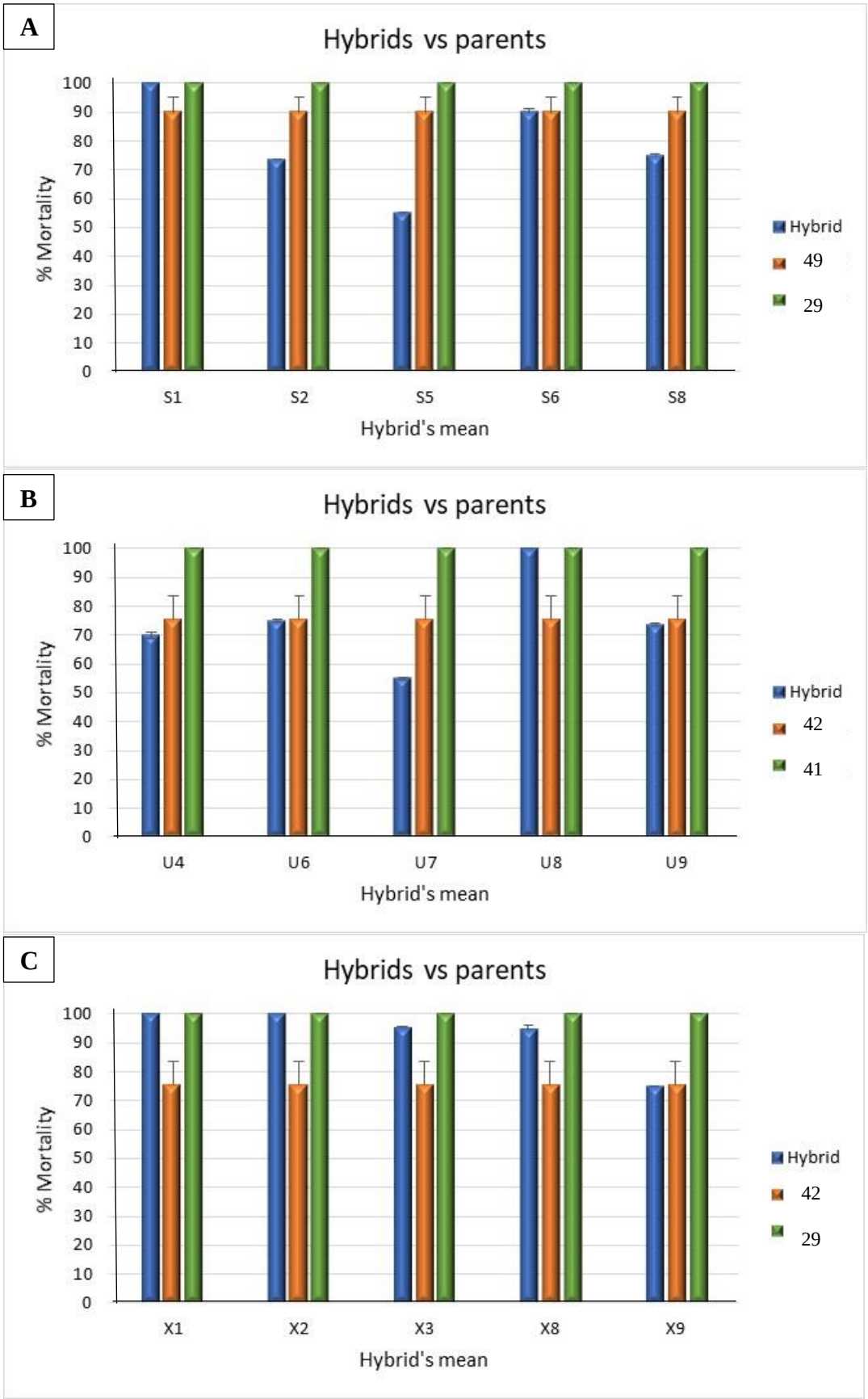


Figure 3.11. Mortality of *Plutella xylostella* larvaes after infection with hybrid strains of *Beauveria*, obtained by protoplast fusion. Combination (A) S (49 x 29). (B) Combination U (42 x 41) and; (C) Combination X (42 x 29).

Genotypic characterization

A phylogenetic tree that included the parental strains and their corresponding offspring were generated for each crossing, with the aim to compare their sequences and search for possible changes that could have emerged as a consequence of genetic recombination processes. Phylogenetic trees are shown in **Figure 3.12**. Results suggested that most of the hybrids obtained from the crossing between strains 29 (*B. pseudobassiana*) and 49 (*B. bassiana*) ("S") might have reverted to the parental wild type 49 strain (**Figure 3.12, A**); whereas the offspring from crosses 41 (*B. bassiana*) with 42 (*B. bassiana*) ("U"), and 42 (*B. bassiana*) with 29 (*B. pseudobassiana*) ("X") seem to be different from their progenitors (**Figure 3.12, B and C**). It was found a variation of 73 nucleotides in combination "S" (see **Appendix 14**), 24 nucleotides in combiantions "U" (see **Appendix 15**) and 81 nucleotides in combiantions "X" (see **Appendix 16**).

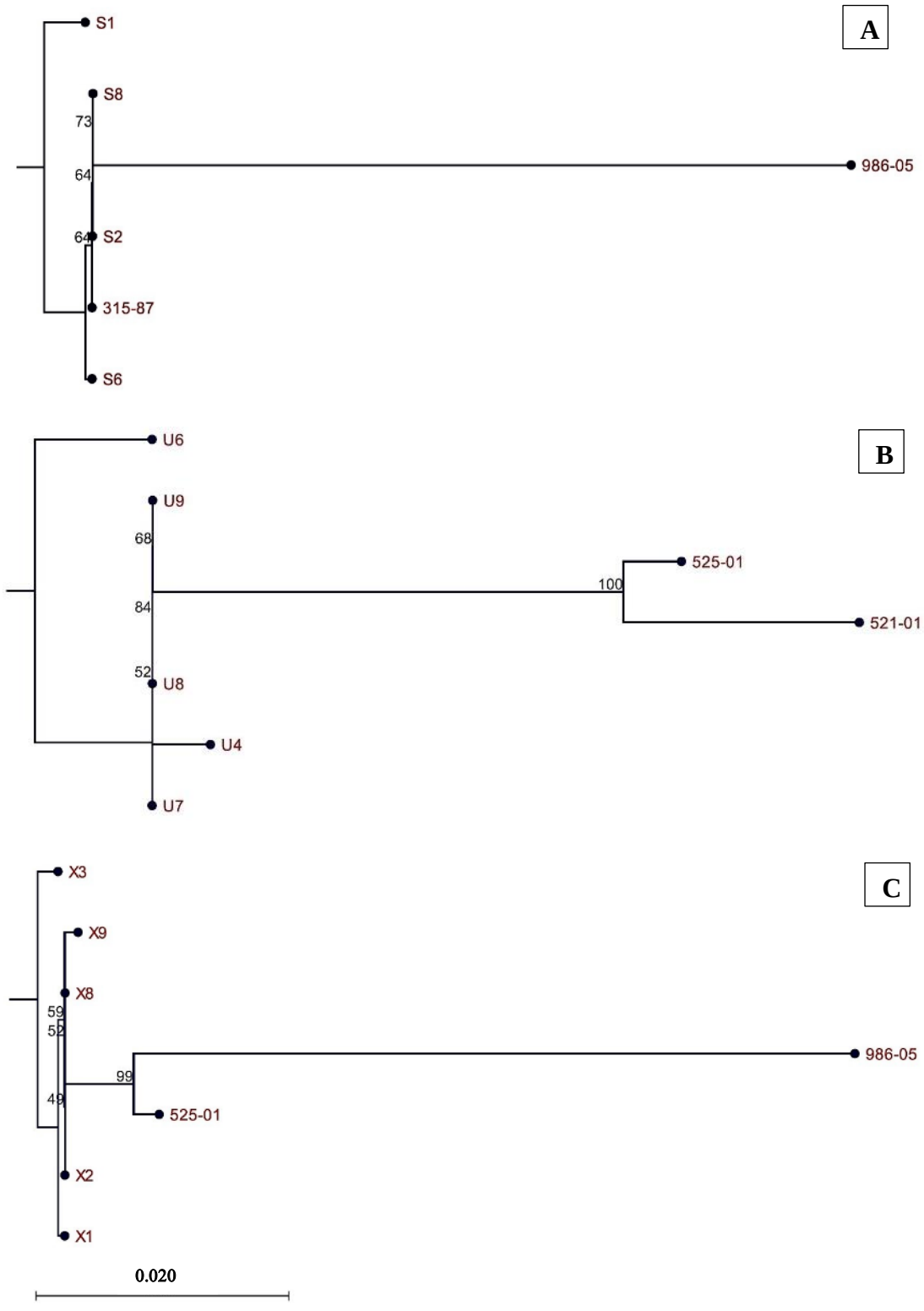


Figure 3.12. Phylogenetic trees of crosses between strains 29 (*B. pseudobassiana*) with 49 (*B. bassiana)* ("S") (A), 42 (*B. bassiana)* with 41 (*B. bassiana)* ("U") (B), and 42 with 29 ("X") (C), generated in CLC Workbench software. Genetic markers used: ITS, Elongation factor, β-tubulin and DNA Lyase.

3.4 Discussion

The anamorphic genera including *Beauveria* occur naturally in a wide range of habitats including farmland, woodland and forests, with the species being normally associated with the soil, and with different *Beauveria* clades having particular habitat preferences (Bidochka *et al.*, 2002; Meyling *et al.*, 2009). The different clades within *Beauveria*, exhibit high genetic diversity, which could suggest a possible parasexual cycle operating in nature as a mechanism for generating genotypic variability, as has been observed under laboratory conditions for *Lecanicillium* (= *Verticillium*) (Karapapa *et al.*, 1997). However, the occurrence of such recombinations in nature has not been reported so far for *Beauveria* or other fungal species within the Hypocreales.

The absence of sexual recombination in anamorphic Ascomycete species with industrial uses has prompted the investigation of heterokaryosis and parasexual recombination as an alternative mechanism for strain breeding programs (Puhalla, 1984). Generation of nitrate non-utilizing (nit) mutants through selection of spontaneous chlorate resistant colonies has become a viable approach, because it is a fast and relatively simple technique, which does not involve the use of mutagens that might introduce unwanted changes into the genome. Although nit mutants cannot use nitrate as the only source of nitrogen, they can grow indefinitely on MM supplemented with nitrate, thus no other media is needed to have a nit mutant stock and it allows the observation of any reversion (Correll *et al.*, 1987; Cove, 1976). Nevertheless, generating sufficient numbers of nit mutants can be a time-consuming endeavour. In the current study, it was challenging to obtain nit mutants in the first round of isolations, and repeated rounds of sub-culturing on chlorate media were needed to purify sectors by isolating single germinating conidia, which has been reported in other studies (Daayf *et al.*, 1995; Joaquim & Rowe, 1991; Strausbaugh *et al.*, 1992; Subbarao *et al.*, 1995). The concentration of chlorate necessary to successfully generate spontaneous mutants is also highly variable between species (Hawthorne & Rees-George, 1996; Kedera *et al.*, 1994; Korolev & Katan, 1997; Newton & Caten, 1988; Puhalla, 1985). For several fungal genera, including

Lecanicillium, *Fusarium, Aspergillus, Verticillium, Metharizium, Neurospora, Colletotrichum*, a chlorate concentration in the media of 1.5 % - 2 % was reportedly enough to generate chlorate-tolerant mutant strains. However, for *Beauveria* genus, this concentration can be 2 – 3 fold higher (Akimov & Portenko, 1996; Castrillo *et al.*, 2004; Chen, 1994; Correll *et al.*, 1988). Therefore, a gradient assay at different concentrations of chlorate, to determine the correct working concentration for a given experiment is appropiate.

In the current study, culturing of *B. bassiana* strains on chlorate medium produced the following mutant phenotype ratios: 55.7% Nit 1, 5.6% Nit M and 13.3% Nit 3 respectively; reversion was present in 25.5% of the strains. These findings agree with a previous study on *Beauveria* in which Nit 1 mutant isolation was predominant: the Nit M mutant phenotype was present for every one out of 10-20 Nit 1 mutants while Nit 3 mutants were rarely observed (> 9%) (Castrillo *et al.*, 2004). Similarly, Couteaudier & Viaud (1977) were only able to obtain Nit 1 and Nit M mutants from among 26 *B. bassiana* strains with no presence of Nit 3 mutants (Couteaudier & Viaud, 1997). It is possible that the ratio of the different nit mutant types is dependent not only on fungal species but also on fungal strain and culture conditions (Korolev & Gindin, 1999). For example, it has been noticed that cultures stored on PDA could give higher proportions of Nit M mutants (Korolev & Gindin, 1999). This may explain why different 'mutant ratios' have been reported in different studies with the same fungal species. For example, Korolev & Katan (1997) reported that over 90% of approximately 3000 mutants of *V. dahlia* were classified as nit 1 while 7% were Nit M, whereas other studies with the same fungal species showed a different distribution, with Nit M mutants reaching percentages as high as 50 % (Chen, 1994; Daayf *et al.*, 1995). In the current study, SDA media was used for Nit mutant generation; however, in future work, it might be worth considering PDA as a potential way to produce more Nit M mutants, allowing the participation of more strains in complementation tests as this media has shown better results in other studies.

Vegetative compatibility is the ability of two strains to fuse their hyphae and through complementation form a heterokaryon (a multinuclear cell with genetically different nuclei), a process that contributes to genetic diversity (Collado-Romero *et al.*, 2010; Katan, 2000; Leslie, 1993). When fungal strains differ in one or more *het* or *vic* loci, then heterokaryosis is restricted and they are vegetatively incompatible (Glass *et al.*, 2000; Xiang & Glass, 2004). Nit mutants are used to determine vegetative compatibility groups (VCG's) among fungal species by testing paired strains with different, complementary auxotrophic mutations (Joaquim & Rowe, 1990; Sugimoto *et al.*, 2003). Identification of VCGs is an effective approach to determine genetic relationships among fungi (Rowe, 1995). In the present research, high diversity was observed among the 50 strains of *Beauveria* which divided into 35 vegetative compatibility groups. This result is comparable to that of Castrillo *et al*. (2004) who detected 23 VCGs in 34 strains of *B. bassiana*, and Couteaudier & Viaud (1997) who determined 14 different VCGs among 26 *B. bassiana* strains examined, and who reported a correlation between VCG and host specificity rather than geographical origin (Couteaudier & Viaud, 1997). In the present study, no relationship was detected between the VCGs and host specificity or geographic origin. The lack of vegetative compatibility between strains has been found in other anamorphic fungal species (Aiuchi *et al.*, 2008b; Joaquim & Rowe, 1990; Sugimoto *et al.*, 2003). For example, in *L. lecanii*, 13 VCGs were reported among 33 strains (Korolev & Gindin, 1999), while in *Colletotrichum spp*. five from 7 strains examined belonged to different VCGs (Brooker *et al.*, 1991). Large numbers of VCGs reflect the phylogenetic diversity of many of these species, which are probably better thought of as species complexes rather than as a single lineage. It has been hypothesized that each vegetative compatibility group could serve as a barrier to genetic exchange in nature, making possible the presence of clonal lineages (Couteaudier & Viaud, 1997).

While exploitation of heterokaryosis and parasexual recombination provides a potentially useful way of breeding different anamorphic fungal strains for industrial strain improvement (Puhalla, 1984), the presence of high numbers of VCG's within a species may represent an important technical barrier, since to proceed with strain improvement by hyphal fusion, both parents should be vegetatively compatible

(Aiuchi *et al.*, 2008b). When the heterokaryon is formed, the anastomosed cells (heterokaryotic cell) are on the colony margin, whereas the rest of the hyphae remain homokaryotic, as was described in *F. oxysporum* (Puhalla, 1985). In fact, at the moment of hyphal fusion it is believed that not only is the heterokaryon present in the dense contact zone between hyphae, but that there are also homokaryons (self-fusing hyphae) as well as Nit mutant parents (non-fusing hyphae) (Aiuchi *et al.*, 2008b). Prototrophic growth is a signal of recombination, which is thought initially to result from diploid formation via karyogamy of the heterokaryon cell; however this diploid cell is quite unstable and after a mitotic crossing over, haploidization occurs and a prototrophic haploid is formed (Crawford *et al.*, 1986). An unstable diploid nucleus after recombination has been reported not only in *B. bassiana* but also in other filamentous species such as *Verticillum spp*. (Hastie & Heale, 1984), *A. niger* (Bonatelli *et al.*, 1983), *M. anisopliae* (Bagagli *et al.*, 1991; Silveira & Azevedo, 1987), and *Trichoderma pseudokoningii* (Furlaneto & Pizzirani-Kleiner, 1992).

Protoplast fusion mediated by polyethylene glycol (PEG) was selected as the technique to overcome the incompatibility found in three out of the seven strains selected for parasexual recombination. This method produces a good yield of viable cells and has been employed for strain improvement for a large number of filamentous fungi, particularly where there is a need to overcome cellular incompatibility issues (Paris, 1977; Kawamoto & Aizawa, 1986; Liu & Friesen, 2012; Martín, 2015). The production of sufficient quantities of protoplasts for experiments is affected by a range of factors such as enzyme concentration and osmotic stabilisers, as well as the fungal species used (Zhang *et al.*, 2016). In the current project, protoplast fusion of complementary nit mutants of different *B. bassiana* strains resulted in stable recombinants with different phenotypes from the wild type parents. The aim was to develop hybrid strains with improved virulence to DBM, greater thermotolerance and tolerance of UV-B radiation. Although recombinant strains with improvement in all three of these phenotypes were not produced, some of the hybrid strains (X1, X2, X3, X8) showed a significant improvement in thermotolerance, whereas tolerance to UV-B radiation and virulence of the hybrids was quite similar to the wild type parents. On the other hand, two recombinant

strains (U3, U10) had a diminished performance compared with their respective parental strains with respect to colony growth, UV-B tolerance and virulence to DBM. This behaviour has been reported in other published work (Couteaudier *et al.*, 1996) in which recombinant strains between *B. sulfurescens* and *B. bassiana*, produced using protoplast fusion and confirmed through RFLP profiling, exhibited different phenotypes to the parent strains, including both increased and decreased virulence against *Ostrinia nubilalis* and *Leptinotarsa decemlineata*. Elsewhere, increase of conidial size and production was observed in *L. lecanii* hybrids after protoplast fusion (Aiuchi *et al.*, 2008a), while recombinants in *Lecanicillium* sp. produced by hyphal anastomosis were reported to show an increase in conidial production, with the authors concluding that this change in phenotype may have been caused by mitotic crossing over or chromosome re-assortments events (Drummond & Heale, 1988). Similarly, *Trichoderma reesei* hybrids produced through protoplast fusion showed an improvement in growth and conidia production compared with wild type parents (Prabavathy *et al.*, 2006). In the data obtained from the *B. bassiana* hybrids in the current research it was found that at least part of the genome of both parents were present in the 15 hybrids. Diploid formation could not be demonstrated in this research, nevertheless as recombination of genetic characteristics from both wild type parents was observed, it is clear that parasexual recombination had taken place. Maybe after protoplast fusion the whole genomes from the mutant parents did not integrate successfully and that is why some of them had the same phenotype of one parent. In this study it was necessary to go through multiple rounds of protoplast fusion to get an improved strain; in crop breeding, for example, they have multiple rounds of crosses and development of new varieties takes a long time.

4 Sexual recombination

4.1 Introduction

Fungi are complex organisms not only for their wide range of phenotypes and lifestyles but also for their diverse methods of reproduction (Dyer & Paoletti, 2005). They can have both, sexual and asexual reproduction depending on the fungus, and in at least 20% of all fungal species, a sexual state has not been observed (Dyer & O'Gorman, 2011). It is not clear why these fungi have lost the ability to undergo meiosis and now they only reproduce asexually. Sexual reproduction has advantages such as reasserting genetic diversity that might allow selection of favourable genes for evolutionary purposes (Geiser *et al.*, 1996; Heitman, 2010). Recent studies have found an unidentified cryptic sexual stage in some filamentous fungi (e.g. *Aspergillus* and *Penicillium*) suggesting that these asexual species might have a hidden potential for sexual reproduction (Gow, 2005; Heitman, 2010; Kück & Pöggeler, 2009). In the genus *Aspergillus* 75 genes has been reportedly involved in sexual reproduction with functions such as mating, formation of fruiting bodies and ascospore production (Dyer & O'gorman, 2012). *Penicillium marneffei* as well as *Aspergillus* spp., for instance, showed apparently functional genes that could play a role in mating and meiosis (Woo *et al.*, 2006). Among these sex-related genes, mating type (MAT) genes have generated a high interest for researchers due to their key role in sexual identity and sexual development (Debuchy *et al.*, 2010). These MAT genes have two structurally unrelated allelic variants, MAT1-1 and MAT1-2, and due to their high divergence are called idiomorphs rather than alleles to denote that they may contain multiple genes and that the genes of alternate mating types have no allelic relationship to one another (Bushley *et al.*, 2013; Kronstad & Staben, 1997). Despite several genes related to sexual reproduction have been found in some fungi, it is also important to consider that there are some unidentified genes related with this cycle. For instance, in the asexual genus *Candida* it was not possible to find genes relating to mating or meiosis. However, some *Candida* species might be able to undergo meiosis with a different sexual pathway (Butler *et al.*, 2009). Some fungi have three different strategies to reproduce (Coppin *et al.*, 1997), which are listed below:

1. **Homothallism:** When both mating type idiomorphs are present in a single fungal strain. These idiomorphs are normally referred to as MAT1-1 and MAT1-2 (informally called MAT1 and MAT2). Each idiomorph encodes more than one gene; for example the MAT1-2 idiomorph in *Erysiphe necator* has 2 genes, MAT1-2-1 and MAT 1-2-2, while the MAT1-1 idiomorph has 3 genes – MAT1-1-1, 1-1-2, and 1-1-3 (Brewer *et al.*, 2011).

2. **Heterothallism:** when a fungal strain contains a single idiomorph (MAT1-1 or MAT1-2) in their nucleus and require a compatible fungus for sexual reproduction (e.g. *B. bassiana, M. anisopliae, Cordyceps militaris, Ophiocordyceps sinensis, Fusarium* spp.) (Bushley *et al.*, 2013; Wilson *et al.*, 2015).

3. **Pseudohomothallism:** intermediate between heterothallism and homothallism, these fungi need two compatible fungi to mate, nevertheless could produce a self-fertile mycelium with the two different nuclei from the parents (heterokaryon) or mycelium with a single nucleus containing one idiomorph capable of outcrossing (e.g. *P. anserina*) (Bushley *et al.*, 2013; Grognet & Silar, 2015).

In many anamorphic fungi their mating systems have not been determined so far. In one study, the development of nucleotide primers designed to align with the conserved domains named a1 and HMG, of the MAT1 and MAT2 genes have helped to amplify fragments of the mating type genes from 41 species across the order Hypocreales (Bushley *et al.*, 2013). The MAT genes function is not restricted only to reproduction, but they have also been involved in the generation of bioactive molecules, and anamorph-teleomorph connections, therefore mating type assays could help to understand how these process occurs (Yokoyama *et al.*, 2004).

As mentioned before, in heterothallic fungi it is necessary to have complementary mating types (MAT1-1 and MAT1-2) to have sexual reproduction (Rydholm *et al.*, 2007). Within a MAT gene there is a high mobility group (HMG) involved in sexual identity due to the presence of highly dissimilar genes (Debuchy *et al.*, 2010). The MAT1-1 allele open reading frame (ORF) encodes a protein with an alpha domain MATa_HMG and MAT2 allele single ORF encodes a protein with a MATA_HMG domain (Debuchy *et al.*, 2010; Martin *et al.*, 2010; Turgeon & Yoder,

2000). The link between mating and incompatibility is another interesting fact whose occurrence is not clear. It has been found that MAT proteins could act as a transcription factor to allow the sexual cycle (Wirsel *et al.*, 1998). For instance, in *N. crassa* the MAT idiomorphs are responsible for mating as well as vegetative incompatibility (Coppin *et al.*, 1997). Mutants of this fungus have lost their function of incompatibility, but the ability to produce fertile perithecia remains intact (Newmeyer *et al.*, 1973). Thus, whether fertility and incompatibility is dominated by two close genes or a single gene with two different functions is still unanswered (Coppin *et al.*, 1997). The proportions in which MAT1 and MAT2 are contained in fungal genomes is dependent on the fungal species and could be highly variable. In some *Aspergillus* (*A. oryzae, A. flavus, A. parasiticus* and *A. niger*) and *Penicillium* (*P. marneffei, P. chrysogenum,* and *P. dipodomyis*) species the ratio of MAT genes present was 1:1 (Henk *et al.*, 2011; Henk & Fisher, 2011; Hoff *et al.*, 2008; Paoletti *et al.*, 2005; Ramirez-Prado *et al.*, 2008; Woo *et al.*, 2006); whereas in *B. bassiana* a prevalence of MAT 1-1 over MAT 1-2 with a range of 31:2 respectively was found (Meyling *et al.*, 2009).

Following the discovery of this important types of genes for sexual reproduction, another significant finding was the observation of a complete sexual cycle in presumed asexual fungi such as *A. fumigatus* and *P. pinophilum*, through induction (Paoletti *et al.*, 2005). Directed crosses between MAT1-1 and MAT1-2 strains were performed resulting in the formation of cleistothecia and recombinant ascospores which were identified by PCR diagnostics (Horn *et al.*, 2009; López-Villavicencio *et al.*, 2010; O'Gorman *et al.*, 2009; Ramirez-Prado *et al.*, 2008). Therefore, strain improvement in ascomycetes fungi through induction of sexual reproduction in the laboratory is now a viable option. *P. chrysogenum* is a good example since it was known as a strict asexual fungus. However, in a recent study they were able to obtain recombinant strains on oatmeal agar that were sterile, but also one hybrid with viable ascospores that increased penicillin production. Moreover, in one hybrid the lack of an undesired pigment (chrysogenin) was found, which contaminates penicillin when it is produced, resulting in a great advantage for industrial purposes (Böhm *et al.*, 2013). This fertile hybrid was obtained in oatmeal

agar supplemented with 0.065 mg/L of biotin, which has been previously described as necessary to develop sexual structures and to fulfil the sexual cycle in other fungi such as *Sordaria, Hirsutella* and *Chaetomium* species (Loughheed, 1961; Molowitz *et al.*, 1976). In addition, there is direct evidence for fitness associated sex (FAS), knowledge that has been applied to induce sexual reproduction in some fungi (Böhm *et al.*, 2013), which shows how sexual reproduction occurs often under stress conditions (Schoustra *et al.*, 2010). This phenomena has been observed in other organisms as well, such as bacteria, which show sex induction under starvation conditions and in spring wheat, where sexual reproduction occurred more often under high cell density (Foster, 2005; Liu *et al.*, 2008). In *A. nidulans*, this condition has been observed suggesting that FAS have evolved in this fungus as a strategy to surpass environmental stress (Osiewacz, 2002). An advantage of asexual conidiospores is the higher dispersal rates than ascospores derived from sexual reproduction (Adams *et al.*, 1998). Sexual reproduction can also have some disadvantages compared with asexual reproduction, including an extra cost of energy associated with acquiring a complementary mate and meiosis (Coppin *et al.*, 1997).

Phylogenetic studies have demonstrated a connection between *Beauveria* anamorphs and *Cordyceps* teleomorphs, which suggest a hidden potential for sexual reproduction in *Beauveria* strains (Rehner & Buckley, 2005). The whole genome of a strains of *B. bassiana* (ARSEF 2860) has been sequenced confirming the presence of both MAT 1 or MAT 2 genes, which means that this organism is heterothallic and outcrossing (Xiao *et al.*, 2012; Yokoyama *et al.*, 2006). A syntenic analysis showed highly conserved genes flanking the mating type locus (Xiao *et al.*, 2012). Furthermore, sex related genes found previously in *A. nidulans* and *N. crassa* were examined in *B. bassiana* and genes functioning in the mating processes, meiosis, karyogamy and development of fruiting bodies that were present in both fungi were discovered (Dyer & O'gorman, 2012; Xiao *et al.*, 2012; Yokoyama *et al.*, 2006). Further studies of *Beauveria* have identified the presence of a *Spo11* gen which was found to be crucial for initiating meiotic recombination (Panizza *et al.*, 2011; Valero-Jiménez *et al.*, 2016).

The production of sexual structures in ascomycetes fungi is not rare. *C. bassiana* the teleomorph of *B. bassiana*, produces synnemata more frequently than perithecial stromata, maybe because specific environmental and nutritional conditions are needed to form fertile fruiting bodies (Lee *et al.*, 2007; Lee *et al.*, 2010a). A field collected tick was found to be infected with both mycelium of *C. bassiana* and *B. bassiana* and it was suggested that the *Beauveria* found over the dead insect could have developed from the *Cordyceps* infecting the tick (Sung *et al.*, 2006). Synnemata have been obtained in other entomopathogenic fungi such us *Hirsutella thompsonii* or *Paecilomyces tenuipes* (Peck) Samson. The question that answer weather if it is possible to find the correct conditions to reproduce these results in *Beauveria* and have a more clear understanding of the teleomorph-anamorph connection (Sung *et al.*, 2006).

The knowledge of the mating type genes represents an alternative strategy for strain improvement in EPF by induction of sexual recombination (i.e. the teleomorph state) in anamorphic strains used for biocontrol. This has been done recently with a number of ascomycete fungi for industrial uses, including *Penicillium* (Böhm *et al.*, 2013) but it has been rarely investigated in EPFs used for biocontrol. The development of a sexual recombination system for the anamorphic hypocrealean EPF could have many applications, including strain improvement, understanding the genetic basis of virulence, and in providing basic information on the anamorph-teleomorph connections in different taxonomic groups (Yokoyama *et al.*, 2004). The teleomorph-anamorph connections in the ascomycete EPF have only recently become apparent, largely as a result of molecular phylogenies constructed from multilocus nucleotide sequencing (Nunes *et al.*, 2013).

The aim of this chapter was to investigate the potential of a sexual recombination in nine isolates of *B. bassiana* with different MAT genes in their genome with the view of improving strains for biocontrol. Three different media, at three different temperatures and two different concentrations of biotin, were performed in order to examine the potential formation of any sexual structures such as cleistothecia or fruiting bodies.

4.2 Methodology

The same 50 fungal isolates used in **Section 2.2.1** (see **Table 2.1**) were used to obtain the information on mating types needed for this section. Storage, growth and DNA extraction methodology are described in **Section 2.2.2**.

4.2.1 Polymerase Chain Reaction (PCR)

The primers used for PCR amplification were purchased from Sigma-Adrich, USA, the sequences are listed in table **Table 4.1**.

Table 4.1. List of primers used for amplification of MAT genes (Sigma-Adrich, UK).

Primer		Name	Sequence
MAT 1	Forward	MAT112.F4	CAG CTC TCC GTC TGC CGA GTT
	Reverse	MAT111.R5	TAG TGA GAA AGC CTG ACG CGG
MAT 2	Forward	MAT2.F4	RTC AGC GTC GGC ATC AAC CCA TT
	Reverse	MAT2.R5	GAA AAY TCG CTG CCA GTC ATR AT

All PCR reactions were performed in a total volume of 25 µL per tube. To set up the reactions, the conditions described in **Section 2.2.2** were used.

The conditions used for PCR amplifications were:

- Mating type 1 gene: 2 minutes denaturation at 95 °C; 30 amplification cycles, each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, 2 minutes extension at 72°C, and a final 7 minutes extension at 72°C.
- Mating type 2 gene: 2 minutes denaturation at 95 °C.; 40 amplification cycles each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 54°C, 1 minute extension at 72°C and a final 7 minutes extension at 72°C (Meyling *et al.*, 2009).

PCR products were loaded onto a 1.2% (w/v) agarose gel (Sigma-Aldrich, USA) in TAE buffer adding gel red dye (2ul/ml). Electrophoresis was carried out for 90 minutes at 90V. PCR products were cleaned up using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol and sequenced by the GATC Biotech Company using the forward primer (5 µM) for each molecular marker.

4.2.2 Fungal selection for sexual recombination

A total of nine strains were selected to perform strain improvement assays through sexual recombination (**Table 4.2**). The strains were selected based on the presence of MAT 1 and MAT 2 genes, and in the analysis of the results obtained in phenotypic experiments (**Section 2.3**).

Table 4.2. Strains selected for sexual recombination

Strain	Origin	Host	Mating Type	Sporulation	Temperature	UV-B	Virulence
23	UK	Diptera	MAT 1	Medium	High	High	Medium
28	UK	Lepidoptera	MAT 1	High	Medium	High	High
4	Commercial, USA	Lepidoptera	MAT 1	Medium	Medium	High	High
42	Kenya	Lepidoptera	MAT 1	High	Low	High	Medium
32	UK	Lepidoptera	MAT 2	Medium	Medium	Low	Medium
30	UK	Lepidoptera	MAT 2	Low	Medium	High	High
21	UK	Lepidoptera	MAT 2	High	Medium	High	Low
41	Kenya	Lepidoptera	MAT 2	Low	Medium	Medium	High
49	Phillipines	Lepidoptera	MAT 1	High	High	High	High

4.2.3 Development of crosses between fungal strains

Five wild type strains containing MAT 1 gene were crossed with four strains containing MAT 2 gene, in 20 different combinations (**Table 4.3**). Conidial suspensions of each fungal strain used in this experiment (5 x 10^5 conidia/ml) were prepared from seven-day-old cultures, following the methodology described in **Section 2.2.1**. Two aliquots of 10 μL from each conidial suspension were separately pipetted onto the agar plate, about 4 cm apart and perpendicular to aliquots of conidia of the opposite mating type in three different media and in all possible combinations. This configuration was aimed to create four interaction zones once colonies grew. Plates were sealed with one layer of Parafilm and crosses were examined for cleistothecia formation periodically over 6 to 9 months with an Olympus SZH10 Stereo microscope.

Preliminary experiments to determine optimal conditions were performed with three combinations (42 x 41, 4 x 32, 49 x 21). These experiments were

performed on three different media (**Appendix 10**): oatmeal agar (OA, Quaker oats), malt extract agar (MEA, 2% Oxoid, UK), and Czapek Dox agar (CZA, Sigma-Aldrich, USA), varying temperatures (20 °C, 25 °C, 30 °C) and biotin concentrations (0.0, 0.05, and 0.5 mg/L). Plates under different conditions mentioned before were incubated in the dark to allow the formation of fertile sexual structures (Böhm *et al.*, 2013; Dyer & O'gorman, 2012; Houbraken *et al.*, 2008; Roca *et al.*, 2003). Additional crosses were only conducted at 25 °C and 0.5 mg/L, as the best conditions, the remaining combinations were carried out using these biotin values in the three media previously described, for each combination.

Table 4.3. Combination of *B. bassiana* strains with opposite mating types (MAT1 x MAT2), for induction of sexual reproduction

MAT1 x MAT2	MAT1 x MAT2	MAT1 x MAT2	MAT1 x MAT2	MAT1 x MAT2
23 x 32	28 x 32	**4 x 32***	42 x 32*	49 x 32
23 x 30	28 x 30	4 x 30	42 x 30	49 x 30
23 x 21	28 x 21	4 x 21	42 x 21*	**49 x 21**
23 x 41	28 x 41	4 x 41	**42 x 41***	49 x 41

In bold the three strains used to set up the conditions for sexual recombination experiments. * Strains used for *in vivo* assays.

4.2.4 *In vivo* bioassays by injection of fungal strains on *Galleria mellonella*

The influence of injection of conidial suspensions from four combinations (4 x 32, 42 x 32, 42 x 41, 42 x 21) in *Galleria mellonella* (Wazp Brand UK Ltda.) was evaluated (**Table 4.3**). A 1 x 10^7 (conidia/mL) conidial suspension of each strain was prepared as described in **Section 2.2**. A combined conidia suspension prepared after mixing 1 mL of conidial suspension from a MAT 1 strain with 1 mL from a MAT 1-2 strain. Final instar *G. mellonella* larvae were cooled on ice (to have decrease the movability of the larvae during the experiment) briefly prior to injection into the right front proleg using a 0.3 ml microfine syringe (BD, USA). Aliquots (30 μL) of the mixed suspension were injected in five *Galleria mellonella* larvae. The injected larvae were placed in a 9cm Petri dishes lined with filter paper and incubated at 25 °C, (16:8 LD) for six months and assessed twice per month looking for the development of sexual structures using a stereo microscope.

4.3 Results

4.3.1 Presence of Mating type genes

Of the 50 strains assessed for Mating type 1-1 and Mating type 1-2 genes, six strains did not contain either of those. Mating type 1-1 gene was found in 25 strains and showed a band of a length of ≈1500 bp. Mating type 1-2 gene was found in 19 strains and presented a band with a length of ≈650 bp (**Table 4.4**). No correlation was found between mating type and place of origin or host. However, a correlation was found among the two *Beauveria* species found in this research. In strains of *B. bassiana* the ratio of Mat 1-1: Mat 1-2 was 25:15 respectively; whereas, for *B. pseudobassiana* Mat1-1 was not found and only four out of eight strains exhibited Mat 1-2. The other four strains in this group did not showed Mat 1-1 neither Mat 1-2.

Table 4.4. Presence of Mating types genes in the fungal strains collection used in this study (Continues on the next page)

Beauveria strain	Country of origin	Insect host	MAT1	MAT2
43	China	Hemipteran		✓
3	Commercial, USA	Lepidoptera	✓	
4	Commercial, USA	Lepidoptera		✓
5	USA	Hymenoptera	✓	
21	UK	Lepidoptera		✓
1	Canada	Diptera	✓	
35	France	Diptera	✓	
36	France	Diptera	✓	
9	USA	Lepidoptera		
20	Brazil	Diptera	✓	
37	Denmark	Diptera	✓	
40	Kenya	Lepidoptera		✓
41	Kenya	Lepidoptera		✓

Table 4.4. Continued. Presence of Mating types genes in the fungal strains collection used in this study.

Beauveria strain	Country of origin	Insect host	MAT1	MAT2
42	Kenya	Lepidoptera	✓	
24	UK	Lepidoptera		✓
910-05	UK	Lepidoptera		
26	UK	Lepidoptera		
27	UK	Lepidoptera	✓	
28	UK	Lepidoptera	✓	
29	UK	Lepidoptera		
30	UK	Lepidoptera		✓
31	UK	Lepidoptera		✓
32	UK	Lepidoptera		✓
50	Australia	Lepidoptera		✓
44	China	Lepidoptera	✓	
44	Turkey	Lepidoptera		✓
8	USA	Coleoptera	✓	
10	USA	Lepidoptera		✓
2	Canada	Lepidoptera		✓
38	Italy	Lepidoptera		✓
47	Vietnam	coleoptera		✓
45	China	Hemipteran	✓	
46	China	Hemipteran		
18	Brazil	Lepidoptera	✓	
19	Brazil	Coleoptera		✓
48	Thailand	Lepidoptera		✓
17	Colombia	Coleoptera	✓	
49	Phillipines	Lepidoptera	✓	
6	USA	Diptera	✓	✓
7	USA	Diptera	✓	
11	USA	Coleoptera	✓	
12	USA	Homoptera	✓	
20	USA	Homoptera	✓	
14	USA	Homoptera	✓	
15	USA	Homoptera		
16	USA	Homoptera	✓	
22	UK	Lepidoptera		✓
23	UK	Diptera	✓	
34	UK	Lepidoptera		✓
33	USA	Lepidoptera	✓	

4.3.2 Crosses of fungal strains with opposite mating type genes

Interaction between the opposite mating type strains from the first three combinations (42 x 41, 4 x 32, 49 x 21) started after one month´s incubation at 25 °C and 30 °C, but only in the oatmeal agar with and without biotin addition (**Figure 4.1**). At 20 °C growth was slower than 25 °C and 30 °C in OA agar and CPA media.

Figure 4.1. Fungal growth from combinations of 42 x 41 after one month´s incubation at 25 °C. A) Czapek dox agar with no interaction in the contact zone between strains from opposite mating types, B) Oatmeal agar supplemented with biotin (0.5mg/L) with interaction in the contact zone.

In MEA 2%, no growth was observed at any of the biotin concentrations or temperatures tested after 6 months incubation. The mycelial growth was highly restricted with a very thin white colony and slow growth. Little contact was observed between hyphae of all crosses (**Figure 4.2**).

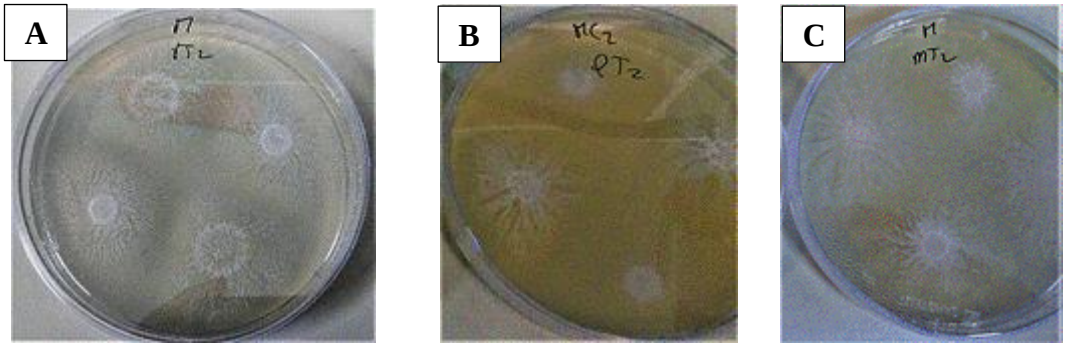


Figure 4.2. Fungal growth after 5 months of incubation at 25 °C on 2% MEA in three different crosses. (A) 42 x 41, (B) 49 x 21 and (C) 4 x 32.

CPA and OA agars supplemented with biotin at either (0.05 and 0.5 mg/L) concentration, supported faster mycelial growth in all of the combinations tested with white and fluffy growth around each strain after the second month (**Figure 4.3**).

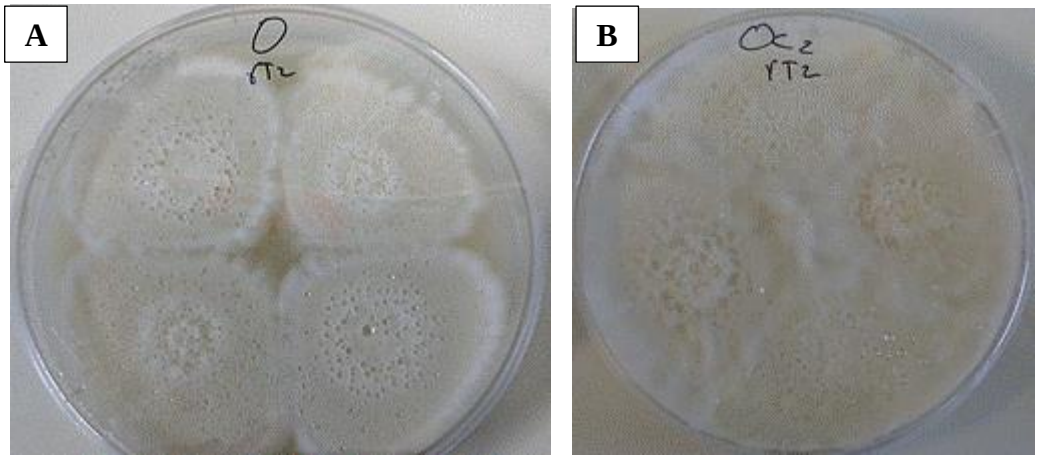


Figure 4.3. Combination 42 x 41 in Oatmeal agar after two months of incubation at 25 °C. (A) Oatmeal agar with no addition of biotin, (B) Oatmeal agar supplemented with biotin (0.5 mg/L).

After six months, only one combination (4 x 32) exhibited a large structure looking like a branch of mycelium and in some cases with a grouping of conidia at the end, resembling synnemata. However, the configuration of these structures was not vertical, they were laying on the surface of the media (**Figure 4.4**). It was not clear if this possible synnemata was a product of an interaction between the two opposite mating type strains because the beginning of the structure was not only in the junction zone, but also on one side of the growing strain. Possible synnemata was present only on OA agar, with or without biotin and at all the three temperatures tested. Nevertheless, at 25 °C the possible synnemata showed the best growth.

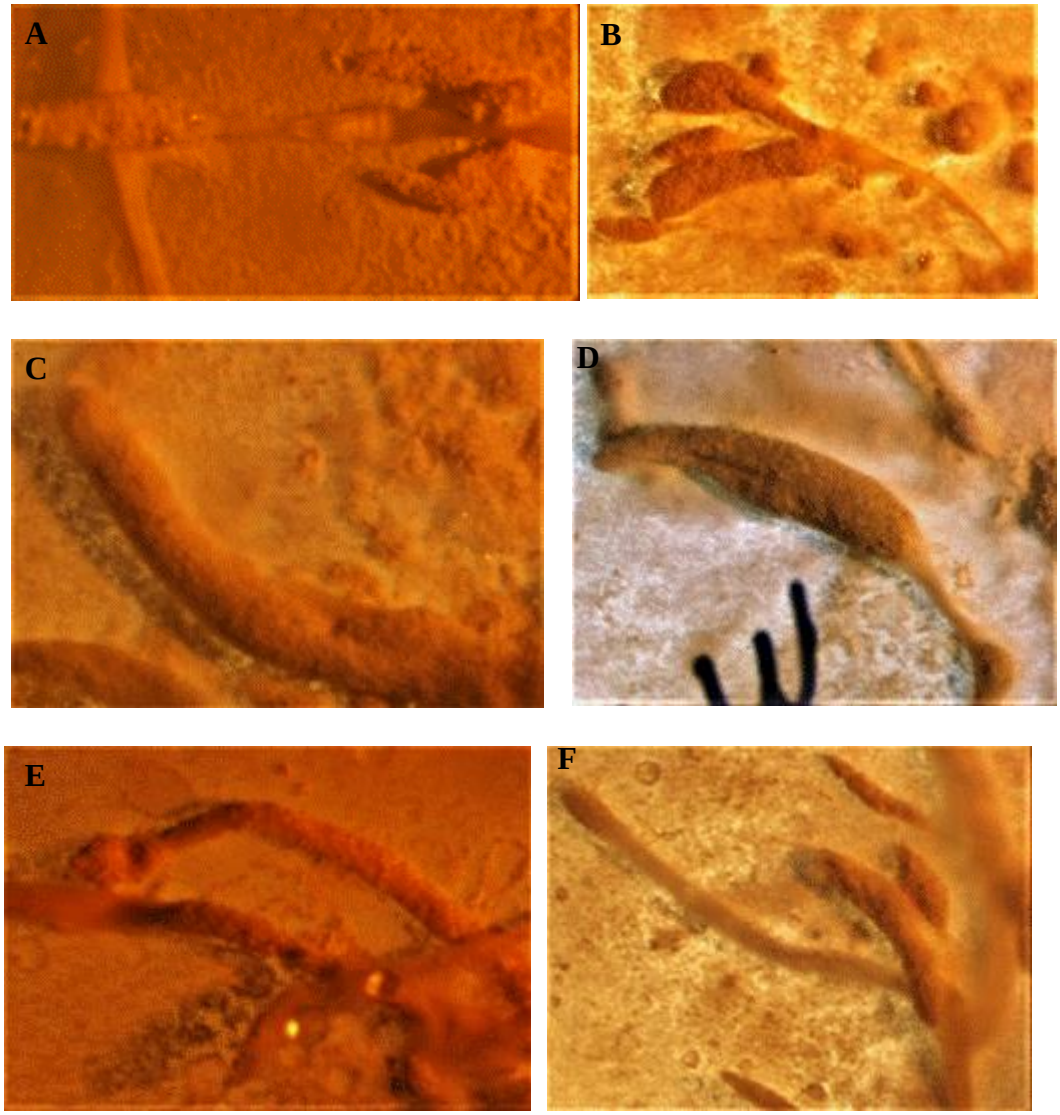


Figure 4.4. Combination of 4 x 32 on oatmeal agar after six months incubation. (A) to (D) growth of structures resembling synnemata. (E) to (F) Possible synnemata growth with accumulation of conidia in the head of the structure in the upper left margin.

In vivo assays by injection of *Galleria mellonella* with fungal strains.

Four days after injection, all larvae died. After three months all dead larvae were covered in spores and white mycelium. Microscope examination showed that only one combination (42 x 21) exhibited evidence of erect structures from larval cadavers as possible synnemata that looked like crystalline branches with an accumulation of material in the top of the structure. Time constraints did not allow a further investigation of these structures (**Figure 4.5**).

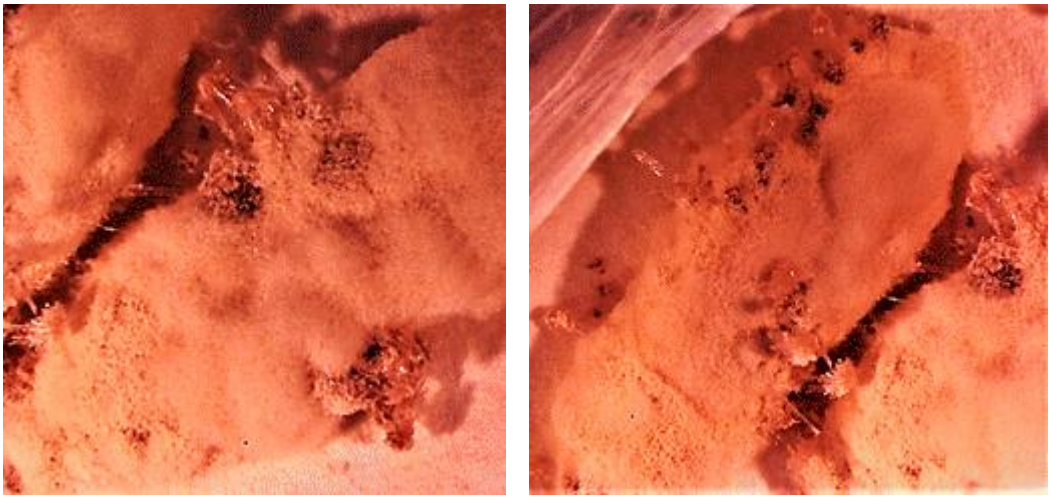


Figure 4.5. Possible synnemata growth over dead *G. mellonella* larvae, covered with white *Beauveria* mycelium, after three months incubation at 25 °C.

4.4 Discussion

In the present study, both MAT1-1 and MAT1-2 idiomorphs were identified in the population of *Beauveria* strains studied in a ratio of 25:19 respectively and 6 strains did not have any MAT genes in their sequence. An interesting fact was that in the *B. bassiana* strains there were a mixed group between MAT1-1 and MAT1-2 idiomorphs (25:15); whereas for *B. pseudobassiana* only MAT 1-2 idiomorph was detected and four out of six strains in this specie did not have any mating type locus. These results underscores the importance in identifying this loci and the necessity for more deeply studies of their genomic distribution (Brewer *et al.*, 2011).

Teleomorphs are able to reproduce sexually through the production of ascospores in relatively large ascocarp fruiting bodies that may persist for several months, while anamorphs undergo asexual reproduction through conidia borne on hyphae from fungus-infected cadavers that are released for a relatively short period after insect host death (Dyer & O'Gorman, 2011). It is believed that fungal species might have evolved to asexuality due to the advantages that this entails; specifically that asexual reproduction reduces the metabolic cost of reproduction for long range dispersal and conserves the ability to have progeny in a wide range of environments (Lee *et al.*, 2010b). The two stages have markedly different morphologies although teleomorphs and anamorphs of the same species are members of the same

genotypic group (Coppin *et al.*, 1997). In filamentous fungi the genes responsible for regulating sexual compatibility and sexual reproduction are MAT genes, which exhibit conserved components related to processes of self-nonself recognition and controlled nuclear migration (Kronstad & Staben, 1997). There are two allelic variants to this locus called idiomoprhs MAT 1-1 and MAT 1-2. In sordariomycetous fungi it is common to find three genes in the MAT1-1 idiomorph, MAT 1-1-1, MAT 1-1-2 and MAT 1-1-3 (Turgeon & Yoder, 2000).

Beauveria is a heterothallic fungus and anamorphic to Cordyceps within the Cordycipitaceae family, although the precise teleomorph connections for all species within *Beauveria* have not been established yet. *C. bassiana*, the reported teleomorph of *B. bassiana*, is thought to occur only within East and Southeast Asia (Sung *et al.*, 2007). The teleomorphic states of the entomopathogenic fungi within the Hypocreales, including *Cordyceps*, are usually found in habitats away from human disturbance which probably favours the growth of fruiting bodies (Boomsma *et al.*, 2014). Characterizing the mating types within a fungal species is a useful tool to help understanding its ecology and evolutionary history. Although species within the ascomycetes generally have two genes in the MAT 1-1 idiomorph, studies with *O. sinensis* and *E. necator* reported another gene in the MAT 1-1 locus (MAT1-1-3) (Brewer *et al.*, 2011; Bushley *et al.*, 2013).

There is limited evidence of sexual reproduction in *Beauveria* species, with the asexually reproducing (i.e. conidiogenous anamorphic) state being the predominant form found in nature. In recent years the teleomorph of *B. bassiana* was described by (Li *et al.*, 2001) as *C. bassiana*. This homothallic fungus is known to grow on dead Lepidoptera larvae and is rare to be in Korea (Lee *et al.*, 2007). Production of fruiting body *in vitro*, can nevertheless, be very difficult. Recent studies have developed a system to successfully produce fruiting bodies from *C. bassiana* in brown rice. They took 10 single ascospore (ascospore = haploid conidia from sexual reproduction) isolates that were obtained from a cross of 2 strains of *C. bassiana*. They then paired different isolates on brown rice medium to see if they would produce fruiting bodies. They observed 2 types of fruiting bodies: synnemata or

perithecial fruiting bodies. Synnematal production was frequent and did not require different isolates to be paired in order to be produced. In contrast, perithecial fruiting body formation was rare and only occurred when isolates with opposite mating type were crossed (Lee *et al.*, 2007; Sung *et al.*, 2006). Interestingly, another study found that *B. bassiana* is capable of forming synnemata. This event appears to be rare, and it is not known if the ability to produce synnemata is related to the ability to produce complex multicellular fruiting bodies (Lee *et al.*, 2010a; Yoon *et al.*, 2003). In the present study, rice or silkworm media was not used after finding the synnemata. Thus, it was not be possible to evaluate how achievable it may be to form a fruiting body or any other sexual structure. It would be very interesting to expand the research using this medium.

Sexual recombination occurrence *in vitro* is more feasible in homothallic fungal strains than in the heterothallic ones because a single individual has both MAT1-1 (alfa domain) and MAT 1-2 (HMG-domain) genes linked at the same locus, hence no mating partner is needed. Besides requirements regarding environmental conditions are less rigid in homothallic than heterothallic species (Dyer & O'Gorman, 2011; Paoletti *et al.*, 2007). Despite the difficulty to set up the correct conditions for sexual reproduction in the laboratory, successful sexual crosses can be used as a tool to develop recombinant strains with improved industrial characteristics in *P. chrysogenum* (Böhm *et al.*, 2013). This approach offers many advantages compared with other genetic recombination technologies for strain improvement (Adrio & Demain, 2006). For instance, recombination in the whole genome provides a significant genetic variation useful at the time of screening. In industry, the optimization of one strain requires that multiple genes have to be studied and manipulated one by one to obtain a novel product, a process that might take a long time before obtaining an applicable result and which could end in genetic instability (Böhm *et al.*, 2013; Schoustra *et al.*, 2010). Moreover, continued random mutagenesis can lead to undesirable deleterious mutations and multiple point mutations. Crossing targeted strains with interesting traits can avoid the need for prior knowledge of the genetic basis, providing a faster and cheaper procedure (Van Den Berg *et al.*, 2008).

There have been some studies conducted in entomopathogenic fungi, like *Ophiocordyceps sinensis* in which the knowledge of the mating type genes has been used as a tool for investigating the mating systems and population structure (Bushley *et al.*, 2013). In contrast to what has happened with other teleomorphic ascomycetes such as *C. militaris* or *C. bassiana*, in *O. sinensis* it has not been possible to have successful mating crosses under laboratory conditions (Bushley *et al.*, 2013). *In vitro* conditions to promote the development of sexual structures as fruiting bodies, stromata or perithecia are quite variable and depend on each fungal strain, making it a difficult procedure to go through, as has been reported for some *Aspergillus* and *Penicillium* (Dyer & O'Gorman, 2011). Following the same conditions used in successful studies for one fungal species or set of strains does not ensure success with other species or strains (Yoon *et al.*, 2003). That is why it is important to find and develop an adequate methodology that fits better and can promote the growth of sexual structures in a specific fungus. For instance, in the entomopathogenic fungus *C. bassiana* there are continuous modifications during the process of fruiting body generation which includes changes in incubation temperature from 25 °C to 20 °C to obtain erect columnar stromata or changes in the media from SDA supplemented with 1% yeast extract (SDAY) to brown rice/silkworm medium because synnemata are not being formed on SDA (Sung *et al.*, 2006). In *S. macrospora*, starch agar allowed vegetative growth, but the addition of corn meal extract induced the formation of fruiting bodies besides the presence of biotin and arginine (Bahn & Hock, 1974; Molowitz *et al.*, 1976). They also found, as for *P. crhysogenum*, that without the presence of biotin in the medium the formation of fruiting bodies does not occur. Arginine as a supplement in *S. mabrospora* cultures had shown to influence on the acceleration of fruiting body formation (Böhm *et al.*, 2013; Dyer & O'gorman, 2012; Dyer & O'Gorman, 2011; Molowitz *et al.*, 1976). The medium and environmental conditions have proved to be critical at the time of induction for sexual reproduction. Oatmeal agar as a basal medium have revealed to be a good source of nutrients for the generation of sexual stages, not only for some species of *Penicillium* and *Aspergillus*, but also for some *Beauveria* species (*B. bassiana* and *B. hoplocheli*) (Böhm *et al.*, 2013; Chase *et al.*, 1986; Fernandes *et al.*, 2006; Robène-Soustrade *et al.*, 2015). This now has been confirmed in the present

research. However, biotin was not a determinant factor at the time of synnemata initiated in the cross 4 x 32. Taking into account that not all crosses in media supplemented with biotin generated synnemata in the present research or in the study done with *P. crhysogenum*. This suggests that fungi considered as strictly asexual could have a continuum for sexual fertility (Böhm *et al.*, 2013; Dyer & Paoletti, 2005).

The time of incubation is another critical factor to obtain structural organs from heterothallic or homothallic fungi. Depending on the fungal species it could takes between 5 weeks and up to 12 months to find any sexual structures. For example, in *C. bassiana* it could take around 3 months to obtain mature stromata (Sung *et al.*, 2006), in *P. chrysogenum* with addition of biotin, cleistothecia with viable ascospores were found after 5 to 6 weeks (Böhm *et al.*, 2013), in *A. fumigatus, A. flavus, A. parasiticus, A. nomius* and *P. tropicoides*, incubation may be as prolonged as 3 to 12 month (Dyer & O'Gorman, 2011; Horn *et al.*, 2009; Houbraken *et al.*, 2008; O'Gorman *et al.*, 2009). In the present study with *B. bassiana* 6 months was needed to observe branch growth and possible synnemata within the OA medium only in one cross (4 x 32) and three months after injected *Galleria* larvae, only in one cross as well (42 x 21).

Regarding vegetative compatibility, in general this is not related with sexual compatibility and they are mostly independent at each other, although in some fungi (e.g. *N. crassa*) it is known that the pairing of both idiomorphs (MAT 1 and MAT 2) would prevent vegetative hyphal fusion (Glass *et al.*, 2000). In addition, a sexual stage has been found amongst strains that belonged to different VCGs in *A. parasiticus*, which means that despite those strains being vegetatively incompatible, this was not a barrier for genetic exchange to occur (Horn *et al.*, 2009). In contrast, in the strains of the current research this was not evident, because synnemata formation was observed in two crosses, 4 x 32 and 42 x 21, where parental strains involved in the crosses belong to the same VCGs in both cases (i.e., VCG 7).

5 General Discussion

In my opinion, it is possible to obtain more effective biopesticides through the recombination of resilient hybrids of EPF *Beauveria spp.*, using the different approaches described. In order to control insect pest avoiding the use of chemical pesticides in the field, it is necessary a product based on microorganisms able to overcome extreme environmental conditions. High temperatures and high UV-B radiation are responsible for vast majority of the reduction in sporulation and virulence on EPFs. The results herein presented represent a valuable source of information to solve this problem. Recombinant fungal strains with improved characteristics can be selected for further studies involving new approaches, e.g. formulation, in order to develop a better biological product. In the present research the best approach to obtain breeding improved strains was protoplast fusion to cross *B. bassiana* strains with complementary traits. The advantages offered by this method include the elimination of the need for vegetatively compatible strains to generate hybrids. However, the utilization of auxotrophic mutants is still required. Nit-M and Nit-1 mutants isolated from hyphal anastomosis trials were used for protoplast fusion in 8 different combinations (**Table 3.3**). The same criteria used in hyphal anastomosis trials for selection of strains intended for hybrid generation was used in this experiment. Positive results were found, as former auxotrophic strains recovered the wild type phenotype after protoplast fusion, showing aerial growth on media supplemented with nitrate. All combinations yielded prototrophic strains, yet only three combinations (named as S (49 x 29), U (41 x 42) and X (42 x 29)) were selected based on the stability of the colonies formed, to perform a phenotypic and molecular characterization to confirm that a recombination event had indeed occurred. All hybrids analyzed showed a performance like their fungal parents regarding growth and conidia germination at different temperatures, tolerance to UV-B radiation and virulence against *Plutella xylostella*. One hybrid named as X2, obtained from the protoplast fusion of strains 29 (*B. pseudobassiana*) and 42 (*B. bassiana*) showed an improvement in speed of growth at different temperatures (**Figure 3.9, C**), more rapid growth could be important for mass production. Considering that one of the fungal parents (strain 29) was identified as the strain with

the highest virulence, obtaining a hybrid with enhanced growth capacity at optimal temperature certainly represent an interesting discovery and makes it a good candidate for further experiments. The exchange of genetic material in hybrids were evaluated by the utilization of three different molecular markers (elongation factor, DNA lyase and ITS). Results suggest that some of the strains obtained after protoplast fusion had been reverted to the wild type form of one of the progenitors. However, hybrids formed from protoplast fusion of strains 29 and 42 (X2) appeared to be the result of an exchange of genetic material, which would explain the improved growth at optimal temperature observed for hybrid X2 (**Figure 3.12**).

In principle, strain improvement programs may require the analysis of thousands of hybrids in order to identify a small number of individuals with enhanced properties. Thus, the identification of an improved strain is very rare (Aiuchi *et al.*, 2008b; Castrillo *et al.*, 2004; Zhang *et al.*, 2016). Considering the limitations of this study, including the restricted number of hybrids analyzed in experiments, the results obtained in this study can be considered as satisfactory, since two parasexual approaches have been evaluated for their potential to produce improved strains, their feasibility has been demonstrated, the methodology has been set and described, the VCGs and self-compatible characterization of 50 different strains have been obtained. A bank of Nit-mutants for these strains has been created whose phenotype has been already determined, and a hybrid with one enhanced trait has been obtained (X2).

Another approach was investigated with the aim of generating genetic recombination between selected fungal strains. Hyphal anastomosis of a total of 7 fungal strains selected because had desirable traits with respect to virulence to DBM, thermal biology, or tolerance of UV-B. Parasexual crosses were set up between strains that had complementary phenotypes (**Table 3.2**). For instance, strain 29 showed the highest virulence, yet it was sensitive to UV-B radiation, and therefore was a candidate for crossing with strains showing high UV-B tolerance in order to select a recombinant with high virulence and UV tolerance. The fusion between hyphae (hyphal anastomosis) from two parental fungi to create a hybrid cell whose cytoplasmic material has been exchanged and the genetic material could have been fused in one nucleus (unstable diploid strain) or remain as separate nuclei in one cell

(heterokaryon), requires that parental strains must be vegetatively compatible with each other (Castrillo *et al.*, 2004). Even further, for the generation of stable hybrids (stable heterokaryons), the involvement of self-compatible parental fungal strains is critical (Leslie, 1993). Therefore, prior to hyphal fusion trials, an experiment was carried out to determine the vegetative compatibility amongst the selected strains, and self-compatibility of each individual strain used. It should be noted that the possible crosses allowed by hyphal anastomosis were constrained by the results obtained in vegetative compatibility and self-compatibility assays.

Vegetative compatibility and self-compatibility assays require, in the first instance the generation of Nit-mutants (auxotrophic nitrate non-utilizing mutants), which show a non-aerial growth on minimal media supplemented with nitrate as the sole nitrogen source (**Figure 3.1**). Nit-mutants were obtained by the selection of spontaneous mutants of a given strain growing in water agar plates supplemented with 6% of potassium chlorate, which was found to be an optimal chlorate concentration for most of the strains used in this study. This is an uncommonly high concentration of chlorate compared with the 1.5 % to 2 % reported for the generation of Nit-mutants in other studies in *L. lecanni* or *V. dahliae* (Aiuchi *et al.*, 2008b; Korolev & Katan, 1997). A high concentration of potassium chlorate also helped to diminish the number of steps required for isolation and purification of single strains, after Nit-mutant generation. A total of 30 Nit-mutants were collected from each selected strain and they were subjected to a phenotypic characterization to identify them as: Nit-1, Nit-2, Nit-3 or Nit-M mutants, depending on their capability for growth on different media (**Table 3.1** and **Figure 3.4**); since it has been reported that the formation of stable heterokaryons only occurs when a Nit-M mutant is fused with either a Nit-1 or Nit-3 mutant (Correll *et al.*, 1987). A successful fusion between mutants derived from two different strains will indicate that these strains are vegetatively compatible; whereas a fusion of mutants derived from a single strain will indicate that the strain in question is self-compatible. A positive fusion recovers the lost function of the auxotrophic mutant, resulting in prototrophic aerial growth in the contact zone between the paired strains. Only self-compatible strains can lead to the formation of stable heterokaryons, thus only strains that fulfil these requirements can be used for crosses in hyphal anastomosis experiments.

None of the selected strains were vegetatively compatible when hyphal anastomosis between strains was investigated, leading to the conclusion that all seven *Beauveria* strains were vegetatively incompatible. Therefore, it was decided to extend the work to include all 50 strains of the fungal collection to determine their vegetative compatible groups. The same procedure was followed to generate Nit-mutants and a collection of 30 Nit-mutants were derived from each strain, together with a phenotypic characterization of each mutant and trials of self-compatibility and vegetative compatibility. Results showed the presence of 35 vegetative compatibility groups (VCG's) in the fungal collection (**Figure 3.6**), and 28 self-compatible strains. The information provided from these experiments is highly valuable, since the VCGs have been revealed and the self-compatible strains have been determined in this study, thus allowing further experiments to be conducted in future with these strains. Moreover, the crosses between different strains from the same VCG were able to form stable heterokaryons, indicating that hyphal anastomosis had occurred. Time restrictions did not allow the characterization of these hybrids. However, the basic principles and the feasibility of using this approach to generate hybrids with mixed genetic material was demonstrated. It was observed that formation of Nit-mutants demands a chlorate concentration that can vary depending on each strain, so that screening of this condition is recommended for the generation of Nit-mutants in other fungal collections.

For sexual recombination it is important to mention that in the filamentous ascomycetes is uncommon, albeit that hidden sexual stages have been reported for some species (Gow, 2005). Achieving sexual reproduction for certain fungi can be a challenging endeavour, as this process only occurs under extremely well-defined culture conditions, which tend to be particular and different for each species, and the development of sexual structures can take several months (Dyer & O'Gorman, 2011; O'Gorman *et al.*, 2009). For heterothallic fungi (such as *B. bassiana*), it is known that sexual reproduction requires the involvement of two fungal individuals with opposite mating types. Hence, in this study an identification of Mat genes was carried out for all 50 strains used in this study (**Table 4.4**). Then, several strains were selected and paired together with the aim of observing the production of perithecial (sexual) fruiting bodies (**Table 4.2** and **Table 4.3**). This was done using both *in vitro* and *in vivo*

methods. For *in vitro* trials, pairings of two strains with opposite mating types genes were performed using three different media: oatmeal agar, malt extract agar, and Czapek Dox agar. Cultures were monitored twice per month for morphological changes in the contact zone including the presence of fruiting bodies. As reported before (Böhm *et al.*, 2013; Fernandes *et al.*, 2006) oatmeal agar promoted a better growth of sexual structures whereas the presence of biotin seemed to have a minimal effect in the present study. The formation of perithecial fruiting bodies was not observed, even after 6 months of incubation, however the formation of synnemata was found in only one combination (strain 4 (*B. bassiana*) x strain 32 (*B. bassiana*)) in oatmeal agar (**Figure 4.4**). For the *in vitro* assays, larvae of *Galleria mellonella* were injected with a mixture of conidia suspension of Mat1 x Mat2 strains. In this case, four combinations were evaluated and only one of them (*B. bassiana* strain 11 (Mat1) x *B. bassiana* strain 21 (Mat2)) resulted in the production of synnemata on the surface of an infected *G. mellonella* larvae although no perithecia were observed (**Figure 4.5**). There are little evidences that supports the occurrence of sexual reproduction in *Beauveria* species, and it is unknown whether these fungi are strictly parasexual or if they can undergo sexual reproduction. Conditions for *in vitro* mating of *Beauveria* have not been identified so far, and synnemata in *Beauveria* also appear to be rare. Nevertheless, *B. bassiana* has proved to have the capacity to form complex multicellular structures, which may be a requirement for the production of a sexual fruiting body, as well as the initiation of the sexual cycle (Yoon *et al.*, 2003). *In vitro* sexual reproduction is more achievable in homothallic fungi, because a single homothallic individual does not require a mating partner and also their requirements regarding environmental conditions are less rigid (O'Gorman *et al.*, 2009; Paoletti *et al.*, 2007). Despite the difficulties related with sexual recombination, this methodology has potential to allow the production of improved strains without the need for a previous knowledge of the genetic basis of progenitors, making it a faster and cheaper option. The results showed in this study shed some light on the sexual behavior of *B. bassiana*, provided a mating type profile for 50 strains of *Beauveria* and has shown the possible formation of synnemata in both *in vitro* and *in vivo* assays, increasing the current knowledge of this interesting field of research.

5.1 Conclusions

- *B. bassiana* has been shown to have potential as an entomopathogenic fungus for bioinsecticides formulations, due to its capacity to kill important crop pests. However, different strains perform differently, meaning that a characterization of the most important traits (e.g. tolerance to environmental conditions or virulence) is highly recommended for any strain intended for bioinsecticides production.
- No relationship was found between the geographical origin of *B. bassiana* strains and their tolerance to temperature or UV-B radiation. This observation does not mean that there is no an environmental effect on the phenotype exhibited by a fungal strain. Conversely, it suggests that the collection of fungal isolates should take into account the micro and macro environmental conditions of the sampling location, rather than being limited only to its geographical coordinates.
- Improvement of fungal strains is a challenging endeavour due to the complex life cycles shown by the fungi kingdom. Though hyphal anastomosis has been demonstrated as a feasible technique to obtain parasexual recombinant hybrids, it should be noted that a previous determination of vegetative and self-compatibility groups is required, which tends to be a laborious and a time-consuming task. The protoplast fusion approach was shown to be easier and faster, yet it can be more interspecific, and mutants can revert to a wild type state of one of the progenitors.
- The generation and utilization of Nit-mutants for parasexual recombination is a reliable method to obtain auxotrophic strains, albeit chlorate concentration used for the generation of these mutants were shown to be specific for each strain and tended to be uncommonly high (6%) for most of the *B. bassiana* strains tested. The applications of these mutants reached beyond parasexual recombination, as auxotrophic strains are used in other fields of research, such as biochemical characterization or in molecular biology approaches.

- When paired together, some *B. bassiana* strain combinations were able to form structures resembling synnemata, which might be a previous stage of the production of fruiting bodies (Sung *et al.*, 2006). Structures similar to synnemata were produced both in vivo and in vitro, suggesting that these structures could be more common than previously thought, however more research is needed on this field.

5.2 Future work

The information provided by the characterization of the fungal collection used in this study (50 isolates of *Beauveria*) represent a valuable source of information to carry on future basic and applied research. This includes investigations of other methods of strain improvement, such as through gene editing, gene silencing or through GM approaches, or studies of the potential to apply mixtures of strains with complementary characteristics against pest populations (e.g. a high virulence strain combined with a strain with high conidial production or tolerance to UV-B radiation). Phylogenetic results from this study could be used as input data in bigger alignments of *Beauveria* sequences, than can help to expand and improve the current accuracy of the phylogenetic tree of this interesting fungus.

The methodologies described here for parasexual and sexual recombination can be used for other isolates of *Beauveria* and even in other entomopathogenic fungi, as well the methodology described for the generation of auxotrophic Nit-mutants. The obtained Nit-mutants themselves can be used to perform other combinations using hyphal anastomosis and protoplast fusion approaches, and also by using other methodologies of recombination. Several hybrids were generated but due to time constrains it was not possible to characterize them. Thus, these strains could be characterized to see if any of them show enhanced phenotypes.

Despite being time demanding, the methodology used for sexual recombination and its result (possible synnemata) could be used in further experiments regarding the application of these methodologies with other strains of *Beauveria* and even in other fungi, and in the generation of more of these structures that could be examined in dept, which would help us to better understand the

possible sexual behaviour of this fungus. Formation of synnemata can be also promoted by SDAY and silkworm media. It would thus be interesting to try this methodology with isolates of the fungal collection used in this study. Additionally, it would also be interesting to obtain the sexually reproducing strains of *C. bassiana* from Korea and see if they will produce perithecia when crossed with some of the strains used in the current study.

Technologies based on CRISPR-Cas9 are quickly becoming a commonly used technique in laboratories around the world, as they are simpler and more reliable for genetic engineering of filamentous fungi. CRISPR-Cas9 vectors equipped with fungal markers could be used for engineering of *Beauveria* strains, as it has been already reported for other fungal species including *Aspergillus aculeatus* and *Trichoderma reesei* (Nødvig *et al.*, 2015). Despite the progress made in this study, there is still much work to be done to increase the current knowledge, gain a deeper understanding and develop new techniques that could be applied in the improvement of *B. bassiana* as an entomopathogenic fungus for crop protection.

6 References

Adams, G., Johnson, N., Leslie, J. F. & Hart, L. P. (1987) Heterokaryons of Gibberella zeae formed following hyphal anastomosis or protoplast fusion. *Experimental Mycology*, 11 (4): 339-353.

Adams, T. H., Wieser, J. K. & Yu, J.-H. (1998) Asexual sporulation in Aspergillus nidulans. *Microbiology and molecular biology reviews*, 62 (1): 35-54.

Adane, K., Moore, D. & Archer, S. (1996) Preliminary studies on the use of Beauveria bassiana to control Sitophilus zeamais (Coleoptera: Curculionidae) in the laboratory. *Journal of Stored Products Research*, 32 (2): 105-113.

Adrio, J. L. & Demain, A. L. (2006) Genetic improvement of processes yielding microbial products. *FEMS microbiology reviews*, 30 (2): 187-214.

Aiuchi, D., Baba, Y., Inami, K., Shinya, R., Tani, M. & Koike, M. (2008a) Variation in growth at different temperatures and production and size of conidia in hybrid strains of Verticillium lecanii (Lecanicillium spp.) (Deuteromycotina: Hyphomycetes). *Applied Entomology and Zoology*, 43 427-436.

Aiuchi, D., Inami, K., Sugimoto, M., Shinya, R., Tani, M., Kuramochi, K. & Koike, M. (2008b) A new method for producing hybrid strains of the entomopathogenic fungus Verticillium lecanii (Lecanicillium spp.) through protoplast fusion by using nitrate non-utilizing (nit) mutants. *Micologia Aplicada International*, 20 (1):

Aiuchi, D., Koike, M., Tani, M., Kuramochi, K., Sugimoto, M. & Nagao, H. (2004) Protoplast fusion, using nitrate non-utilizing (nit) mutants in the entomopathogenic fungus Verticillium lecanii (Lecanicillium spp.). *S. Michele all'Adige, Italy*, 27 (8): 127-130.

Akimov, G. & Portenko, L. (1996) Studying vegetative compatibility in Verticillium dahliae Kleb. with non-nitrate-utilizing mutants. *Russian Journal of Genetics*, 32 (2): 184-189.

Altre, J., Vandenberg, J. & Cantone, F. (1999) Pathogenicity ofPaecilomyces fumosoroseusIsolates to Diamondback Moth, Plutella xylostella: Correlation with Spore Size, Germination Speed, and Attachment to Cuticle. *Journal of Invertebrate Pathology*, 73 (3): 332-338.

Amnuaykanjanasin, A., Jirakkakul, J., Panyasiri, C., Panyarakkit, P., Nounurai, P., Chantasingh, D., Eurwilaichitr, L., Cheevadhanarak, S. & Tanticharoen, M. (2013) Infection and colonization of tissues of the aphid Myzus persicae and cassava mealybug Phenacoccus manihoti by the fungus Beauveria bassiana. *BioControl*, 58 379-391.

Andersen, M., Magan, N., Mead, A. & Chandler, D. (2006) Development of a population-based threshold model of conidial germination for analysing the effects of physiological manipulation on the stress tolerance and infectivity of insect pathogenic fungi. *Environmental Microbiology*, 8 (9): 1625-1634.

Asi, M. R., Bashir, M. H., Afzal, M., Zia, K. & Akram, M. (2013) Potential of entomopathogenic fungi for biocontrol of Spodoptera litura fabricius (Lepidoptera: Noctuidae). *Journal of Animal and Plant Sciences*, 23 913-918.

Bagagli, E., Valadares, M. & Azevedo, J. (1991) Parameiosis in the entomopathogenic fungus Metarhizium anisopliae (Metsh.) Sorokin. *Revista Brasileira de Genética*, 14 (2): 261-271.

Bahn, M. & Hock, B. (1974) Morphogenese von Sordaria macrospora: Induktion der Perithezienbildung durch Arginin. *Berichte der Deutschen Botanischen Gesellschaft*, 87 (3): 433-442.

Bayman, P. & Cotty, P. J. (1991) Improved media for selecting nitrate-nonutilizing mutants in Aspergillus flavus. *Mycologia*, 311-316.

Bidochka, M. J. & Khachatourians, G. G. (1990) Identification of Beauveria bassiana extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, Melanoplus sanguinipes. *Journal of Invertebrate Pathology*, 56 (3): 362-370.

Bidochka, M. J., Menzies, F. V. & Kamp, A. M. (2002) Genetic groups of the insect-pathogenic fungus Beauveria bassiana are associated with habitat and thermal growth preferences. *Archives of Microbiology*, 178 (6): 531-537.

Billiard, S., Lopez-Villavicencio, M., Devier, B., Hood, M. E., Fairhead, C. & Giraud, T. (2011) Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biol Rev Camb Philos Soc*, 86 (2): 421-442.

Billiard, S., Lopez-Villavicencio, M., Hood, M. E. & Giraud, T. (2012) Sex, outcrossing and mating types: unsolved questions in fungi and beyond. *J Evol Biol*, 25 (6): 1020-1038.

Bonatelli, R., Azevedo, J. & Valent, G. U. (1983) PARASEXUALITY IN A CITRIC-ACID PRODUCING STRAIN OF ASPERGILLUS-NIGER. *Revista Brasileira de Genética*,

Boomsma, J. J., Jensen, A. B., Meyling, N. V. & Eilenberg, J. (2014) Evolutionary interaction networks of insect pathogenic fungi. *Annual Review of Entomology*, 59 467-485.

Borisade, O. & Magan, N. (2014) Growth and sporulation of entomopathogenic Beauveria bassiana, Metarhizium anisopliae, Isaria farinosa and Isaria fumosorosea

strains in relation to water activity and temperature interactions. *Biocontrol science and technology*, 24 (9): 999-1011.

Braga, G. U., Flint, S. D., Miller, C. D., Anderson, A. J. & Roberts, D. W. (2001) Variability in response to UV-B among species and strains of Metarhizium isolated from sites at latitudes from 61 N to 54 S. *Journal of Invertebrate Pathology*, 78 (2): 98-108.

Brewer, M. T., Cadle-Davidson, L., Cortesi, P., Spanu, P. D. & Milgroom, M. G. (2011) Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi. *Fungal Genetics and Biology*, 48 (7): 704-713.

Brooker, N. L., Leslie, J. F. & Dickman, M. B. (1991) Nitrate non-utilizing mutants of Colletotrichum and their use in studies of vegetative compatibility and genetic relatedness. *Phytopathology*, 81 (6): 672-677.

Bugeme, D. M., Maniania, N. K., Knapp, M. & Boga, H. I. (2008) Effect of temperature on virulence of Beauveria bassiana and Metarhizium anisopliae isolates to Tetranychus evansi. In: *Diseases of Mites and Ticks*. Springer: 275-285.

Bushley, K. E., Li, Y., Wang, W. J., Wang, X. L., Jiao, L., Spatafora, J. W. & Yao, Y. J. (2013) Isolation of the MAT1-1 mating type idiomorph and evidence for selfing in the Chinese medicinal fungus Ophiocordyceps sinensis. *Fungal Biol*, 117 (9): 599-610.

Busvine, J. R. (1967) *A critical review of the techniques for testing insecticides*. Commonwealth Institute Of Entomology; London.

Butler, G., Rasmussen, M. D., Lin, M. F., Santos, M. A., Sakthikumar, S., Munro, C. A., Rheinbay, E., Grabherr, M., Forche, A. & Reedy, J. L. (2009) Evolution of pathogenicity and sexual reproduction in eight Candida genomes. *Nature*, 459 (7247): 657.

Böhm, J., Hoff, B., Gorman, C. M. O., Wolfers, S., Klix, V., Binger, D. & Zadra, I. (2013) Sexual reproduction and mating-type – mediated strain development in the penicillin-producing fungus Penicillium chrysogenum. *Pnas*, 110 1476-1481.

Cabanillas, H. E. & Jones, W. A. (2009) Effects of temperature and culture media on vegetative growth of an entomopathogenic fungus Isaria sp.(Hypocreales: Clavicipitaceae) naturally affecting the whitefly, Bemisia tabaci in Texas. *Mycopathologia*, 167 (5): 263.

Campbell, C., Leslie, J. & Farrokhi-Nejad, R. (1992) Genetic diversity of Fusarium moniliforme in seed from two maize cultivars. *Phytopathology*, 82 1082.

Castrillo, L. A., Griggs, M. H., Ranger, C. M., Reding, M. E. & Vandenberg, J. D. (2011) Virulence of commercial strains of Beauveria bassiana and Metarhizium brunneum (Ascomycota: Hypocreales) against adult Xylosandrus germanus (Coleoptera: Curculionidae) and impact on brood. *Biological Control*, 58 (2): 121-126.

Castrillo, L. A., Griggs, M. H. & Vandenberg, J. D. (2004) Vegetative compatibility groups in indigenous and mass-released strains of the entomopathogenic fungus Beauveria bassiana: likelihood of recombination in the field. *J Invertebr Pathol*, 86 (1-2): 26-37.

Chandler, D. (1994) Cryopreservation of fungal spores using porous beads. *Mycological research*, 98 (5): 525-526.

Chandler, D. (2017) Basic and Applied Research on Entomopathogenic Fungi. In: *Microbial Control of Insect and Mite Pests*. Elsevier: 69-89.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J. & Grant, W. P. (2011) The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 366 (1573): 1987-1998.

Chandler, D., Grant, W. P. & Greaves, J. (2010) *Biopesticides: pest management and regulation*. CABI.

Chase, A., Osborne, L. & Ferguson, V. (1986) Selective isolation of the entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae from an artificial potting medium. *Florida Entomologist*, 285-292.

Chen, W. (1994) Vegetative compatibility groups of Verticillium dahliae from ornamental woody plants. *PHYTOPATHOLOGY-NEW YORK AND BALTIMORE THEN ST PAUL-*, 84 214-214.

Collado-Romero, M., Jiménez-Díaz, R. M. & Mercado-Blanco, J. (2010) DNA sequence analysis of conserved genes reveals hybridization events that increase genetic diversity in Verticillium dahliae. *Fungal biology*, 114 (2-3): 209-218.

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997) Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews*, 61 (4): 411-428.

Copping, L. G. & Menn, J. J. (2000) Biopesticides: a review of their action, applications and efficacy. *Pest Management Science: formerly Pesticide Science*, 56 (8): 651-676.

Correll, J., Gordon, T. & McCain, A. (1988) Vegetative compatibility and pathogenicity of Verticillium albo-atrum. *Phytopathology*, 78 (8): 1017-1021.

Correll, J., Klittich, C. & Leslie, J. (1987) Nitrate nonutilizing mutants of Fusarium oxysporum and their use in vegetative compatibility tests. *Phytopathology*, 77 (12): 1640-1646.

Correll, J. C., Klittich, C. J. & Leslie, J. F. (1989) Heterokaryon self-incompatibility in Gibberella fujikuroi (Fusarium moniliforme). *Mycological Research*, 93 (1): 21-27.

Couteaudier, Y. & Viaud, M. (1997) New insights into population structure of Beauveria bassiana with regard to vegetative compatibility groups and telomeric restriction fragment length polymorphisms. *FEMS Microbiology Ecology*, 22 (3): 175-182.

Couteaudier, Y., Viaud, M. & Riba, G. (1996) Genetic nature, stability, and improved virulence of hybrids from protoplast fusion in Beauveria. *Microbial Ecology*, 32 (1): 1-10.

Cove, D. (1976) Chlorate toxicity in Aspergillus nidulans: the selection and characterisation of chlorate resistant mutants. *Heredity*, 36 (2): 191.

Crawford, M. S., Chumley, F. G., Weaver, C. G. & Valent, B. (1986) Characterization of the heterokaryotic and vegetative diploid phases of Magnaporthe grisea. *Genetics*, 114 (4): 1111-1129.

Daayf, F., Nicole, M. & Geiger, J.-P. (1995) Differentiation ofVerticillium dahliae populations on the basis of vegetative compatibility and pathogenicity on cotton. *European Journal of Plant Pathology*, 101 (1): 69-79.

Davidson, G., Phelps, K., Sunderland, K., Pell, J., Ball, B., Shaw, K. & Chandler, D. (2003) Study of temperature–growth interactions of entomopathogenic fungi with potential for control of Varroa destructor (Acari: Mesostigmata) using a nonlinear model of poikilotherm development. *Journal of Applied Microbiology*, 94 (5): 816-825.

Debuchy, R., Berteaux-Lecellier, V. & Silar, P. (2010) Mating systems and sexual morphogenesis in ascomycetes. In: *Cellular and molecular biology of filamentous fungi*. American Society of Microbiology: 501-535.

Denholm, I. & Rowland, M. W. (1992) Tactics for managing pesticide resistance in arthropods: theory and practice. *Annual review of entomology*, 37 91-112.

Devi, K. U., Sridevi, V., Mohan, C. M. & Padmavathi, J. (2005) Effect of high temperature and water stress on in vitro germination and growth in isolates of the entomopathogenic fungus Beauveria bassiana (Bals.) Vuillemin. *Journal of invertebrate pathology*, 88 (3): 181-189.

Devine, G. J. & Furlong, M. J. (2007) Insecticide use: contexts and ecological consequences. *Agriculture and Human values*, 24 (3): 281-306.

Drummond, J. & Heale, J. (1988) Genetic studies on the inheritance of pathogenicity in Verticillium lecanii against Trialeurodes vaporariorum. *Journal of invertebrate pathology*, 52 (1): 57-65.

Dyer, P. & Paoletti, M. (2005) Reproduction in Aspergillus fumigatus: sexuality in a supposedly asexual species? *Medical mycology*, 43 (sup1): 7-14.

Dyer, P. S. & O'gorman, C. M. (2012) Sexual development and cryptic sexuality in fungi: insights from Aspergillus species. *FEMS microbiology reviews*, 36 (1): 165-192.

Dyer, P. S. & O'Gorman, C. M. (2011) A fungal sexual revolution: Aspergillus and Penicillium show the way. *Current opinion in microbiology*, 14 (6): 649-654.

Edelstein, J. D., Trumper, E. V. & Lecuona, R. E. (2005) Temperature-dependent development of the entomopathogenic fungus Nomuraea rileyi (Farlow) Samson in Anticarsia gemmatalis (Hübner) larvae (Lepidoptera: Noctuidae). *Neotropical entomology*, 34 (4): 593-599.

Ehler, L. E. (2006) Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. *Pest management science*, 62 (9): 787-789.

Esser, K. & Kuenen, R. (2012) *Genetics of fungi*. Springer Science & Business Media.

Esteves, I., Peteira, B., Atkins, S. D., Magan, N. & Kerry, B. (2009) Production of extracellular enzymes by different isolates of Pochonia chlamydosporia. *Mycological Research*, 113 (8): 867-876.

Fang, W., Azimzadeh, P. & Leger, R. J. S. (2012) Strain improvement of fungal insecticides for controlling insect pests and vector-borne diseases. *Current opinion in microbiology*, 15 (3): 232-238.

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., Feng, J., Yang, X., Zhang, Y. & Pei, Y. (2005) Cloning of Beauveria bassiana chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Applied and environmental microbiology*, 71 (1): 363-370.

Fargues, J., Delmas, J. & Lebrun, R. (1994) Leaf consumption by larvae of the Colorado potato beetle (Coleoptera: Chrysomelidae) infected with the entomopathogen, Beauveria bassiana. *Journal of Economic Entomology*, 87 (1): 67-71.

Fargues, J., Goettel, M., Smits, N., Ouedraogo, A. & Rougier, M. (1997) Effect of temperature on vegetative growth of Beauveria bassiana isolates from different origins. *Mycologia*, 383-392.

Fargues, J., Goettel, M., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L., Lomer, C. & Rougier, M. (1996) Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia*, 135 (3): 171-181.

Faria, M. R. D. & Wraight, S. P. (2007) Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*, 43 237-256.

Fernandes, E. K. K., Costa, G. L., Moraes, Á. M. L., Zahner, V. & Bittencourt, V. R. E. P. (2006) Study on morphology, pathogenicity, and genetic variability of Beauveria bassiana isolates obtained from Boophilus microplus tick. *Parasitology research*, 98 (4): 324-332.

Fernandes, É. K. K., Rangel, D. E. N., Moraes, Á. M. L., Bittencourt, V. R. E. P. & Roberts, D. W. (2007) Variability in tolerance to UV-B radiation among Beauveria spp. isolates. *Journal of Invertebrate Pathology*, 96 237-243.

Fernandes, É. K. K., Rangel, D. E. N., Moraes, Á. M. L., Bittencourt, V. R. E. P. & Roberts, D. W. (2008) Cold activity of Beauveria and Metarhizium, and thermotolerance of Beauveria. *Journal of Invertebrate Pathology*, 98 69-78.

Foster, P. L. (2005) Stress responses and genetic variation in bacteria. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 569 (1): 3-11.

Furlaneto, M. C. & Pizzirani-Kleiner, A. A. (1992) Intraspecific hybridisation of Trichoderma pseudokoningii by anastomosis and by protoplast fusion. *FEMS microbiology letters*, 90 (2): 191-196.

Furlong, M. J., Zu-Hua, S., Yin-Quan, L., Shi-Jian, G., Yao-Bin, L., Shu-Sheng, L. & Zalucki, M. P. (2004) Experimental analysis of the influence of pest management practice on the efficacy of an endemic arthropod natural enemy complex of the diamondback moth. *Journal of Economic Entomology*, 97 (6): 1814-1827.

Gadau, M. & Lingg, A. (1992) Protoplast fusion in fungi. *Handbook of applied mycology*, 4 101-129.

Geiser, D. M., Timberlake, W. E. & Arnold, M. L. (1996) Loss of meiosis in Aspergillus. *Molecular Biology and Evolution*, 13 (6): 809-817.

Ghikas, D. V., Kouvelis, V. N. & Typas, M. A. (2010) Phylogenetic and biogeographic implications inferred by mitochondrial intergenic region analyses and ITS1-5.8 S-ITS2 of the entomopathogenic fungi Beauveria bassiana and B. brongniartii. *BMC microbiology*, 10 (1): 174.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L. & Stewart, A. (2012) Have biopesticides come of age? *Trends in biotechnology*, 30 (5): 250-258.

Glass, N. L., Jacobson, D. J. & Shiu, P. K. (2000) The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual review of genetics*, 34 (1): 165-186.

Glass, N. L. & Kuldau, G. A. (1992) Mating type and vegetative incompatibility in filamentous ascomycetes. *Annual review of phytopathology*, 30 (1): 201-224.

Gow, N. A. (2005) Fungal genomics: forensic evidence of sexual activity. *Current biology*, 15 (13): R509-R511.

Griffin, M. R. (2007) Beauveria bassiana, a cotton endophyte with biocontrol activity against seedling disease.

Grognet, P. & Silar, P. (2015) Maintaining heterokaryosis in pseudo-homothallic fungi. *Communicative & integrative biology*, 8 (4): e994382.

Grzywacz, D., Rossbach, A., Rauf, A., Russell, D., Srinivasan, R. & Shelton, A. (2010) Current control methods for diamondback moth and other brassica insect pests and the prospects for improved management with lepidopteran-resistant Bt vegetable brassicas in Asia and Africa. *Crop Protection*, 29 (1): 68-79.

Hallsworth, J. E. & Magan, N. (1999) Water and temperature relations of growth of the entomogenous fungi Beauveria bassiana, Metarhizium anisopliae, and Paecilomyces farinosus. *Journal of invertebrate pathology*, 74 (3): 261-266.

Harcourt, D. (1957) Biology of the diamondback moth, Plutella maculipennis (Curt.)(Lepidoptera: Plutellidae), in eastern Ontario. II. Life-history, behaviour, and host relationships. *The Canadian Entomologist*, 89 (12): 554-564.

Hartl, D. L. & Clark, A. G. (1998) Principles of population genetics.

Hassan, E. (2014) Advances in Plant Biopesticides.

Hassan, E. & Gökçe, A. (2014) Production and consumption of biopesticides. In: *Advances in Plant Biopesticides*. Springer: 361-379.

Hastie, A. & Heale, J. (1984) Genetics of Verticillium. *Phytopathologia Mediterranea*, 130-162.

Hawthorne, B. & Rees-George, J. (1996) Use of nitrate non-utilizing mutants to study vegetative incompatibility in Fusarium solani (Nectria haematococca), especially members of mating populations I, V and VI. *Mycological Research*, 100 (9): 1075-1081.

Heitman, J. (2010) Evolution of eukaryotic microbial pathogens via covert sexual reproduction. *Cell host & microbe*, 8 (1): 86-99.

Henk, D. A., Eagle, C., Brown, K., Van Den Berg, M., Dyer, P., Peterson, S. & Fisher, M. (2011) Speciation despite globally overlapping distributions in Penicillium chrysogenum: the population genetics of Alexander Fleming's lucky fungus. *Molecular Ecology*, 20 (20): 4288-4301.

Henk, D. A. & Fisher, M. C. (2011) Genetic diversity, recombination, and divergence in animal associated Penicillium dipodomyis. *PLoS One*, 6 (8): e22883.

Hoff, B., Pöggeler, S. & Kück, U. (2008) Eighty years after its discovery, Fleming's Penicillium strain discloses the secret of its sex. *Eukaryotic Cell*, 7 (3): 465-470.

Horn, B. W., Ramirez-Prado, J. H. & Carbone, I. (2009) Sexual reproduction and recombination in the aflatoxin-producing fungus Aspergillus parasiticus. *Fungal Genet Biol*, 46 (2): 169-175.

Horrigan, L., Lawrence, R. S. & Walker, P. (2002) How sustainable agriculture can address the environmental and human health harms of industrial agriculture. *Environmental health perspectives*, 110 (5): 445.

Houbraken, J., Varga, J., Rico-Munoz, E., Johnson, S. & Samson, R. A. (2008) Sexual reproduction as the cause of heat resistance in the food spoilage fungus Byssochlamys spectabilis (anamorph Paecilomyces variotii). *Applied and Environmental Microbiology*, 74 (5): 1613-1619.

Huang, B. F. & Feng, M. G. (2009) Comparative tolerances of various Beauveria bassiana isolates to UV-B irradiation with a description of a modeling method to assess lethal dose. *Mycopathologia*, 168 (3): 145-152.

Hussein, K. A., Abdel-Rahman, M. A., Abdel-Mallek, A. Y., El-Maraghy, S. S. & Joo, J. H. (2012) Pathogenicity of Beauveria bassiana and Metarhizium anisopliae against Galleria mellonella. *Phytoparasitica*, 40 (2): 117-126.

Inglis, G. D., Johnson, D. L. & Goettel, M. S. (1996) Effects of temperature and thermoregulation on mycosis by Beauveria bassiana in grasshoppers. *Biological Control*, 7 (2): 131-139.

Isman, M. B. (2006) Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu. Rev. Entomol.*, 51 45-66.

James, R., Croft, B., Shaffer, B. & Lighthart, B. (1998) Impact of temperature and humidity on host–pathogen interactions between Beauveria bassiana and a coccinellid. *Environmental Entomology*, 27 (6): 1506-1513.

Joaquim, T. & Rowe, R. (1991) Vegetative compatibility and virulence of strains of Verticillium dahliae from soil and potato plants. *Phytopathology*, 81 (5): 552-558.

Joaquim, T. R. & Rowe, R. C. (1990) Reassessment of vegetative compatibility relationships among strains of Verticillium dahliae using nitrate-nonutilizing mutants. *reactions*, 4 (40): 41.

Kaaya, G. P. & Hassan, S. (2000) Entomogenous fungi as promising biopesticides for tick control. *Experimental & applied acarology*, 24 (12): 913-926.

Karapapa, V., Bainbridge, B. & Heale, J. (1997) Morphological and molecular characterization of Verticillium longisporum comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101 (11): 1281-1294.

Katan, T. (2000) Vegetative compatibility in populations of Verticillium—an overview. *Advances in Verticillium research and disease management*, 69-86.

Kedera, C., Leslie, J. & Claflin, L. (1994) Genetic diversity of Fusarium section Liseola (Gibberella fujikuroi) in individual maize stalks. *Phytopathology (USA)*,

Kepler, R. M., Humber, R. A., Bischoff, J. F. & Rehner, S. A. (2014) Clarification of generic and species boundaries for Metarhizium and related fungi through multigene phylogenetics. *Mycologia*, 106 (4): 811-829.

Khachatourians, G. G. & Qazi, S. S. (2008) Entomopathogenic fungi: biochemistry and molecular biology. In: *Human and Animal Relationships*. Springer: 33-61.

Kim, J. S., Skinner, M., Gouli, S. & Parker, B. L. (2011) Generating thermotolerant colonies by pairing Beauveria bassiana isolates. *FEMS Microbiology Letters*, 324 165-172.

Klass, J. I., Blanford, S. & Thomas, M. B. (2007) Development of a model for evaluating the effects of environmental temperature and thermal behaviour on biological control of locusts and grasshoppers using pathogens. *Agricultural and Forest Entomology*, 9 (3): 189-199.

Kocacevik, S., Sevim, A., EROĞLU, M., DEMİRBAĞ, Z. & Demir, I. (2016) Virulence and horizontal transmission of Beauveria pseudobassiana SA Rehner & Humber in Ips sexdentatus and Ips typographus (Coleoptera: Curculionidae). *Turkish Journal of Agriculture and Forestry*, 40 (2): 241-248.

Korolev, N. & Gindin, G. (1999) Vegetative compatibility in the entomopathogen Verticillium lecanii. *Mycological research*, 103 (7): 833-840.

Korolev, N. & Katan, T. (1997) Improved medium for selecting nitrate-nonutilizing (nit) mutants of Verticillium dahliae. *Phytopathology*, 87 (10): 1067-1070.

Kronstad, J. & Staben, C. (1997) Mating type in filamentous fungi. *Annual review of genetics*, 31 (1): 245-276.

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, 33 (7): 1870-1874.

Kück, U. & Pöggeler, S. (2009) Cryptic sex in fungi. *Fungal Biology Reviews*, 23 (3): 86-90.

Lacey, L. A. (2016) *Microbial control of insect and mite pests: From theory to practice*. Academic Press.

Lacey, L. A., Frutos, R., Kaya, H. & Vail, P. (2001) Insect pathogens as biological control agents: do they have a future? *Biological control*, 21 (3): 230-248.

Lechenet, M., Bretagnolle, V., Bockstaller, C., Boissinot, F., Petit, M. S., Petit, S. & Munier-Jolain, N. M. (2014) Reconciling pesticide reduction with economic and environmental sustainability in arable farming. *PLoS ONE*, 9 1-10.

Lee, J.-O., Shrestha, B., Kim, T.-W., Sung, G.-H. & Sung, J.-M. (2007) Stable formation of fruiting body in Cordyceps bassiana. *Mycobiology*, 35 (4): 230-234.

Lee, J.-O., Shrestha, B., Sung, G.-H., Han, S.-K., Kim, T.-W. & Sung, J.-M. (2010a) Cultural characteristics and fruiting body production in Cordyceps bassiana. *Mycobiology*, 38 (2): 118-121.

Lee, S. C., Ni, M., Li, W., Shertz, C. & Heitman, J. (2010b) The evolution of sex: a perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews*, 74 (2): 298-340.

Lepre, A., Sutherland, J., Trunk, J. & Sutherland, B. (1998) A robust, inexpensive filter for blocking UVC radiation in broad-spectrum 'UVB'lamps. *Journal of Photochemistry and Photobiology B: Biology*, 43 (1): 34-40.

Leslie, J. F. (1993) Fungal vegetative compatibility. *Annual review of phytopathology*, 31 (1): 127-150.

Li, H. X. & Lee, B. H. (2014) Enhanced conidial thermotolerance and field efficacy of Beauveria bassiana by saccharides. *Biotechnol Lett*, 36 (12): 2481-2488.

Li, Z., Li, C., Huang, B. & Fan, M. (2001) Discovery and demonstration of the teleomorph ofBeauveria bassiana (Bals.) Vuill., an important entomogenous fungus. *Chinese Science Bulletin*, 46 (9): 751-753.

Lindquist, S. & Kim, G. (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proceedings of the National Academy of Sciences*, 93 (11): 5301-5306.

Liu, J., Wang, G. X., Wei, L. & Wang, C. M. (2008) Reproductive allocation patterns in different density populations of spring wheat. *Journal of integrative plant biology*, 50 (2): 141-146.

Loughheed, T. (1961) The effect of nutrition on synnemata formation in Hirsutella gigantea Petch. *Canadian Journal of Botany*, 39 (4): 865-873.

López-Villavicencio, M., Aguileta, G., Giraud, T., de Vienne, D., Lacoste, S., Couloux, A. & Dupont, J. (2010) Sex in Penicillium: Combined phylogenetic and experimental approaches. *Fungal genetics and biology*, 47 (8): 693-706.

Magan, N. (2001) Physiological approaches to improving the ecological fitness of fungal biocontrol agents. *Fungi as biocontrol agents*, 239-252.

Martin, T., Lu, S.-W., van Tilbeurgh, H., Ripoll, D. R., Dixelius, C., Turgeon, B. G. & Debuchy, R. (2010) Tracing the origin of the fungal α1 domain places its ancestor in the HMG-box superfamily: implication for fungal mating-type evolution. *PloS one*, 5 (12): e15199.

Meyling, N. V., Lubeck, M., Buckley, E. P., Eilenberg, J. & Rehner, S. A. (2009) Community composition, host range and genetic structure of the fungal entomopathogen Beauveria in adjoining agricultural and seminatural habitats. *Mol Ecol*, 18 (6): 1282-1293.

Molnar, A., Sulyok, L. & Hornok, L. (1990) Parasexual recombination between vegetatively incompatible strains in Fusarium oxysporum. *Mycological Research*, 94 (3): 393-398.

Molowitz, R., Bahn, M. & Hock, B. (1976) The control of fruiting body formation in the ascomycete Sordaria macrospora Auersw. by arginine and biotin: a two-factor analysis. *Planta*, 128 (2): 143-148.

Moose, S. P. & Mumm, R. H. (2008) Molecular plant breeding as the foundation for 21st century crop improvement. *Plant physiology*, 147 (3): 969-977.

Newmeyer, D., Howe Jr, H. B. & Galeazzi, D. R. (1973) A search for complexity at the mating-type locus of Neurospora crassa. *Canadian Journal of Genetics and Cytology*, 15 (3): 577-585.

Newton, A. & Caten, C. (1988) Auxotrophic mutants of Septoria nodorum isolated by direct screening and by selection for resistance to chlorate. *Transactions of the British Mycological Society*, 90 (2): 199-207.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. (2000) Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and molecular biology reviews*, 64 (3): 548-572.

Noventa-Jordão, M. A. n., Couto, R. M., Goldman, M. H. S., Aguirre, J., Iyer, S., Caplan, A., Terenzi, H. F. & Goldman, G. H. (1999) Catalase activity is necessary for heat-shock recovery in Aspergillus nidulans germlings. *Microbiology*, 145 (11): 3229-3234.

Nunes, J., Jorge, J. A., Henrique, L., Guimarães, S. & Al, E. T. (2013) Production of Invertases by Anamorphic (Aspergillus nidulans) and Teleomorphic (Emericela nidulans) Fungi under Submerged Fermentation Using Rye Flour as Carbon Source. 2013 421-429.

Nødvig, C. S., Nielsen, J. B., Kogle, M. E. & Mortensen, U. H. (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One*, 10 (7): e0133085.

Oerke, E.-C. (2006) Crop losses to pests. *The Journal of Agricultural Science*, 144 31.

Oerke, E. C. & Dehne, H. W. (2004) Safeguarding production - Losses in major crops and the role of crop protection. *Crop Protection*, 23 275-285.

Ogoshi, A. (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of Rhizoctonia solani Kuhn. *Annual Review of phytopathology*, 25 (1): 125-143.

Orr, D. & Lahiri, S. (2014) Biological control of insect pests in crops. In: *Integrated Pest Management*. Elsevier: 531-548.

Osiewacz, H. D. (2002) Sexual development in Ascomycetes fruit body formation of Aspergillus nidulans. In: *Molecular biology of fungal development*. CRC Press: 212-238.

O'Gorman, C. M., Fuller, H. T. & Dyer, P. S. (2009) Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. *Nature*, 457 (7228): 471.

Paccola-Meirelles, L. & Azevedo, J. (1991) Parasexuality in Beauveria bassiana. *Journal of Invertebrate Pathology*, 57 (2): 172-176.

Pal, K. K. & Gardener, B. M. (2006) Biological control of plant pathogens. *The plant health instructor*, 2 1117-1142.

Panizza, S., Mendoza, M. A., Berlinger, M., Huang, L., Nicolas, A., Shirahige, K. & Klein, F. (2011) Spo11-accessory proteins link double-strand break sites to the chromosome axis in early meiotic recombination. *Cell*, 146 (3): 372-383.

Paoletti, M., Rydholm, C., Schwier, E. U., Anderson, M. J., Szakacs, G., Lutzoni, F., Debeaupuis, J.-P., Latgé, J.-P., Denning, D. W. & Dyer, P. S. (2005) Evidence for sexuality in the opportunistic fungal pathogen Aspergillus fumigatus. *Current Biology*, 15 (13): 1242-1248.

Paoletti, M., Seymour, F. A., Alcocer, M. J., Kaur, N., Calvo, A. M., Archer, D. B. & Dyer, P. S. (2007) Mating type and the genetic basis of self-fertility in the model fungus Aspergillus nidulans. *Current Biology*, 17 (16): 1384-1389.

Patel, R. (2013) The long green revolution. *The Journal of Peasant Studies*, 40 (1): 1-63.

Piazena, H. (1996) The effect of altitude upon the solar UV-B and UV-A irradiance in the tropical Chilean Andes. *Solar energy*, 57 (2): 133-140.

Pimentel, D. (2009) Pesticides and pest control. In: *Integrated pest management: innovation-development process*. Springer: 83-87.

Pimentel, D. & Burgess, M. (2014) Environmental and economic costs of the application of pesticides primarily in the United States. In: *Integrated pest management*. Springer: 47-71.

Pontecorvo, G. (1956) The parasexual cycle in fungi. *Annual Reviews in Microbiology*, 10 (1): 393-400.

Potter, C. (1952) An improved laboratory apparatus for applying direct sprays and surface films, with data on the electrostatic charge on atomized spray fluids. *Annals of Applied Biology*, 39 (1): 1-28.

Prabavathy, V., Mathivanan, N., Sagadevan, E., Murugesan, K. & Lalithakumari, D. (2006) Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in Trichoderma reesei. *Enzyme and Microbial Technology*, 38 (5): 719-723.

Puhalla, J. E. (1984) A visual indicator of heterokaryosis in Fusarium oxysporum from celery. *Canadian journal of botany*, 62 (3): 540-545.

Puhalla, J. E. (1985) Classification of strains of Fusarium oxysporum on the basis of vegetative compatibility. *Canadian Journal of Botany*, 63 (2): 179-183.

Ramirez-Prado, J. H., Moore, G. G., Horn, B. W. & Carbone, I. (2008) Characterization and population analysis of the mating-type genes in Aspergillus flavus and Aspergillus parasiticus. *Fungal Genetics and Biology*, 45 (9): 1292-1299.

Rangel, D. E., Braga, G. U., Anderson, A. J. & Roberts, D. W. (2005) Influence of growth environment on tolerance to UV-B radiation, germination speed, and morphology of Metarhizium anisopliae var. acridum conidia. *Journal of invertebrate pathology*, 90 (1): 55-58.

Rangel, D. E., Braga, G. U., Flint, S. D., Anderson, A. J. & Roberts, D. W. (2004) Variations in UV-B tolerance and germination speed of Metarhizium anisopliae conidia produced on insects and artificial substrates. *Journal of Invertebrate Pathology*, 87 (2-3): 77-83.

Ravensberg, W. J. (2011a) Critical factors in the successful commercialization of microbial pest control products. In: *A roadmap to the successful development and commercialization of microbial pest control products for control of arthropods*. Springer: 295-356.

Ravensberg, W. J. (2011b) A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products for Control of Arthropods. *Pest Control*, 235-293.

Rehner, S. A. & Buckley, E. (2005) A Beauveria phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia*, 97 (1): 84-98.

Rehner, S. a., Minnis, A. M., Sung, G.-H., Luangsa-Ard, J. J., Devotto, L. & Humber, R. a. (2011) Phylogeny and systematics of the anamorphic, entomopathogenic genus Beauveria. *Mycologia*, 103 1055-1073.

Rehner, S. A., Posada, F., Buckley, E. P., Infante, F., Castillo, A. & Vega, F. E. (2006) Phylogenetic origins of African and Neotropical Beauveria bassiana sl pathogens of the coffee berry borer, Hypothenemus hampei. *Journal of Invertebrate Pathology*, 93 (1): 11-21.

Roberts, D. W. & Hajek, A. E. (1992) Entomopathogenic fungi as bioinsecticides. In: *Frontiers in industrial Mycology*. Springer: 144-159.

Robène-Soustrade, I., Jouen, E., Pastou, D., Payet-Hoarau, M., Goble, T., Linderme, D., Lefeuvre, P., Calmès, C., Reynaud, B. & Nibouche, S. (2015) Description and phylogenetic placement of Beauveria hoplocheli sp. nov. used in the biological control of the sugarcane white grub, Hoplochelus marginalis, on Reunion Island. *Mycologia*, 107 (6): 1221-1232.

Roca, M. G., Davide, L. C., Mendes-Costa, M. C. & Wheals, A. (2003) Conidial anastomosis tubes in Colletotrichum. *Fungal Genetics and Biology*, 40 (2): 138-145.

Rowe, R. C. (1995) Recent progress in understanding relationships betweenVerticillium species and subspecific groups. *Phytoparasitica*, 23 (1): 31.

Roy, M., Brodeur, J. & Cloutier, C. (2002) Relationship between temperature and developmental rate of Stethorus punctillum (Coleoptera: Coccinellidae) and its prey Tetranychus mcdanieli (Acarina: Tetranychidae). *Environmental Entomology*, 31 (1): 177-187.

Rydholm, C., Dyer, P. & Lutzoni, F. (2007) DNA sequence characterization and molecular evolution of MAT1 and MAT2 mating-type loci of the self-compatible ascomycete mold Neosartorya fischeri. *Eukaryotic cell*, 6 (5): 868-874.

Samuels, K., Heale, J. & Llewellyn, M. (1989) Characteristics relating to the pathogenicity of Metarhizium anisopliae toward Nilaparvata lugens. *Journal of Invertebrate Pathology*, 53 (1): 25-31.

Sarfraz, M., Keddie, A. B. & Dosdall, L. M. (2005) Biological control of the diamondback moth, Plutella xylostella: a review. *Biocontrol Science and Technology*, 15 (8): 763-789.

Schoustra, S., Rundle, H. D., Dali, R. & Kassen, R. (2010) Fitness-associated sexual reproduction in a filamentous fungus. *Current Biology*, 20 (15): 1350-1355.

Silveira, W. & Azevedo, J. (1987) Protoplast fusion and genetic recombination in Metarhizium anisopliae. *Enzyme and microbial technology*, 9 (3): 149-152.

Singh, V., Hussein, M., Singh, A., Hassan, M. & Gupta, P. (2015) Histological and immunohistochemical changes in cerebellum of chick embryos after exposure to neonicotinoid insecticide imidacloprid. *Journal of the Anatomical Society of India*, 64 (2): 122-127.

Skinner, M., Parker, B. L. & Kim, J. S. (2014) Role of entomopathogenic fungi in integrated pest management. In: *Integrated pest management*. Elsevier: 169-191.

Smits, N., BriÈRe, J.-F. & Fargues, J. (2003) Comparison of non-linear temperature-dependent development rate models applied to in vitro growth of entomopathogenic fungi. *Mycological research*, 107 (12): 1476-1484.

St Leger, R. J., Allee, L., May, B., Staples, R. C. & Roberts, D. W. (1992) World-wide distribution of genetic variation among isolates of Beauveria spp. *Mycological Research*, 96 (12): 1007-1015.

Steinmann, K. P., Zhang, M., Grant, J. a., Pickel, C., Goodhue, R. E. & Klonsky, K. (2010) Quantifying economic and environmental tradeoffs of walnut arthropod pest management. *Agricultural Systems*, 103 294-306.

Strausbaugh, C., Schroth, M., Weinhold, A. & Hancock, J. (1992) Assessment of vegetative compatibility of Verticillium dahliae tester strains and isolates from California potatoes. *Phytopathology (USA)*,

Subbarao, K., Chassot, A., Gordon, T., Hubbard, J., Bonello, P., Mullin, R., Okamoto, D., Davis, R. & Koike, S. (1995) Genetic relationships and cross pathogenicities of Verticillium dahliae isolates from cauliflower and other crops. *Phytopathology*, 85 (10): 1105-1112.

Sugimoto, M., Koike, M., Nagao, H., Okumura, K., Tani, M. & Kuramochi, K. (2003) Genetic diversity of the entomopathogenVerticillium lecanii on the basis of vegetative compatibility. *Phytoparasitica*, 31 (5): 450-457.

Sung, G.-H., Hywel-Jones, N. L., Sung, J.-M., Luangsa-ard, J. J., Shrestha, B. & Spatafora, J. W. (2007) Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. *Studies in mycology*, 57 5-59.

Sung, G.-H., Spatafora, J. W., Zare, R., Hodge, K. T. & Gams, W. (2001) A revision of Verticillium sect. Prostrata. II. Phylogenetic analyses of SSU and LSU nuclear rDNA sequences from anamorphs and teleomorphs of the Clavicipitaceae. *Nova Hedwigia*, 72 (3-4): 311-328.

Sung, J.-M., Lee, J.-O., Humber, R. A., Sung, G.-H. & Shrestha, B. (2006) Cordyceps bassiana and production of stromata in vitro showing Beauveria anamorph in Korea. *Mycobiology*, 34 (1): 1-6.

Talekar, N. & Shelton, A. (1993) Biology, ecology, and management of the diamondback moth. *Annual review of entomology*, 38 (1): 275-301.

Terashima, Y. & Fujiie, A. (2007) Comparison of conditions for mycelial growth of Lepista sordida causing fairy rings on Zoysia matrella turf to those on Agrostis palustris turf. *Mycoscience*, 48 (6): 365-372.

Tumuhaise, V., Ekesi, S., Maniania, N., Tonnang, H., Tanga, C., Ndegwa, P., Irungu, L., Srinivasan, R. & Mohamed, S. (2018) Temperature-dependent growth and virulence, and mass production potential of two candidate isolates of Metarhizium anisopliae (Metschnikoff) Sorokin for managing Maruca vitrata Fabricius (Lepidoptera: Crambidae) on cowpea. *African Entomology*, 26 (1): 73-83.

Turgeon, B. G. & Yoder, O. (2000) Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology*, 31 (1): 1-5.

Valero-Jiménez, C. A., Faino, L., Spring, D., Smit, S., Zwaan, B. J. & van Kan, J. A. (2016) Comparative genomics of Beauveria bassiana: uncovering signatures of virulence against mosquitoes. *BMC genomics*, 17 (1): 986.

Van Den Berg, M. A., Albang, R., Albermann, K., Badger, J. H., Daran, J.-M., Driessen, A. J., Garcia-Estrada, C., Fedorova, N. D., Harris, D. M. & Heijne, W. H. (2008) Genome sequencing and analysis of the filamentous fungus Penicillium chrysogenum. *Nature biotechnology*, 26 (10): 1161.

Vandenberg, J., Shelton, A., Wilsey, W. & Ramos, M. (1998) Assessment of Beauveria bassiana sprays for control of diamondback moth (Lepidoptera: Plutellidae) on crucifers. *Journal of Economic Entomology*, 91 (3): 624-630.

Verkerk, R. H. & Wright, D. J. (1996) Multitrophic interactions and management of the diamondback moth: a review. *Bulletin of Entomological Research*, 86 (3): 205-216.

Vidal, C., Fargues, J. & Lacey, L. A. (1997) Intraspecific variability ofPaecilomyces fumosoroseus: effect of temperature on vegetative growth. *Journal of Invertebrate Pathology*, 70 (1): 18-26.

Villaverde, J. J., Sevilla-Morán, B., Sandín-España, P., López-Goti, C. & Alonso-Prados, J. L. (2014) Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. *Pest management science*, 70 (1): 2-5.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18 (1): 315-322.

Wilson, A. M., Wilken, P. M., van der Nest, M. A., Steenkamp, E. T., Wingfield, M. J. & Wingfield, B. D. (2015) Homothallism: an umbrella term for describing diverse sexual behaviours. *IMA fungus*, 6 (1): 207-214.

Wirsel, S., Horwitz, B., Yamaguchi, K., Yoder, O. & Turgeon, B. (1998) Single mating type-specific genes and their 3' UTRs control mating and fertility in Cochliobolus heterostrophus. *Molecular and General Genetics MGG*, 259 (3): 272-281.

Woo, P. C., Chong, K. T., Tse, H., Cai, J. J., Lau, C. C., Zhou, A. C., Lau, S. K. & Yuen, K.-y. (2006) Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermal dimorphic fungus Penicillium marneffei. *FEBS letters*, 580 (14): 3409-3416.

Wraight, S. & Ramos, M. (2015) Delayed efficacy of Beauveria bassiana foliar spray applications against Colorado potato beetle: impacts of number and timing of applications on larval and next-generation adult populations. *Biological Control*, 83 51-67.

Wraight, S., Ramos, M., Avery, P., Jaronski, S. & Vandenberg, J. (2010) Comparative virulence of Beauveria bassiana isolates against lepidopteran pests of vegetable crops. *Journal of Invertebrate Pathology*, 103 (3): 186-199.

Wright, M. G. (2014) Biological control of invasive insect pests. In: *Integrated Pest Management*. Elsevier: 267-281.

Xiang, Q. & Glass, N. L. (2004) The control of mating type heterokaryon incompatibility by vib-1, a locus involved in het-c heterokaryon incompatibility in Neurospora crassa. *Fungal Genetics and Biology*, 41 (12): 1063-1076.

Xiao, G., Ying, S.-H., Zheng, P., Wang, Z.-L., Zhang, S., Xie, X.-Q., Shang, Y., St. Leger, R. J., Zhao, G.-P., Wang, C. & Feng, M.-G. (2012) Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana. *Scientific Reports*, 2

Ying, S.-H. & Feng, M.-G. (2011) A conidial protein (CP15) of Beauveria bassiana contributes to the conidial tolerance of the entomopathogenic fungus to thermal and oxidative stresses. *Applied microbiology and biotechnology*, 90 (5): 1711-1720.

Yokoyama, E., Arakawa, M., Yamagishi, K. & Hara, A. (2006) Phylogenetic and structural analyses of the mating-type loci in Clavicipitaceae. *FEMS microbiology letters*, 264 (2): 182-191.

Yokoyama, E., Yamagishi, K. & Hara, A. (2004) Development of a PCR-based mating-type assay for Clavicipitaceae. *FEMS Microbiol Lett*, 237 (2): 205-212.

Yoon, C.-S., Yu, K.-W., Bae, S.-H., Song, H.-H., Park, H.-S. & Lee, C. (2003) Chemical properties and physiological activities of synnemata of Beauveria bassiana. *Journal of microbiology and biotechnology*, 13 (1): 125-133.

Zalucki, M. P., Shabbir, A., Silva, R., Adamson, D., Shu-Sheng, L. & Furlong, M. J. (2012) Estimating the economic cost of one of the world's major insect pests, Plutella xylostella (Lepidoptera: Plutellidae): just how long is a piece of string? *Journal of Economic Entomology*, 105 (4): 1115-1129.

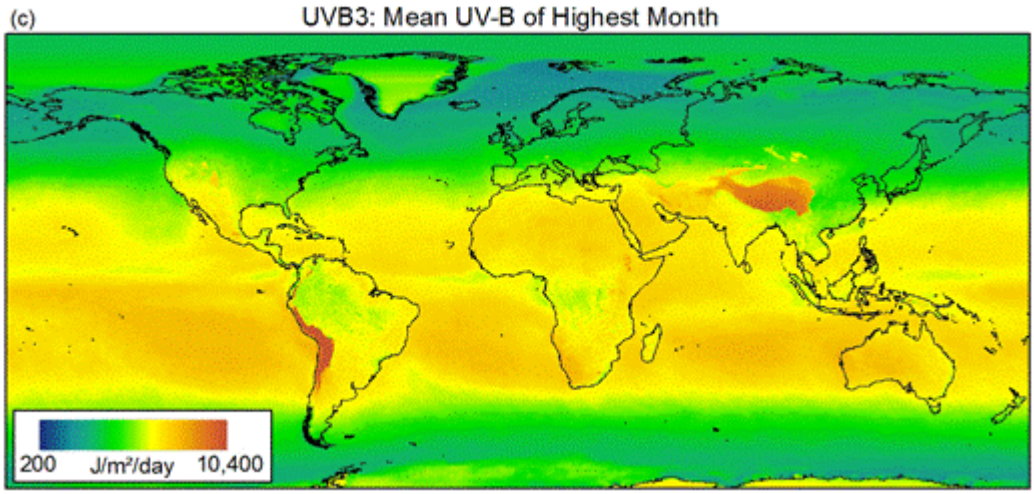
Zhang, Y. J., Xie, M., Zhang, X. L., Peng, D. L., Yu, W. B., Li, Q., Li, Q., Zhao, J. J. & Zhang, Z. R. (2016) Establishment of polyethylene-glycol-mediated protoplast transformation for Lecanicillium lecanii and development of virulence-enhanced strains against Aphis gossypii. *Pest management science*, 72 (10): 1951-1958.

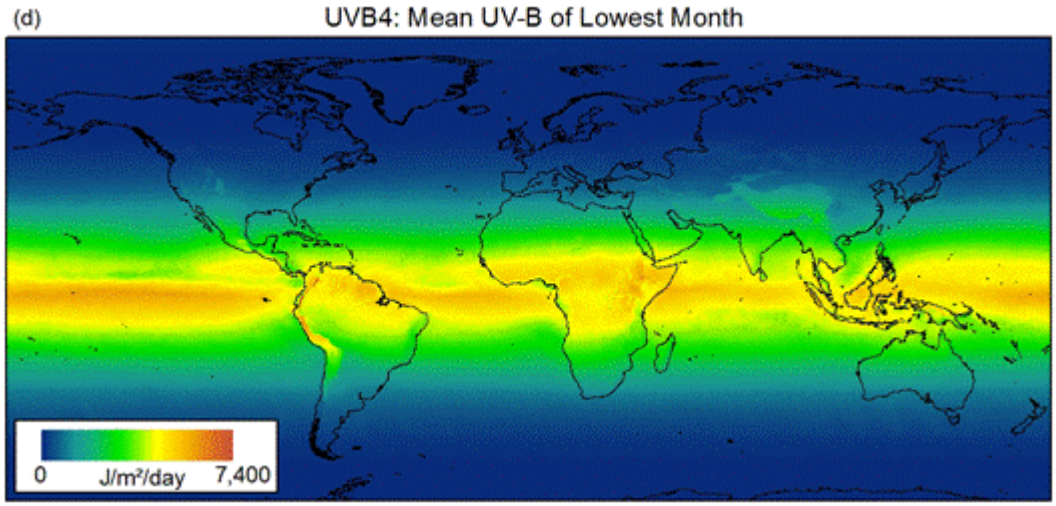
Álvarez-Baz, G., Fernández-Bravo, M., Pajares, J. & Quesada-Moraga, E. (2015) Potential of native Beauveria pseudobassiana strain for biological control of Pine Wood Nematode vector Monochamus galloprovincialis. *Journal of invertebrate pathology*, 132 48-56.

7 Appendix

Appendix 1

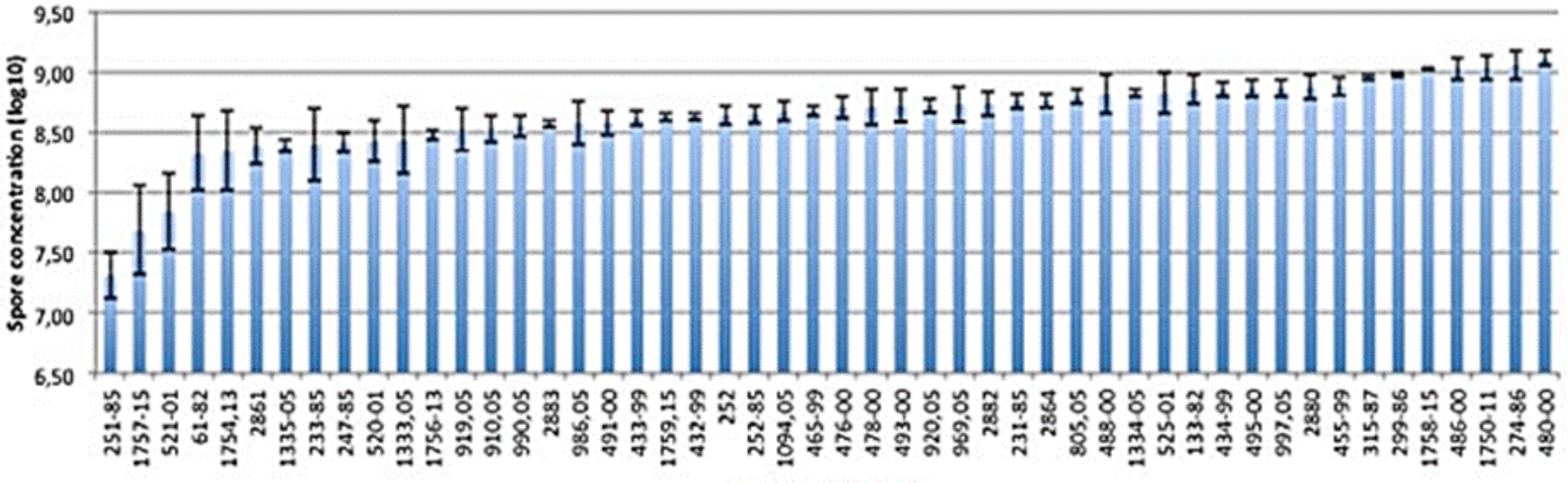
Global UV-B radiation in the Highest Month of the year and in the Lowest Month of the year (UFZ, 2014).





Appendix 2

Mean conidia concentration (log 10/m) after 13 days of incubation at 22 °C. Error bars are standard error of the mean, *n* = 3.



Appendix 3

ANOVA One way for colony extension rate in 50 strains of *Beauveria* and Tukey test (p>0,05) at 10 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	229.99	49	4.69	10.98	<0.0001
Strain	229.99	49	4.69	10.98	<0.0001
Error	42.73	100	0.43		
Total	272.72	149			

Test:Tukey Alpha:=0.05 LSD:=2.20161

Error: 0.4273 df: 100

Strain	Means	n	S.E.		
7	0.00	3	0.38	A	
37	0.00	3	0.38	A	
40	0.00	3	0.38	A	
1	0.00	3	0.38	A	
23	0.00	3	0.38	A	
38	0.00	3	0.38	A	
433-94	0.00	3	0.38	A	
24	0.00	3	0.38	A	
20	0.00	3	0.38	A	
6	0.00	3	0.38	A	
22	0.00	3	0.38	A	
28	0.00	3	0.38	A	
27	0.00	3	0.38	A	
41	0.00	3	0.38	A	
25	0.00	3	0.38	A	
49	0.00	3	0.38	A	
16	0.00	3	0.38	A	
5	0.00	3	0.38	A	
35	0.00	3	0.38	A	
36	0.00	3	0.38	A	
29	0.00	3	0.38	A	
20	0.00	3	0.38	A	
48	0.00	3	0.38	A	
32	0.00	3	0.38	A	
50	0.00	3	0.38	A	
10	0.00	3	0.38	A	
45	0.00	3	0.38	A	
46	0.00	3	0.38	A	
2	0.00	3	0.38	A	
44	0.00	3	0.38	A	
47	0.00	3	0.38	A	
34	0.00	3	0.38	A	
19	0.00	3	0.38	A	
11	0.00	3	0.38	A	
44	0.00	3	0.38	A	
8	0.00	3	0.38	A	
9	0.00	3	0.38	A	
18	0.00	3	0.38	A	
43	0.00	3	0.38	A	
42	0.23	3	0.38	A	
15	0.23	3	0.38	A	
31	0.43	3	0.38	A	
30	0.53	3	0.38	A	
17	1.07	3	0.38	A	
14	1.33	3	0.38	A	
12	1.50	3	0.38	A	
21	1.63	3	0.38	A	
33	1.93	3	0.38	A	
26	5.80	3	0.38		B
3	6.30	3	0.38		B

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for Colony growth in 50 strains of *Beauveria* and Tukey test (p>0,05) at 15 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	40622.51	49	829.03	4.69	<0.0001
Strain	40622.51	49	829.03	4.69	<0.0001
Error	17669.87	100	176.70		
Total	58292.38	149			

Test:Tukey Alpha:=0.05 LSD:=44.76872

Error: 176.6987 df: 100

Strain	Means	n	S.E.			
20	0.93	3	7.67	A		
44	1.20	3	7.67	A		
41	1.83	3	7.67	A		
47	2.00	3	7.67	A		
28	2.03	3	7.67	A		
9	2.07	3	7.67	A		
48	2.27	3	7.67	A		
34	2.30	3	7.67	A		
7	2.33	3	7.67	A		
45	3.60	3	7.67	A		
36	4.37	3	7.67	A		
11	4.67	3	7.67	A		
44	4.80	3	7.67	A		
32	4.83	3	7.67	A		
1	5.13	3	7.67	A		
35	5.13	3	7.67	A		
25	5.27	3	7.67	A		
10	6.03	3	7.67	A		
6	6.43	3	7.67	A		
22	6.47	3	7.67	A		
38	7.43	3	7.67	A		
8	7.47	3	7.67	A		
5	8.00	3	7.67	A		
19	8.53	3	7.67	A		
42	10.13	3	7.67	A		
50	10.20	3	7.67	A		
27	10.43	3	7.67	A		
24	14.13	3	7.67	A		
2	14.43	3	7.67	A		
37	17.77	3	7.67	A	B	
20	18.47	3	7.67	A	B	
23	20.67	3	7.67	A	B	
29	22.30	3	7.67	A	B	
18	22.37	3	7.67	A	B	
46	27.40	3	7.67	A	B	C
40	27.63	3	7.67	A	B	C
14	27.93	3	7.67	A	B	C
15	28.57	3	7.67	A	B	C
49	28.93	3	7.67	A	B	C
30	31.07	3	7.67	A	B	C
26	37.03	3	7.67	A	B	C
33	37.07	3	7.67	A	B	C
31	37.67	3	7.67	A	B	C
17	37.90	3	7.67	A	B	C
3	38.40	3	7.67	A	B	C
16	40.73	3	7.67	A	B	C
12	41.07	3	7.67	A	B	C
43	42.93	3	7.67	A	B	C
21	61.77	3	7.67		B	C
433-94	68.30	3	7.67			C

ANOVA One way for Colony growth in 50 strains of *Beauveria* and Tukey test (p>0,05) at 20 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	39371.23	49	803.49	2.80	<0.0001
Strain	39371.23	49	803.49	2.80	<0.0001
Error	28713.79	100	287.14		
Total	68085.02	149			

Test:Tukey Alpha:=0.05 LSD:=57.06939

Error: 287.1379 df: 100

Strain	Means	n	S.E.			
20	34.67	3	9.78	A		
9	36.80	3	9.78	A	B	
11	48.67	3	9.78	A	B	C
7	54.70	3	9.78	A	B	C
8	55.60	3	9.78	A	B	C
41	63.90	3	9.78	A	B	C
44	68.53	3	9.78	A	B	C
36	69.30	3	9.78	A	B	C
28	70.80	3	9.78	A	B	C
25	71.03	3	9.78	A	B	C
47	71.13	3	9.78	A	B	C
44	74.40	3	9.78	A	B	C
34	74.60	3	9.78	A	B	C
1	77.83	3	9.78	A	B	C
38	78.10	3	9.78	A	B	C
22	78.13	3	9.78	A	B	C
6	78.97	3	9.78	A	B	C
48	80.87	3	9.78	A	B	C
23	83.70	3	9.78	A	B	C
10	85.47	3	9.78	A	B	C
30	87.30	3	9.78	A	B	C
35	88.67	3	9.78	A	B	C
18	89.67	3	9.78	A	B	C
49	90.13	3	9.78	A	B	C
31	91.00	3	9.78	A	B	C
42	91.27	3	9.78	A	B	C
17	91.57	3	9.78	A	B	C
32	91.87	3	9.78		B	C
45	92.43	3	9.78		B	C
14	92.80	3	9.78		B	C
26	93.13	3	9.78		B	C
24	93.70	3	9.78		B	C
50	93.80	3	9.78		B	C
40	94.80	3	9.78			C
29	95.30	3	9.78			C
27	95.73	3	9.78			C
5	96.10	3	9.78			C
2	96.10	3	9.78			C
37	96.23	3	9.78			C
16	96.77	3	9.78			C
19	97.23	3	9.78			C
12	97.47	3	9.78			C
33	97.63	3	9.78			C
20	97.97	3	9.78			C
46	98.47	3	9.78			C
15	98.70	3	9.78			C
21	98.93	3	9.78			C
3	99.47	3	9.78			C
43	99.57	3	9.78			C
433-94	100.00	3	9.78			C

ANOVA One way for Colony growth in 50 strains of *Beauveria* and Tukey test (p>0,05) at 25 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	9714.21	49	198.25	3.90	<0.0001
Strain	9714.21	49	198.25	3.90	<0.0001
Error	5082.16	100	50.82		
Total	14796.37	149			

Test:Tukey Alpha:=0.05 LSD:=24.00944

Error: 50.8216 df: 100

Strain	Means	n	S.E.				
9	63.00	3	4.12	A			
20	71.03	3	4.12	A	B		
11	76.20	3	4.12	A	B	C	
41	76.30	3	4.12	A	B	C	
7	83.80	3	4.12	A	B	C	
8	84.60	3	4.12	A	B	C	
10	84.87	3	4.12	A	B	C	
28	88.23	3	4.12		B	C	
47	88.63	3	4.12		B	C	
44	90.13	3	4.12		B	C	
44	90.60	3	4.12		B	C	
34	91.83	3	4.12		B	C	
29	92.60	3	4.12		B	C	
22	94.13	3	4.12		B	C	
26	94.33	3	4.12		B	C	
25	94.40	3	4.12		B	C	
23	94.47	3	4.12		B	C	
19	95.40	3	4.12			C	
36	96.37	3	4.12			C	
1	96.53	3	4.12			C	
18	96.60	3	4.12			C	
38	96.63	3	4.12			C	
17	96.67	3	4.12			C	
27	96.77	3	4.12			C	
48	97.20	3	4.12			C	
6	97.63	3	4.12			C	
42	97.90	3	4.12			C	
32	98.33	3	4.12			C	
49	98.37	3	4.12			C	
45	98.37	3	4.12			C	
2	98.47	3	4.12			C	
35	98.50	3	4.12			C	
40	98.60	3	4.12			C	
30	98.93	3	4.12			C	
33	99.13	3	4.12			C	
5	99.23	3	4.12			C	
14	99.33	3	4.12				C
24	99.53	3	4.12			C	
21	99.60	3	4.12			C	
20	99.77	3	4.12				C
15	99.77	3	4.12				C
31	99.77	3	4.12			C	
12	99.80	3	4.12				C
37	99.90	3	4.12			C	
43	99.90	3	4.12			C	
3	100.00	3	4.12			C	
16	100.00	3	4.12				C
50	100.00	3	4.12			C	
46	100.00	3	4.12			C	
433-94	100.00	3	4.12				C

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for Colony growth in 50 strains of *Beauveria* and Tukey test (p>0,05) at 30 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	15136.20	49	308.90	3.70	<0.0001
Strain	15136.20	49	308.90	3.70	<0.0001
Error	8351.13	100	83.51		
Total	23487.33	149			

Test:Tukey Alpha:=0.05 LSD:=30.77731

Error: 83.5113 df: 100

Strain	Means	n	S.E.				
9	50.97	3	5.28	A			
25	59.20	3	5.28	A	B		
41	72.40	3	5.28	A	B	C	
44	76.70	3	5.28	A	B	C	
11	79.37	3	5.28	A	B	C	
10	85.60	3	5.28		B	C	
20	86.00	3	5.28		B	C	
28	89.50	3	5.28		B	C	
7	90.53	3	5.28			C	
34	90.60	3	5.28			C	
26	92.50	3	5.28			C	
29	94.00	3	5.28			C	
38	94.20	3	5.28			C	
30	94.30	3	5.28			C	
31	94.90	3	5.28			C	
44	94.97	3	5.28			C	
27	96.17	3	5.28			C	
36	96.33	3	5.28			C	
19	96.43	3	5.28			C	
8	96.50	3	5.28			C	
47	96.83	3	5.28			C	
40	96.87	3	5.28			C	
49	97.00	3	5.28			C	
32	97.50	3	5.28			C	
42	97.60	3	5.28			C	
22	97.93	3	5.28			C	
37	97.97	3	5.28			C	
2	98.27	3	5.28			C	
24	98.37	3	5.28			C	
17	99.03	3	5.28			C	
6	99.03	3	5.28			C	
18	99.17	3	5.28			C	
1	99.17	3	5.28			C	
48	99.23	3	5.28			C	
50	99.30	3	5.28			C	
45	99.57	3	5.28			C	
23	99.57	3	5.28			C	
14	99.77	3	5.28			C	
21	99.77	3	5.28			C	
5	99.77	3	5.28			C	
35	99.90	3	5.28			C	
33	99.90	3	5.28			C	
16	100.00	3	5.28			C	
433-94	100.00	3	5.28			C	
3	100.00	3	5.28			C	
20	100.00	3	5.28			C	
12	100.00	3	5.28			C	
15	100.00	3	5.28			C	
43	100.00	3	5.28			C	
46	100.00	3	5.28			C	

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for Colony growth in 50 strains of *Beauveria* and Tukey test (p>0,05) at 33 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	4.28	49	0.09	4.28	<0.0001
Strain	4.28	49	0.09	4.28	<0.0001
Error	2.04	100	0.02		
Total	6.32	149			

Test:Tukey Alpha:=0.05 LSD:=0.48103

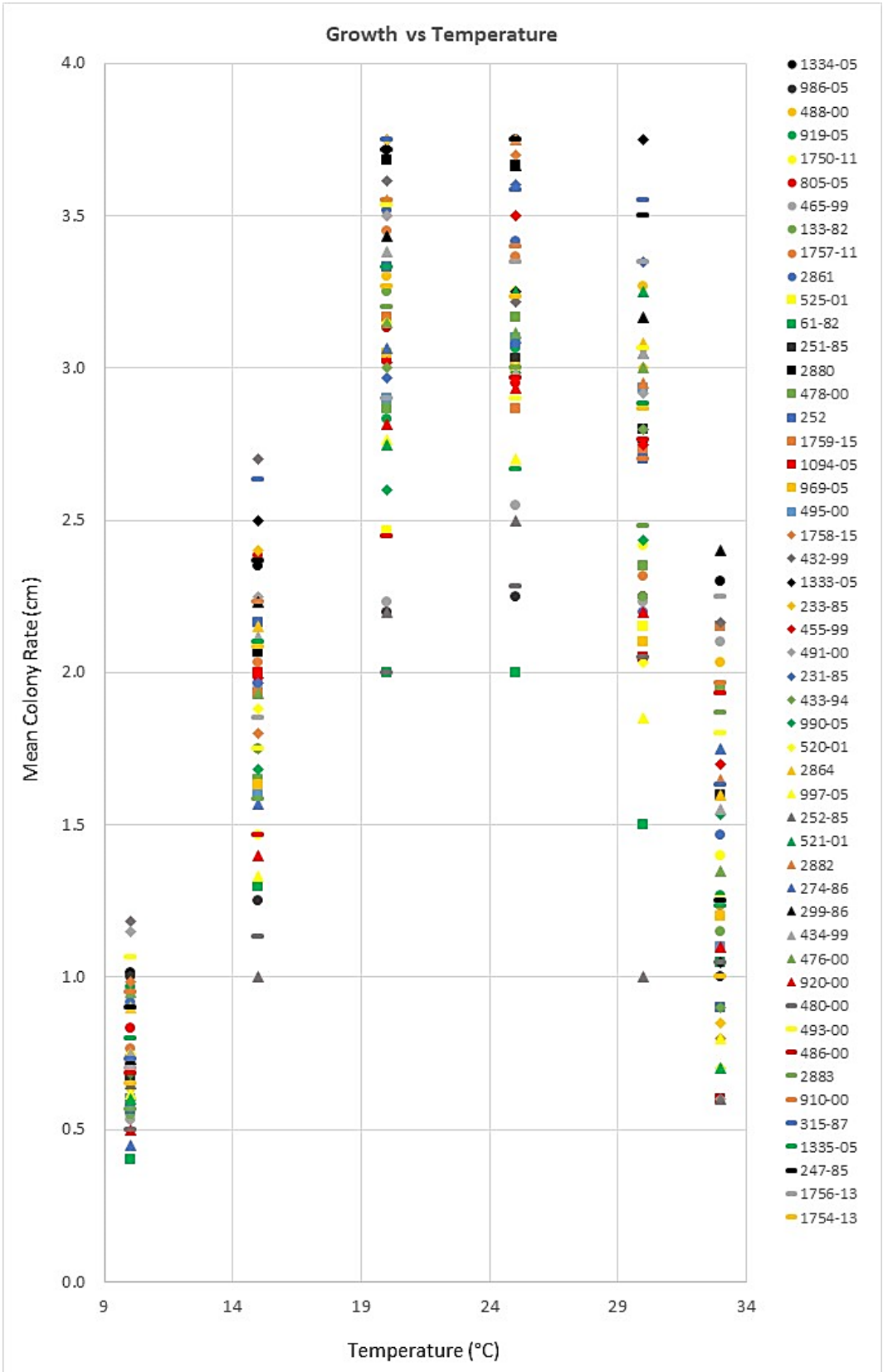
Error: 0.0204 df: 100

Strain	Means	n	S.E.	
31	0.00	3	0.08	A
20	0.00	3	0.08	A
20	0.00	3	0.08	A
24	0.00	3	0.08	A
42	0.00	3	0.08	A
30	0.00	3	0.08	A
7	0.00	3	0.08	A
37	0.00	3	0.08	A
3	0.00	3	0.08	A
22	0.00	3	0.08	A
1	0.00	3	0.08	A
40	0.00	3	0.08	A
26	0.00	3	0.08	A
28	0.00	3	0.08	A
38	0.00	3	0.08	A
29	0.00	3	0.08	A
49	0.00	3	0.08	A
27	0.00	3	0.08	A
6	0.00	3	0.08	A
41	0.00	3	0.08	A
23	0.00	3	0.08	A
25	0.00	3	0.08	A
36	0.00	3	0.08	A
35	0.00	3	0.08	A
45	0.00	3	0.08	A
46	0.00	3	0.08	A
34	0.00	3	0.08	A
18	0.00	3	0.08	A
11	0.00	3	0.08	A
44	0.00	3	0.08	A
8	0.00	3	0.08	A
2	0.00	3	0.08	A
9	0.00	3	0.08	A
10	0.00	3	0.08	A
15	0.00	3	0.08	A
50	0.00	3	0.08	A
32	0.00	3	0.08	A
16	0.00	3	0.08	A
48	0.00	3	0.08	A
47	0.00	3	0.08	A
12	0.00	3	0.08	A
44	0.00	3	0.08	A
17	0.00	3	0.08	A
5	0.00	3	0.08	A
19	0.00	3	0.08	A
43	0.00	3	0.08	A
21	0.23	3	0.08	A
33	0.33	3	0.08	A
14	0.37	3	0.08	A
433-94	1.10	3	0.08	B

Means with a common letter are not significantly different (p > 0.05)

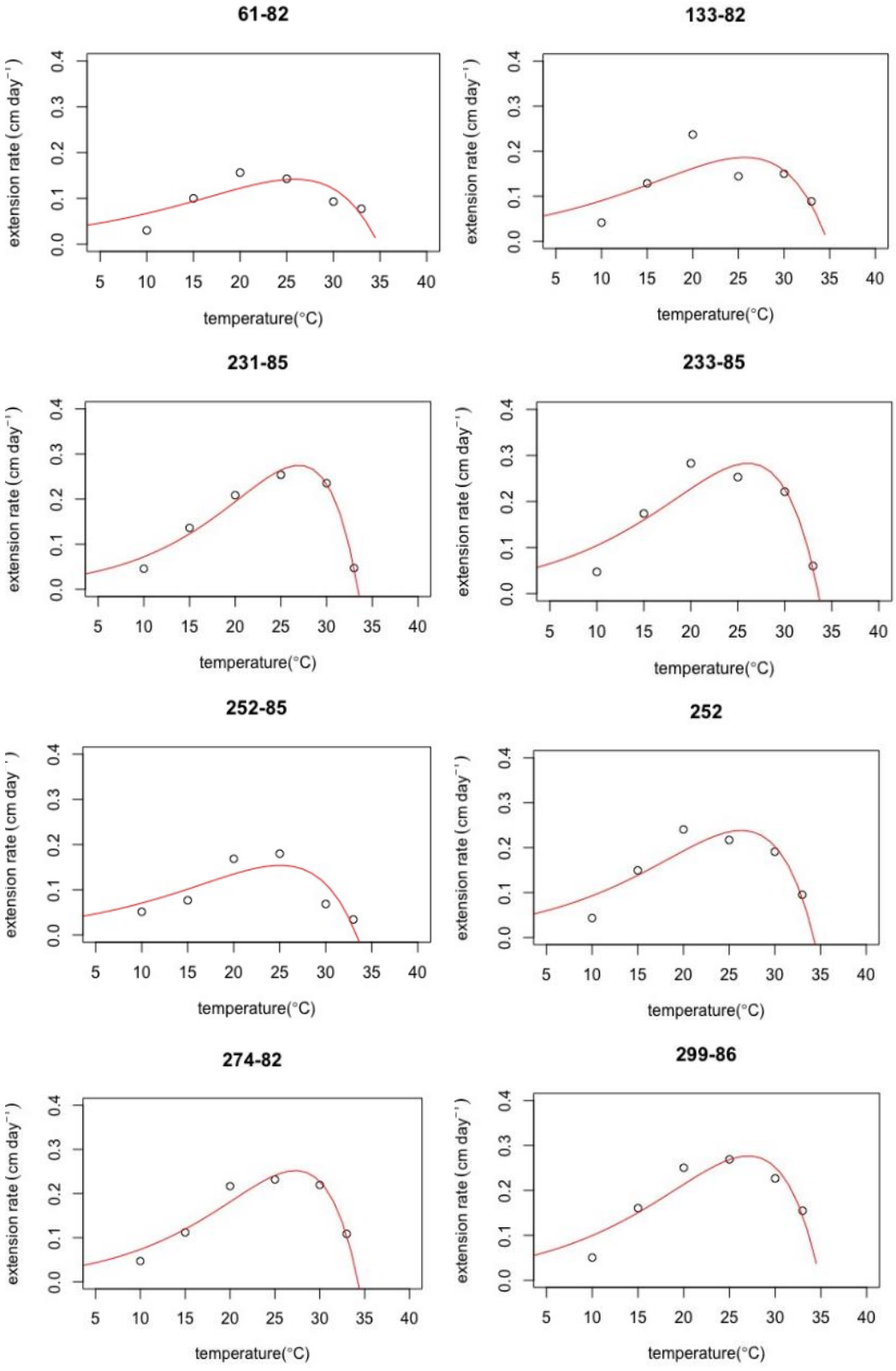
Appendix 4

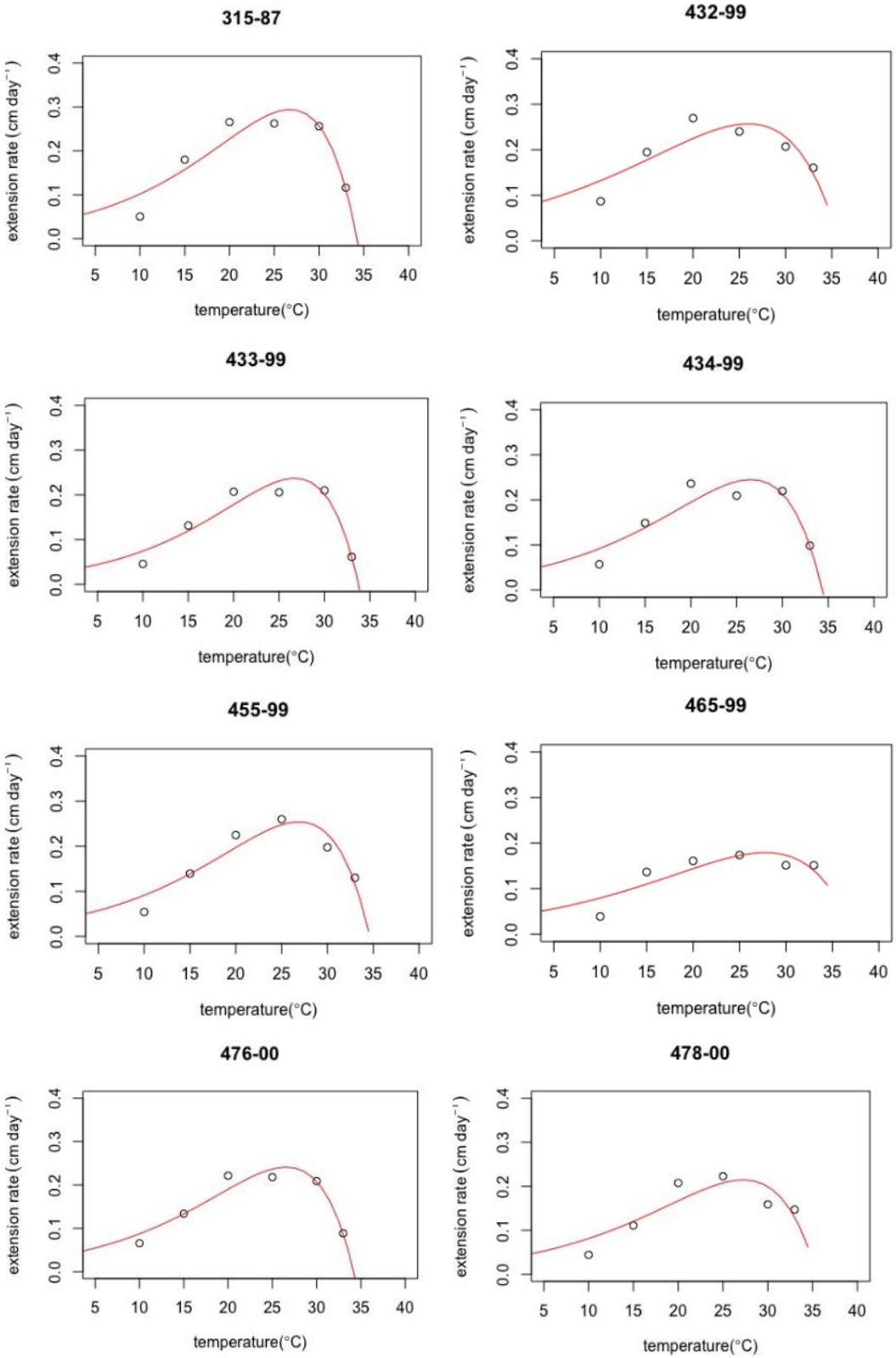
Fungal growth after 4 weeks of incubation of each strain used in this study, at six different temperatures.

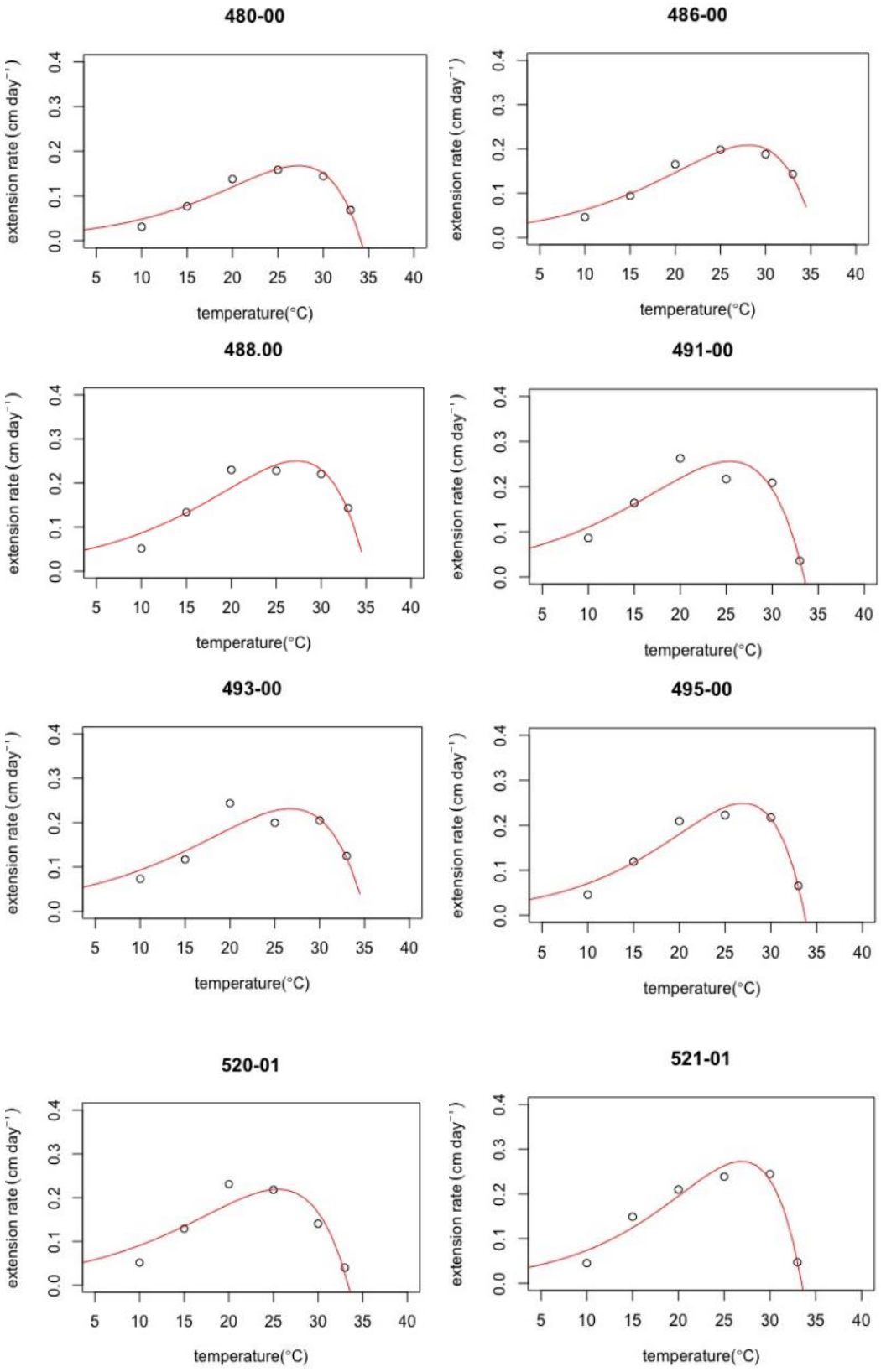


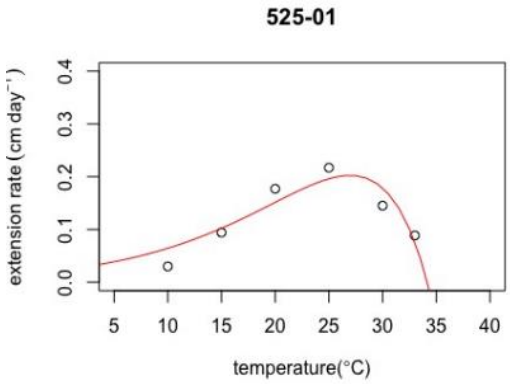
Appendix 5

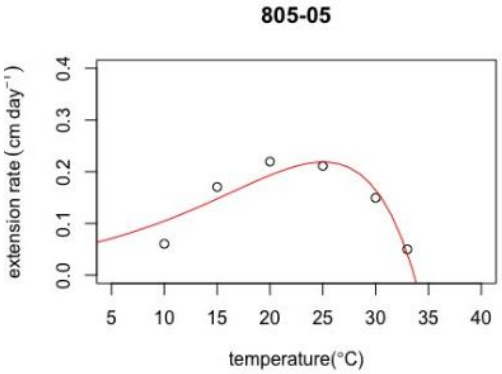
Lactin-1 non-linear model fitted to mean colony extension rate (cm/day) plotted against six temperatures () for fifty strains of *Beauveria*.

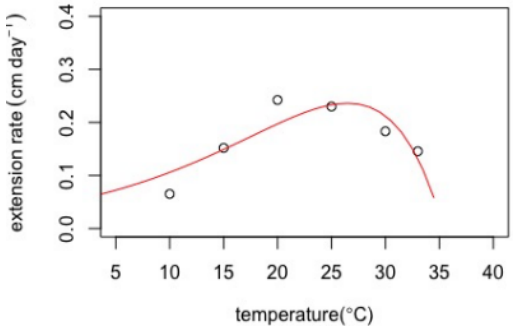


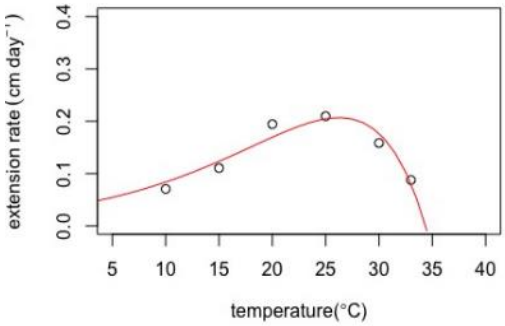

315-87
extension rate (cm day⁻¹)
temperature(°C)
432-99
433-99
434-99
455-99
465-99
476-00
478-00

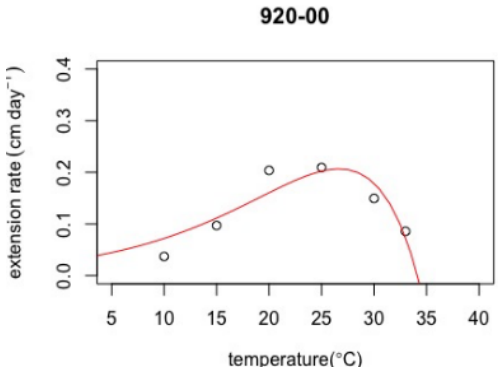

480-00
486-00
488.00
491-00
493-00
495-00
520-01
521-01
extension rate (cm day⁻¹)
temperature(°C)
0.0
0.1
0.2
0.3
0.4
5
10
15
20
25
30
35
40

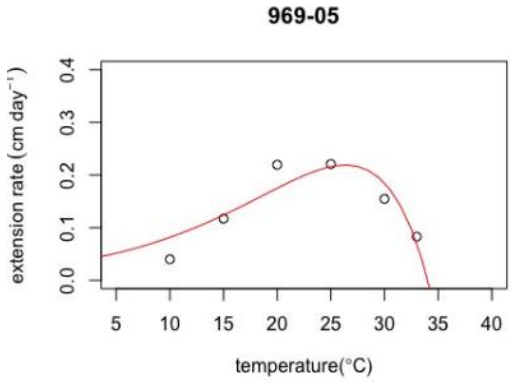

525-01
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

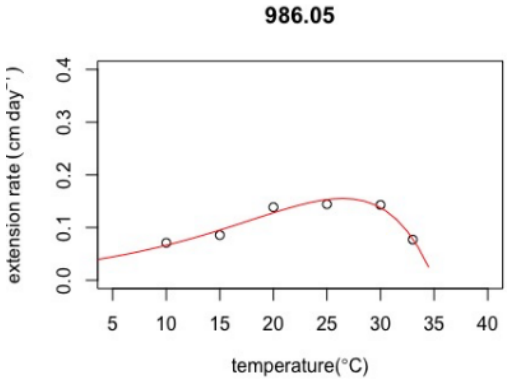

805-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

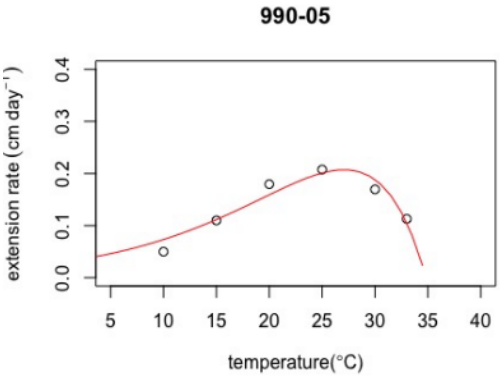

910-00
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

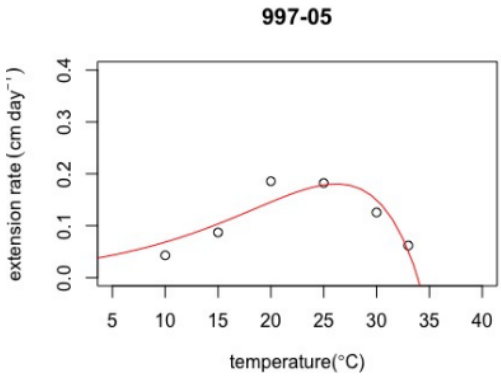

919.05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

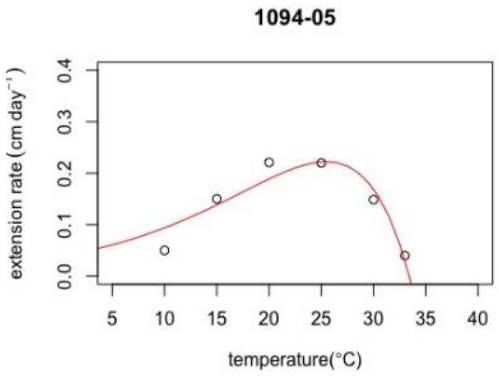

920-00
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

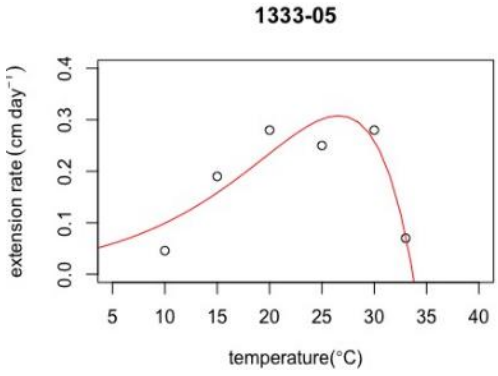

969-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

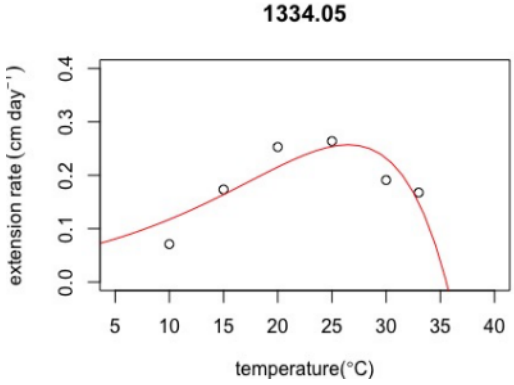

986.05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

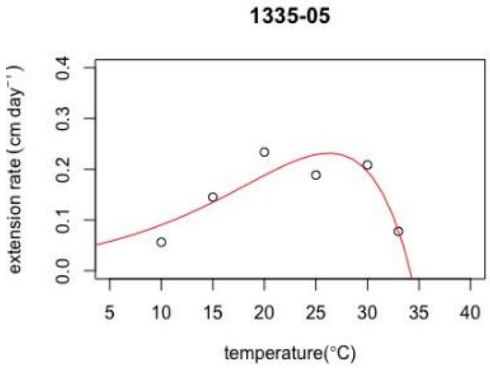

990-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

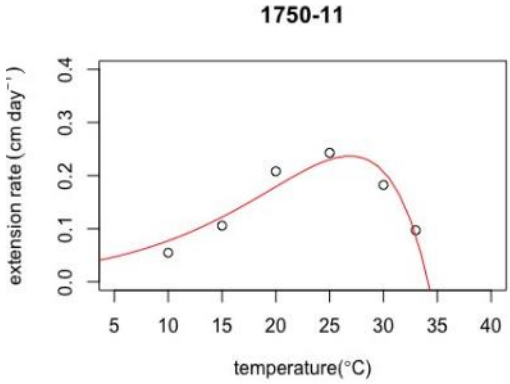

997-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

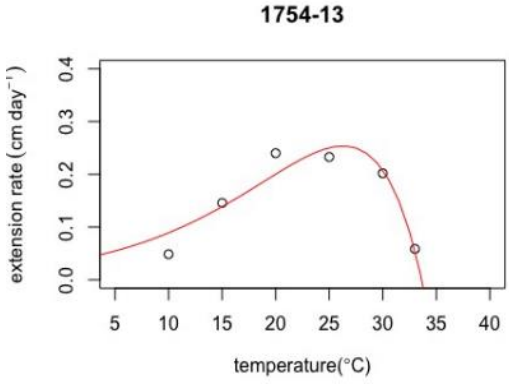

1094-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

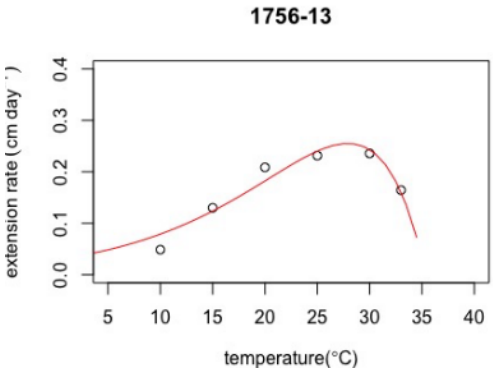

1333-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

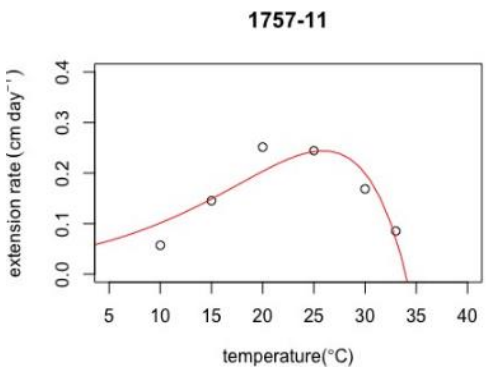

1334.05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

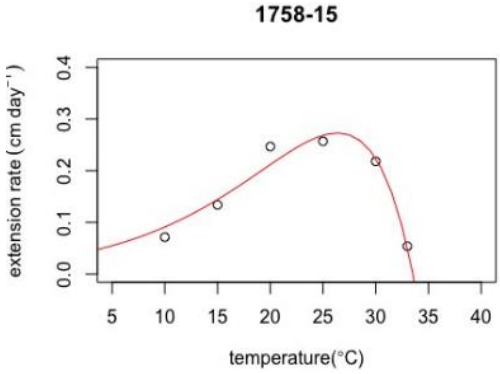

1335-05
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

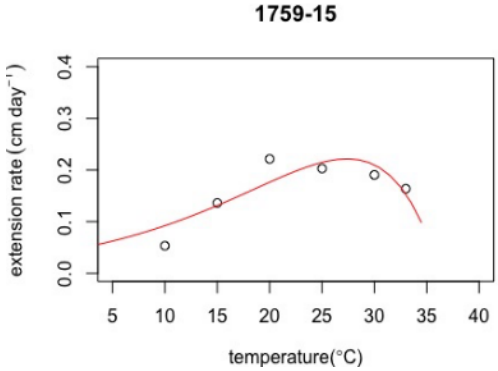

1750-11
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

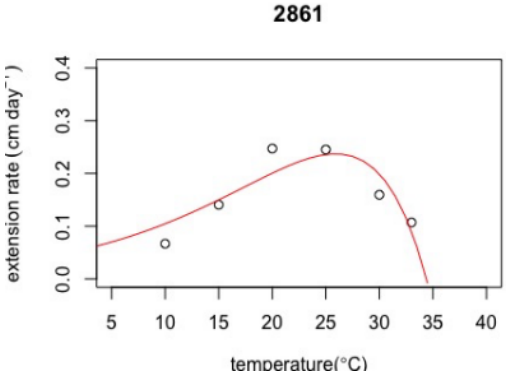

1754-13
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

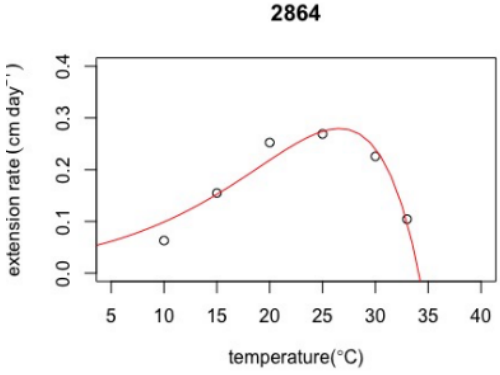

1756-13
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

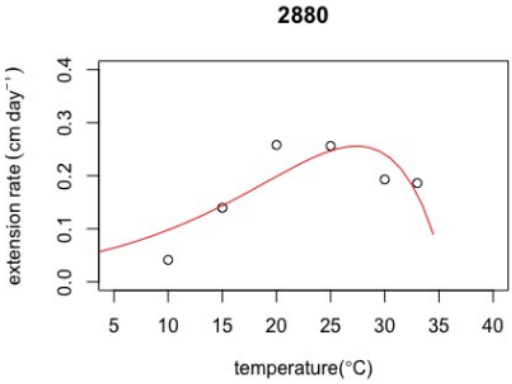

1757-11
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

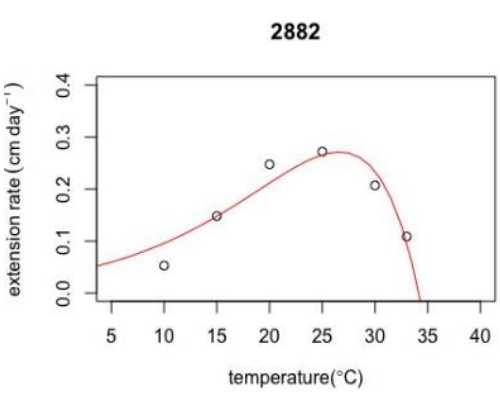

1758-15
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

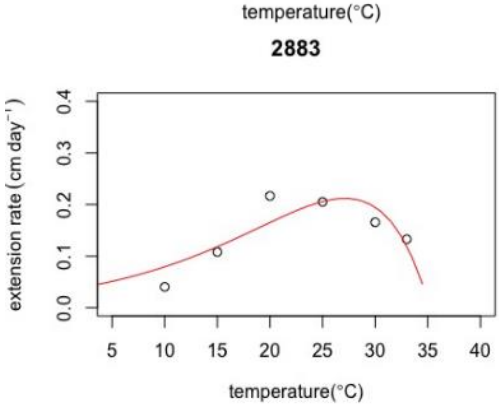

1759-15
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)


2861
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)


2864
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)


2880
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)


2882
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)


2883
extension rate (cm day⁻¹)
0.0 0.1 0.2 0.3 0.4
5 10 15 20 25 30 35 40
temperature(°C)

Appendix 6

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	229.99	49	4.69	10.98	<0.0001
Strain	229.99	49	4.69	10.98	<0.0001
Error	42.73	100	0.43		
Total	272.72	149			

Test:Tukey Alpha:=0.05 LSD:=2.20161

Error: 0.4273 df: 100

Strain	Means	n	S.E.		
7	0.00	3	0.38	A	
37	0.00	3	0.38	A	
40	0.00	3	0.38	A	
1	0.00	3	0.38	A	
23	0.00	3	0.38	A	
38	0.00	3	0.38	A	
433-94	0.00	3	0.38	A	
24	0.00	3	0.38	A	
20	0.00	3	0.38	A	
6	0.00	3	0.38	A	
22	0.00	3	0.38	A	
28	0.00	3	0.38	A	
27	0.00	3	0.38	A	
41	0.00	3	0.38	A	
25	0.00	3	0.38	A	
49	0.00	3	0.38	A	
16	0.00	3	0.38	A	
5	0.00	3	0.38	A	
35	0.00	3	0.38	A	
36	0.00	3	0.38	A	
29	0.00	3	0.38	A	
20	0.00	3	0.38	A	
48	0.00	3	0.38	A	
32	0.00	3	0.38	A	
50	0.00	3	0.38	A	
10	0.00	3	0.38	A	
45	0.00	3	0.38	A	
46	0.00	3	0.38	A	
2	0.00	3	0.38	A	
44	0.00	3	0.38	A	
47	0.00	3	0.38	A	
34	0.00	3	0.38	A	
19	0.00	3	0.38	A	
11	0.00	3	0.38	A	
44	0.00	3	0.38	A	
8	0.00	3	0.38	A	
9	0.00	3	0.38	A	
18	0.00	3	0.38	A	
43	0.00	3	0.38	A	
42	0.23	3	0.38	A	
15	0.23	3	0.38	A	
31	0.43	3	0.38	A	
30	0.53	3	0.38	A	
17	1.07	3	0.38	A	
14	1.33	3	0.38	A	
12	1.50	3	0.38	A	
21	1.63	3	0.38	A	
33	1.93	3	0.38	A	
26	5.80	3	0.38		B
3	6.30	3	0.38		B

ANOVA One way for conidia germination in 50 strains of *Beauveria bassiana* and Tukey test (p>0,05) at 15 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	40622.51	49	829.03	4.69	<0.0001
Strain	40622.51	49	829.03	4.69	<0.0001
Error	17669.87	100	176.70		
Total	58292.38	149			

Test:Tukey Alpha:=0.05 LSD:=44.76872

Error: 176.6987 df: 100

Strain	Means	n	S.E.			
20	0.93	3	7.67	A		
44	1.20	3	7.67	A		
41	1.83	3	7.67	A		
47	2.00	3	7.67	A		
28	2.03	3	7.67	A		
9	2.07	3	7.67	A		
48	2.27	3	7.67	A		
34	2.30	3	7.67	A		
7	2.33	3	7.67	A		
45	3.60	3	7.67	A		
36	4.37	3	7.67	A		
11	4.67	3	7.67	A		
44	4.80	3	7.67	A		
32	4.83	3	7.67	A		
1	5.13	3	7.67	A		
35	5.13	3	7.67	A		
25	5.27	3	7.67	A		
10	6.03	3	7.67	A		
6	6.43	3	7.67	A		
22	6.47	3	7.67	A		
38	7.43	3	7.67	A		
8	7.47	3	7.67	A		
5	8.00	3	7.67	A		
19	8.53	3	7.67	A		
42	10.13	3	7.67	A		
50	10.20	3	7.67	A		
27	10.43	3	7.67	A		
24	14.13	3	7.67	A		
2	14.43	3	7.67	A		
37	17.77	3	7.67	A	B	
20	18.47	3	7.67	A	B	
23	20.67	3	7.67	A	B	
29	22.30	3	7.67	A	B	
18	22.37	3	7.67	A	B	
46	27.40	3	7.67	A	B	C
40	27.63	3	7.67	A	B	C
14	27.93	3	7.67	A	B	C
15	28.57	3	7.67	A	B	C
49	28.93	3	7.67	A	B	C
30	31.07	3	7.67	A	B	C
26	37.03	3	7.67	A	B	C
33	37.07	3	7.67	A	B	C
31	37.67	3	7.67	A	B	C
17	37.90	3	7.67	A	B	C
3	38.40	3	7.67	A	B	C
16	40.73	3	7.67	A	B	C
12	41.07	3	7.67	A	B	C
43	42.93	3	7.67	A	B	C
21	61.77	3	7.67		B	C
433-94	68.30	3	7.67			C

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for conidia germination in 50 strains of *Beauveria bassiana* and Tukey test (p>0,05) at 20 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	39371.23	49	803.49	2.80	<0.0001
Strain	39371.23	49	803.49	2.80	<0.0001
Error	28713.79	100	287.14		
Total	68085.02	149			

Test:Tukey Alpha:=0.05 LSD:=57.06939

Error: 287.1379 df: 100

Strain	Means	n	S.E.							
20	34.67	3	9.78	A						
9	36.80	3	9.78	A	B					
11	48.67	3	9.78	A	B	C				
7	54.70	3	9.78	A	B	C				
8	55.60	3	9.78	A	B	C				
41	63.90	3	9.78	A	B	C				
44	68.53	3	9.78	A	B	C				
36	69.30	3	9.78	A	B	C				
28	70.80	3	9.78	A	B	C				
25	71.03	3	9.78	A	B	C				
47	71.13	3	9.78	A	B	C				
44	74.40	3	9.78	A	B	C				
34	74.60	3	9.78	A	B	C				
1	77.83	3	9.78	A	B	C				
38	78.10	3	9.78	A	B	C				
22	78.13	3	9.78	A	B	C				
6	78.97	3	9.78	A	B	C				
48	80.87	3	9.78	A	B	C				
23	83.70	3	9.78	A	B	C				
10	85.47	3	9.78	A	B	C				
30	87.30	3	9.78	A	B	C				
35	88.67	3	9.78	A	B	C				
18	89.67	3	9.78	A	B	C				
49	90.13	3	9.78	A	B	C				
31	91.00	3	9.78	A	B	C				
42	91.27	3	9.78	A	B	C				
17	91.57	3	9.78	A	B	C				
32	91.87	3	9.78		B	C				
45	92.43	3	9.78		B	C				
14	92.80	3	9.78		B	C				
26	93.13	3	9.78		B	C				
24	93.70	3	9.78		B	C				
50	93.80	3	9.78		B	C				
40	94.80	3	9.78			C				
29	95.30	3	9.78			C				
27	95.73	3	9.78			C				
5	96.10	3	9.78			C				
2	96.10	3	9.78			C				
37	96.23	3	9.78			C				
16	96.77	3	9.78			C				
19	97.23	3	9.78			C				
12	97.47	3	9.78			C				
33	97.63	3	9.78			C				
20	97.97	3	9.78			C				
46	98.47	3	9.78			C				
15	98.70	3	9.78			C				
21	98.93	3	9.78			C				
3	99.47	3	9.78			C				
43	99.57	3	9.78			C				
433-94	100.00	3	9.78							C

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for conidia germination in 50 strains of *Beauveria bassiana* and Tukey test (p>0,05) at 25 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	9714.21	49	198.25	3.90	<0.0001
Strain	9714.21	49	198.25	3.90	<0.0001
Error	5082.16	100	50.82		
Total	14796.37	149			

Test:Tukey Alpha:=0.05 LSD:=24.00944

Error: 50.8216 df: 100

Strain	Means	n	S.E.				
9	63.00	3	4.12	A			
20	71.03	3	4.12	A	B		
11	76.20	3	4.12	A	B	C	
41	76.30	3	4.12	A	B	C	
7	83.80	3	4.12	A	B	C	
8	84.60	3	4.12	A	B	C	
10	84.87	3	4.12	A	B	C	
28	88.23	3	4.12		B	C	
47	88.63	3	4.12		B	C	
44	90.13	3	4.12		B	C	
44	90.60	3	4.12		B	C	
34	91.83	3	4.12		B	C	
29	92.60	3	4.12		B	C	
22	94.13	3	4.12		B	C	
26	94.33	3	4.12		B	C	
25	94.40	3	4.12		B	C	
23	94.47	3	4.12		B	C	
19	95.40	3	4.12			C	
36	96.37	3	4.12			C	
1	96.53	3	4.12			C	
18	96.60	3	4.12			C	
38	96.63	3	4.12			C	
17	96.67	3	4.12			C	
27	96.77	3	4.12			C	
48	97.20	3	4.12			C	
6	97.63	3	4.12			C	
42	97.90	3	4.12			C	
32	98.33	3	4.12			C	
49	98.37	3	4.12			C	
45	98.37	3	4.12			C	
2	98.47	3	4.12			C	
35	98.50	3	4.12			C	
40	98.60	3	4.12			C	
30	98.93	3	4.12			C	
33	99.13	3	4.12			C	
5	99.23	3	4.12			C	
14	99.33	3	4.12				C
24	99.53	3	4.12			C	
21	99.60	3	4.12			C	
20	99.77	3	4.12				C
15	99.77	3	4.12				C
31	99.77	3	4.12			C	
12	99.80	3	4.12				C
37	99.90	3	4.12			C	
43	99.90	3	4.12			C	
3	100.00	3	4.12			C	
16	100.00	3	4.12				C
50	100.00	3	4.12			C	
46	100.00	3	4.12			C	
433-94	100.00	3	4.12				C

ANOVA One way for conidia germination in 50 strains of *Beauveria bassiana* and Tukey test (p>0,05) at 30 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	15136.20	49	308.90	3.70	<0.0001
Strain	15136.20	49	308.90	3.70	<0.0001
Error	8351.13	100	83.51		
Total	23487.33	149			

Test:Tukey Alpha:=0.05 LSD:=30.77731

Error: 83.5113 df: 100

Strain	Means	n	S.E.			
9	50.97	3	5.28	A		
25	59.20	3	5.28	A	B	
41	72.40	3	5.28	A	B	C
44	76.70	3	5.28	A	B	C
11	79.37	3	5.28	A	B	C
10	85.60	3	5.28		B	C
20	86.00	3	5.28		B	C
28	89.50	3	5.28		B	C
7	90.53	3	5.28			C
34	90.60	3	5.28			C
26	92.50	3	5.28			C
29	94.00	3	5.28			C
38	94.20	3	5.28			C
30	94.30	3	5.28			C
31	94.90	3	5.28			C
44	94.97	3	5.28			C
27	96.17	3	5.28			C
36	96.33	3	5.28			C
19	96.43	3	5.28			C
8	96.50	3	5.28			C
47	96.83	3	5.28			C
40	96.87	3	5.28			C
49	97.00	3	5.28			C
32	97.50	3	5.28			C
42	97.60	3	5.28			C
22	97.93	3	5.28			C
37	97.97	3	5.28			C
2	98.27	3	5.28			C
24	98.37	3	5.28			C
17	99.03	3	5.28			C
6	99.03	3	5.28			C
18	99.17	3	5.28			C
1	99.17	3	5.28			C
48	99.23	3	5.28			C
50	99.30	3	5.28			C
45	99.57	3	5.28			C
23	99.57	3	5.28			C
14	99.77	3	5.28			C
21	99.77	3	5.28			C
5	99.77	3	5.28			C
35	99.90	3	5.28			C
33	99.90	3	5.28			C
16	100.00	3	5.28			C
433-94	100.00	3	5.28			C
3	100.00	3	5.28			C
20	100.00	3	5.28			C
12	100.00	3	5.28			C
15	100.00	3	5.28			C
43	100.00	3	5.28			C
46	100.00	3	5.28			C

Means with a common letter are not significantly different (p > 0.05)

ANOVA One way for conidia germination in 50 strains of *Beauveria bassiana* and Tukey test (p>0,05) at 33 °C

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	4.28	49	0.09	4.28	<0.0001
Strain	4.28	49	0.09	4.28	<0.0001
Error	2.04	100	0.02		
Total	6.32	149			

Test:Tukey Alpha:=0.05 LSD:=0.48103

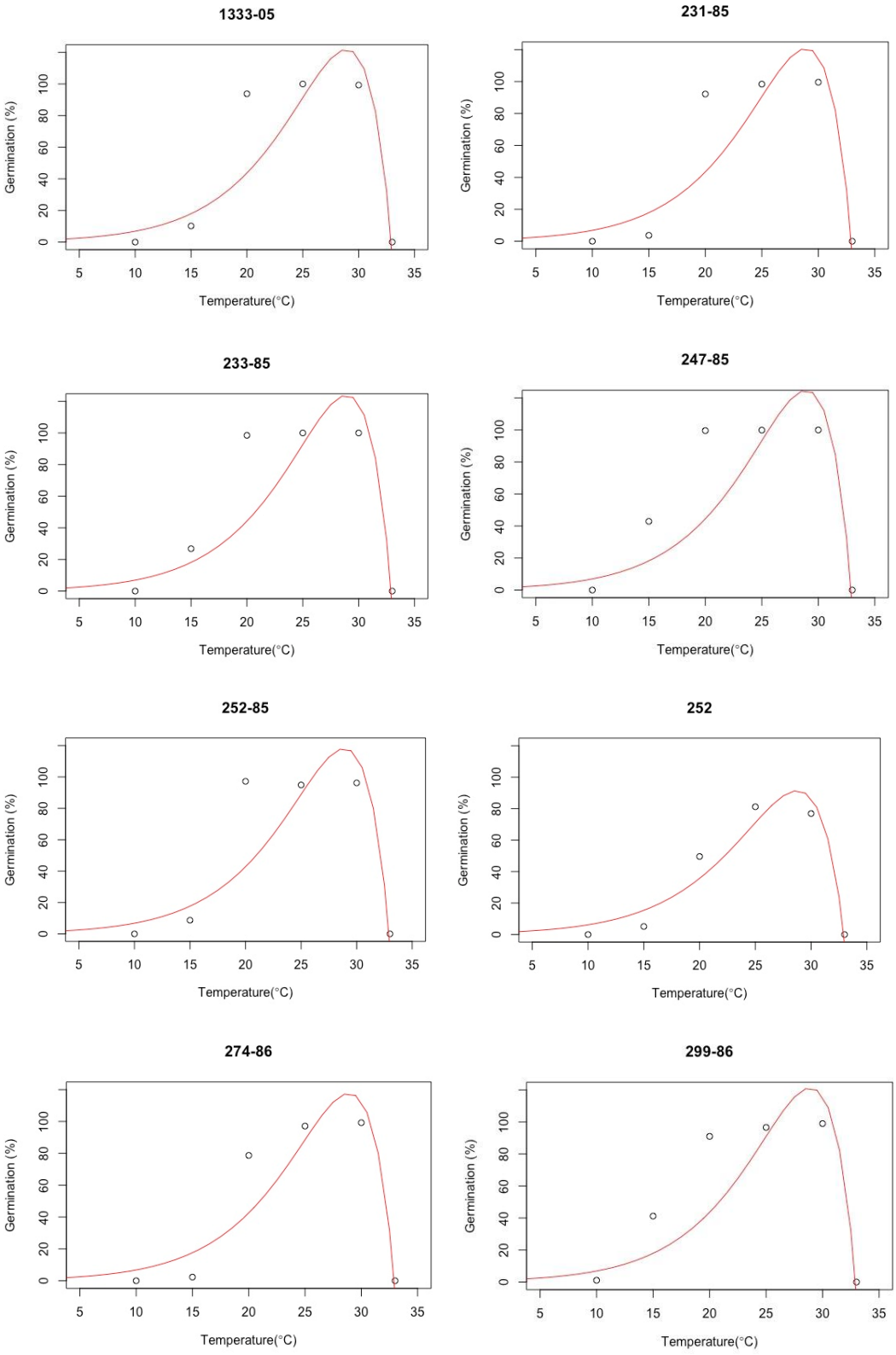
Error: 0.0204 df: 100

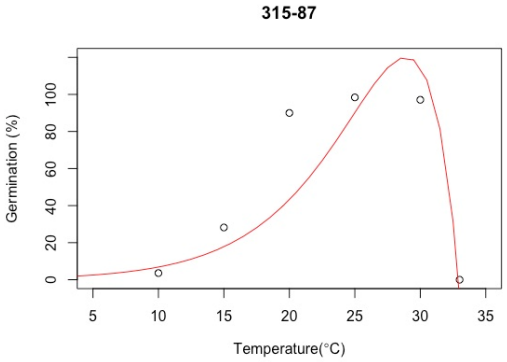
Strain	Means	n	S.E.	
31	0.00	3	0.08	A
20	0.00	3	0.08	A
20	0.00	3	0.08	A
24	0.00	3	0.08	A
42	0.00	3	0.08	A
30	0.00	3	0.08	A
7	0.00	3	0.08	A
37	0.00	3	0.08	A
3	0.00	3	0.08	A
22	0.00	3	0.08	A
1	0.00	3	0.08	A
40	0.00	3	0.08	A
26	0.00	3	0.08	A
28	0.00	3	0.08	A
38	0.00	3	0.08	A
29	0.00	3	0.08	A
49	0.00	3	0.08	A
27	0.00	3	0.08	A
6	0.00	3	0.08	A
41	0.00	3	0.08	A
23	0.00	3	0.08	A
25	0.00	3	0.08	A
36	0.00	3	0.08	A
35	0.00	3	0.08	A
45	0.00	3	0.08	A
46	0.00	3	0.08	A
34	0.00	3	0.08	A
18	0.00	3	0.08	A
11	0.00	3	0.08	A
44	0.00	3	0.08	A
8	0.00	3	0.08	A
2	0.00	3	0.08	A
9	0.00	3	0.08	A
10	0.00	3	0.08	A
15	0.00	3	0.08	A
50	0.00	3	0.08	A
32	0.00	3	0.08	A
16	0.00	3	0.08	A
48	0.00	3	0.08	A
47	0.00	3	0.08	A
12	0.00	3	0.08	A
44	0.00	3	0.08	A
17	0.00	3	0.08	A
5	0.00	3	0.08	A
19	0.00	3	0.08	A
43	0.00	3	0.08	A
21	0.23	3	0.08	A
33	0.33	3	0.08	A
14	0.37	3	0.08	A
433-94	1.10	3	0.08	B

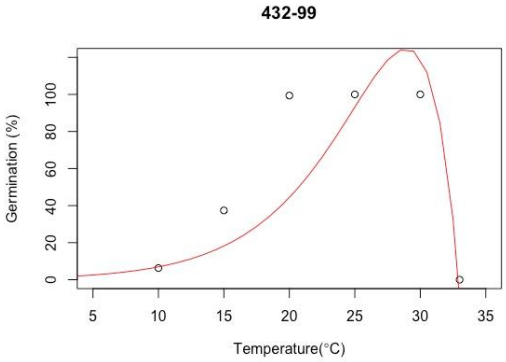
Means with a common letter are not significantly different (p > 0.05)

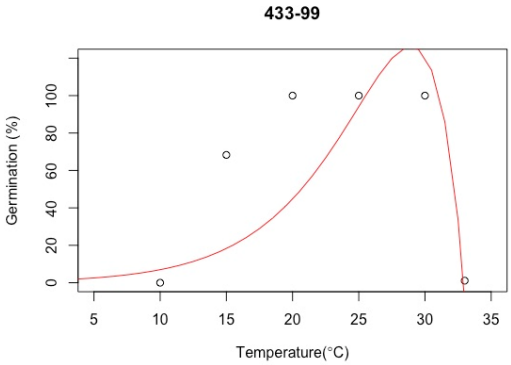
Appendix 7

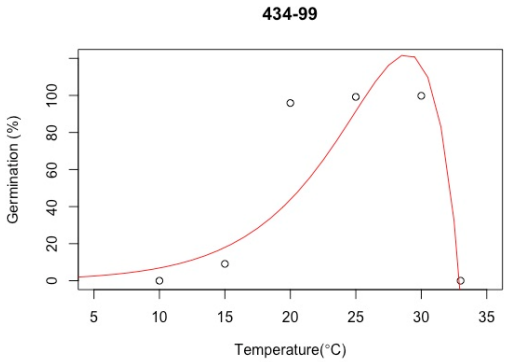
Lactin-1 non-linear model fitted to mean of percentage of germination plotted against six temperatures for fifty strains of *Beauveria*.

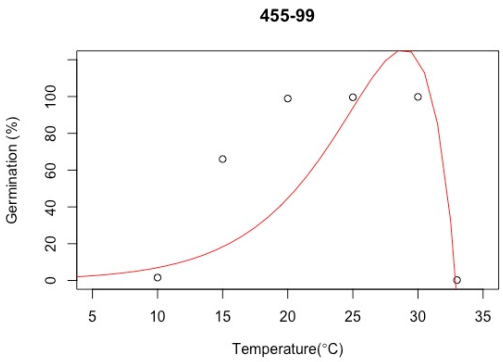


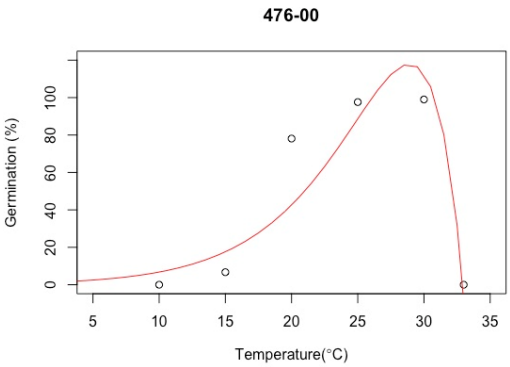

315-87
Germination (%)
Temperature(°C)

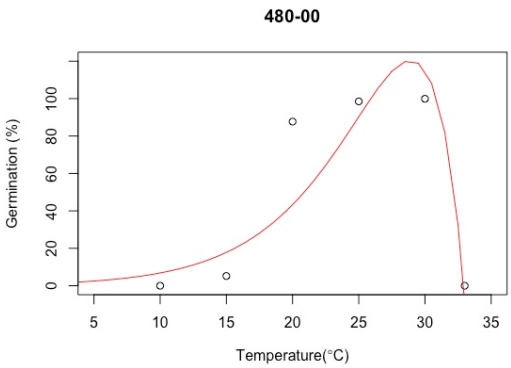

432-99
Germination (%)
Temperature(°C)

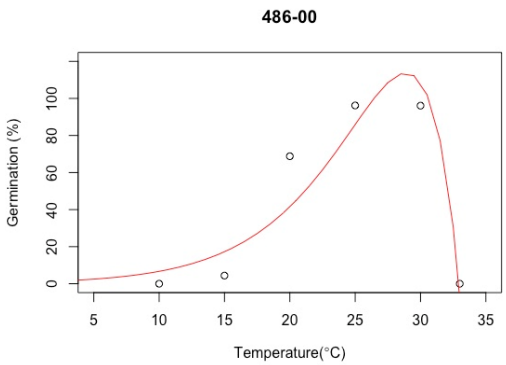

433-99
Germination (%)
Temperature(°C)

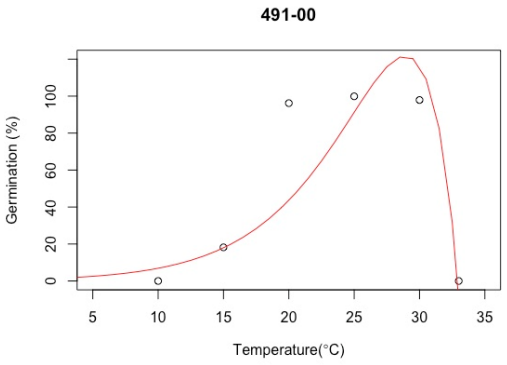

434-99
Germination (%)
Temperature(°C)

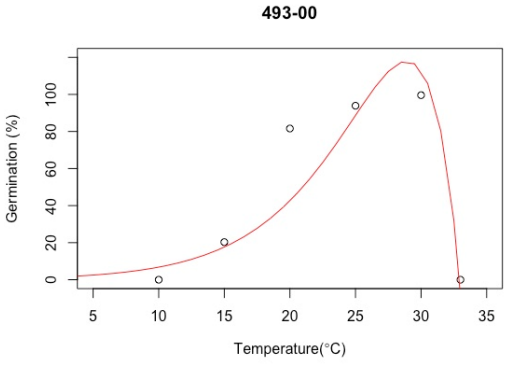

455-99
Germination (%)
Temperature(°C)

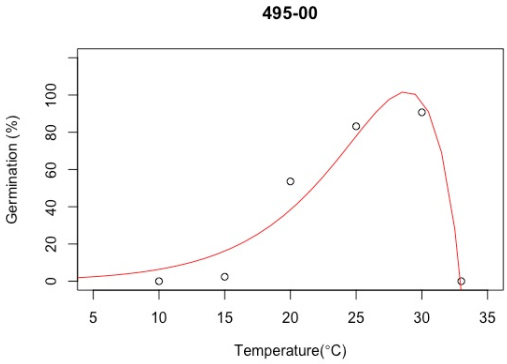

476-00
Germination (%)
Temperature(°C)

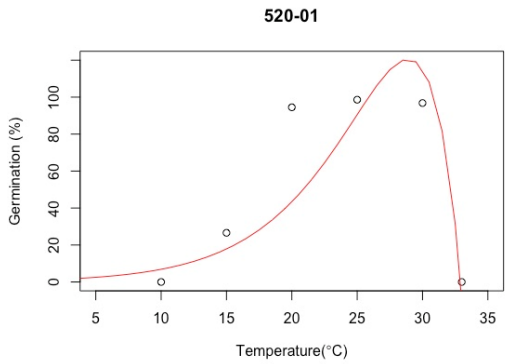

480-00
Germination (%)
Temperature(°C)

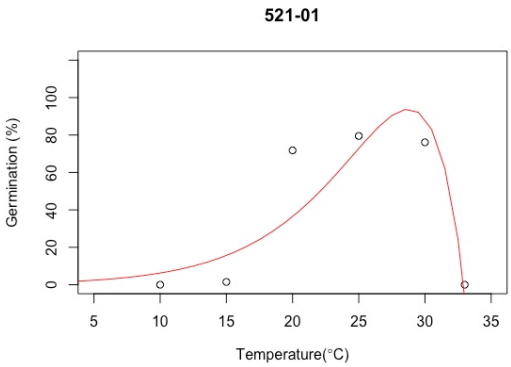

486-00
Germination (%)
Temperature(°C)

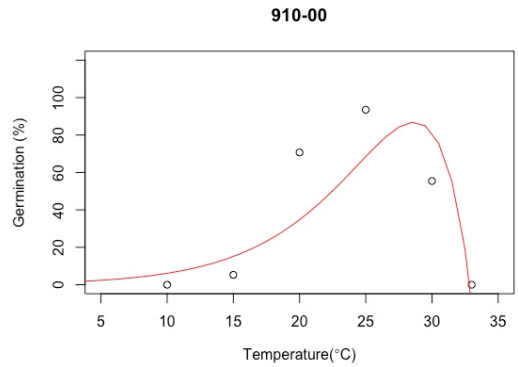

491-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

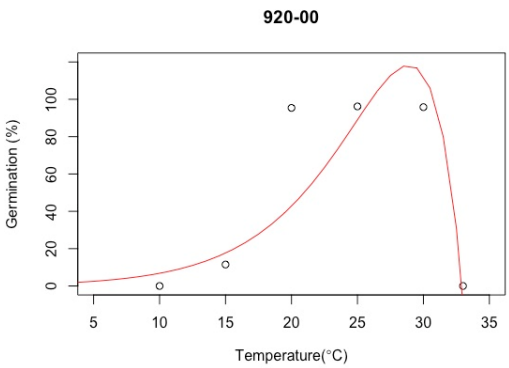

493-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

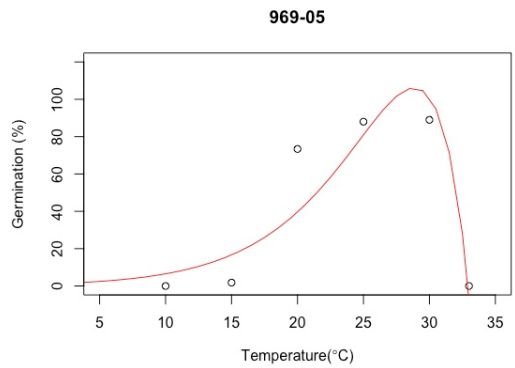

495-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

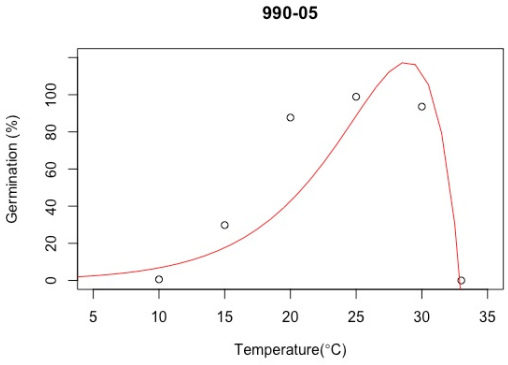

520-01
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

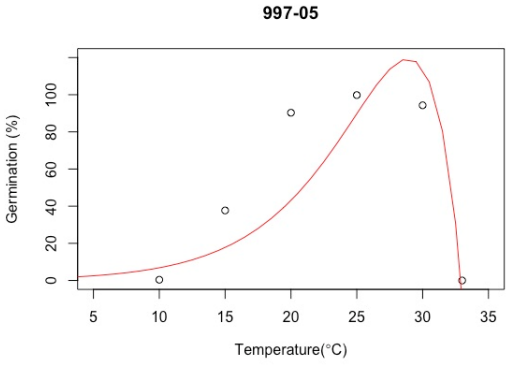

521-01
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

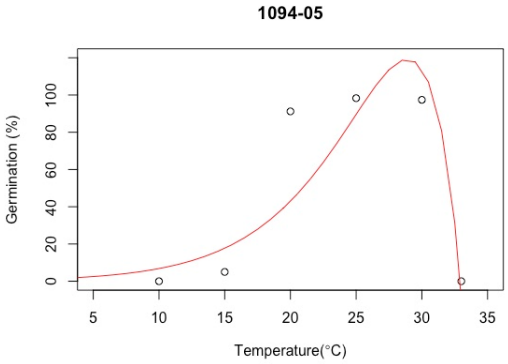

910-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

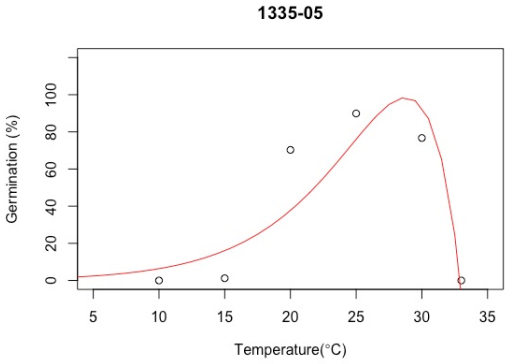

920-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

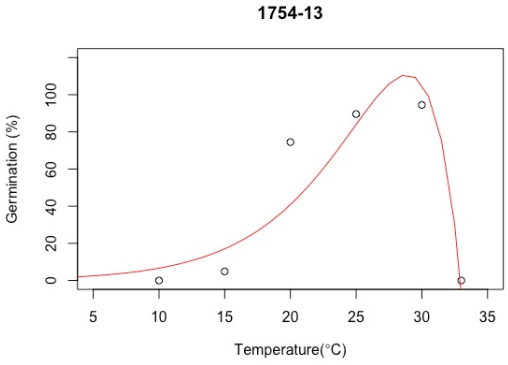

969-05
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

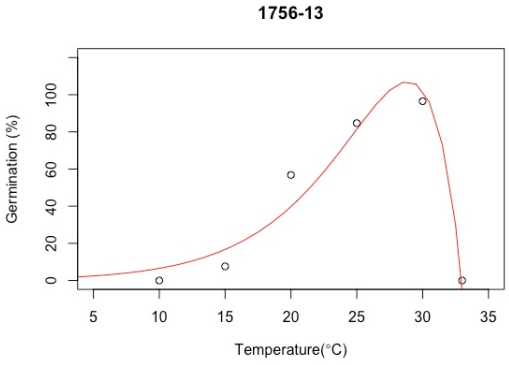

990-05
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

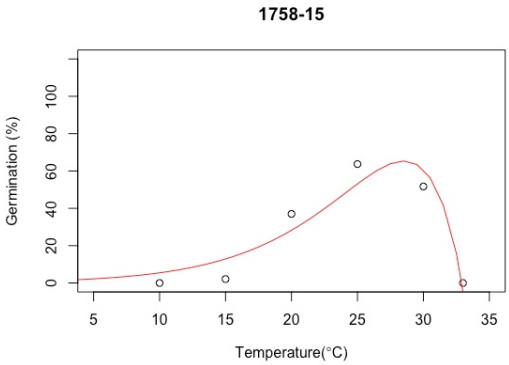

997-05
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

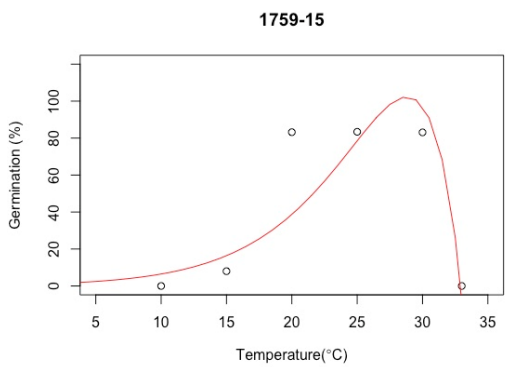

1094-05
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

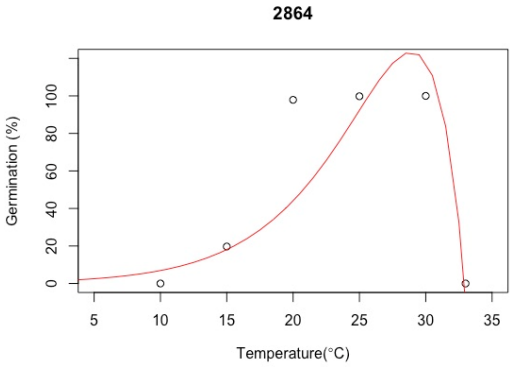

1335-05
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

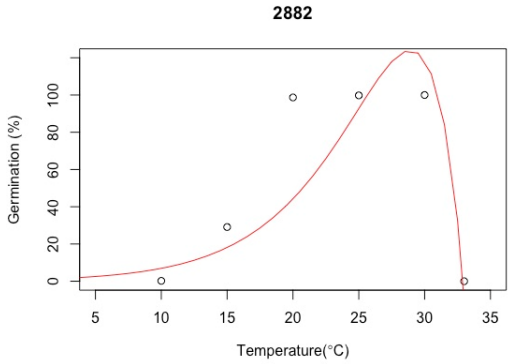

1754-13
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

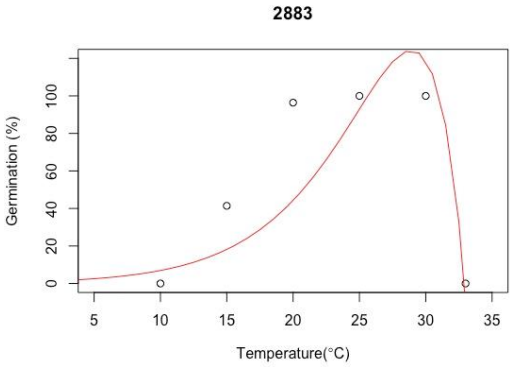

1756-13
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

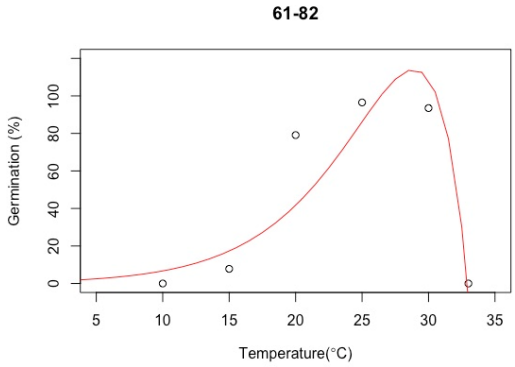

1758-15
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

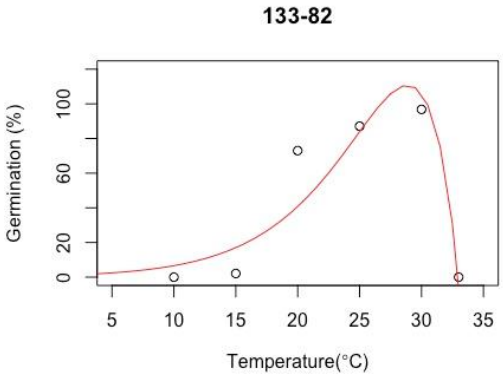

1759-15
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

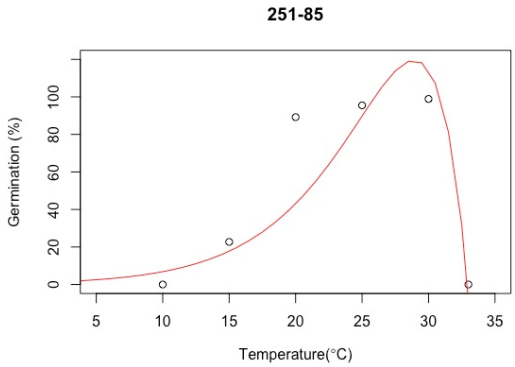

2864
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

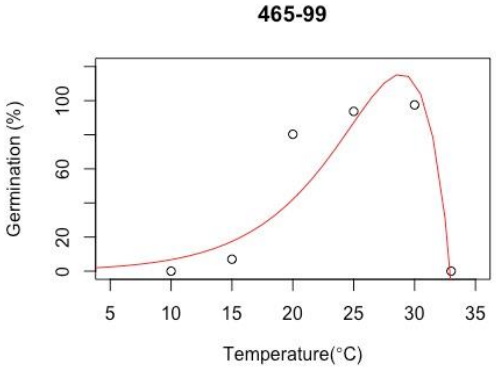

2882
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

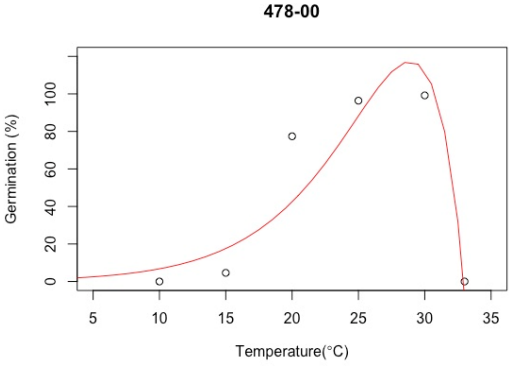

2883
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

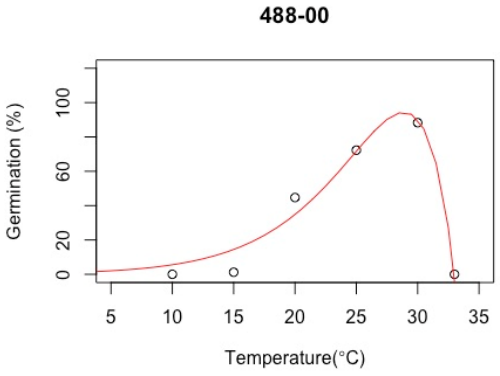

61-82
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

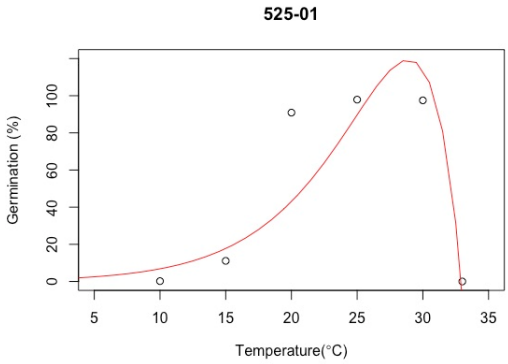

133-82
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)

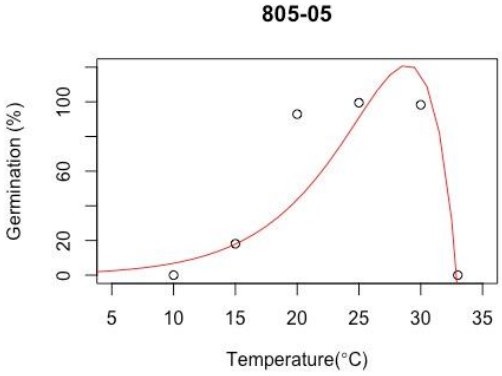

251-85
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

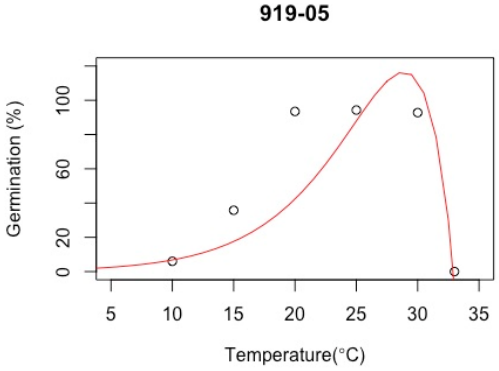

465-99
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)

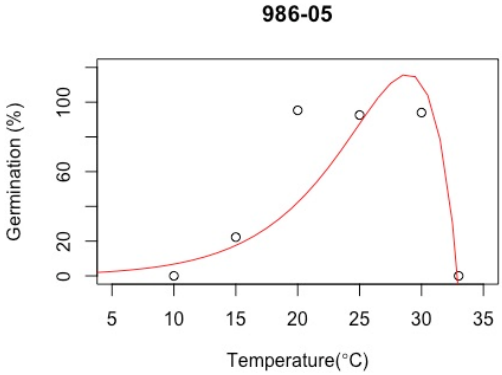

478-00
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

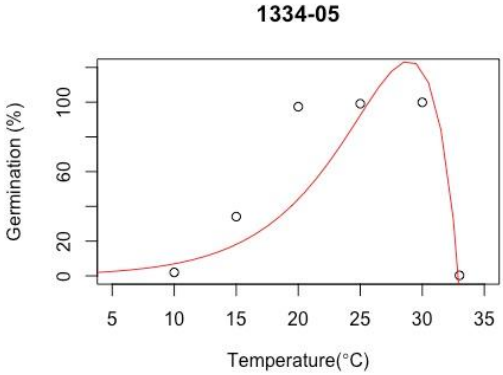

488-00
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)

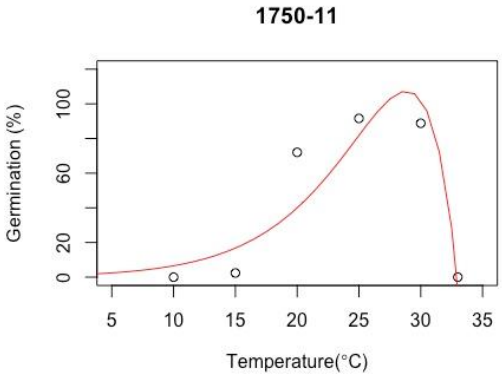

525-01
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

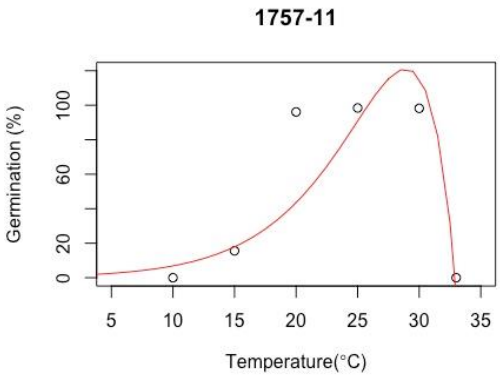

805-05
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)

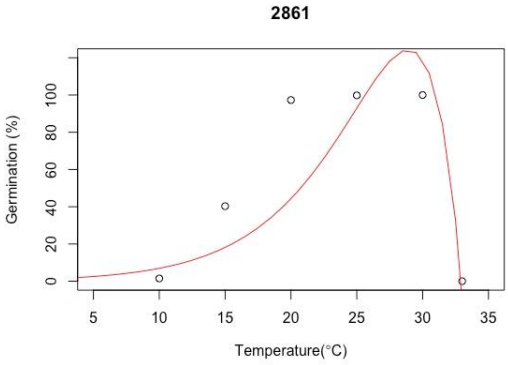

919-05
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)

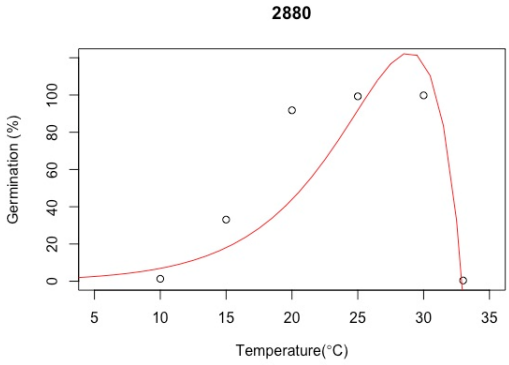

986-05
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)


1334-05
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)


1750-11
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)


1757-11
Germination (%)
0
20
60
100
5
10
15
20
25
30
35
Temperature(°C)


2861
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)


2880
Germination (%)
0
20
40
60
80
100
5
10
15
20
25
30
35
Temperature(°C)

Appendix 8

ANOVA One way and Tukey test (p>0,05) for conidia germination in 50 strains of *Beauveria* after 90 minutes of UV-B Radiation.

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	93668.86	49	1911.61	4.77	<0.0001
Sample	93668.86	49	1911.61	4.77	<0.0001
Error	40103.05	100	401.03		
Total	133771.90	149			

Test:Tukey Alpha:=0.05 LSD:=67.44451

Error: 401.0305 df: 100

Sample	Means	n	S.E.									
29	0.00	3	11.56	A								
43	4.73	3	11.56	A	B							
41	13.47	3	11.56	A	B	C						
27	14.90	3	11.56	A	B	C	D					
46	18.63	3	11.56	A	B	C	D	E				
47	19.00	3	11.56	A	B	C	D	E				
2	23.97	3	11.56	A	B	C	D	E	F			
11	25.33	3	11.56	A	B	C	D	E	F			
7	25.57	3	11.56	A	B	C	D	E	F			
32	28.27	3	11.56	A	B	C	D	E	F			
38	29.33	3	11.56	A	B	C	D	E	F			
910-01	29.73	3	11.56	A	B	C	D	E	F			
44	30.60	3	11.56	A	B	C	D	E	F			
18	31.40	3	11.56	A	B	C	D	E	F			
42	31.47	3	11.56	A	B	C	D	E	F			
8	32.87	3	11.56	A	B	C	D	E	F			
36	34.33	3	11.56	A	B	C	D	E	F			
9	35.40	3	11.56	A	B	C	D	E	F			
20	36.70	3	11.56	A	B	C	D	E	F			
34	38.17	3	11.56	A	B	C	D	E	F			
22	40.10	3	11.56	A	B	C	D	E	F			
6	43.57	3	11.56	A	B	C	D	E	F			
37	45.40	3	11.56	A	B	C	D	E	F			
10	48.60	3	11.56	A	B	C	D	E	F			
19	49.57	3	11.56	A	B	C	D	E	F			
1	55.03	3	11.56	A	B	C	D	E	F			
3	56.10	3	11.56	A	B	C	D	E	F			
17	57.47	3	11.56	A	B	C	D	E	F			
274-96	58.17	3	11.56	A	B	C	D	E	F			
30	60.90	3	11.56	A	B	C	D	E	F			
805-01	64.70	3	11.56	A	B	C	D	E	F			
4	65.47	3	11.56	A	B	C	D	E	F			
5	65.60	3	11.56	A	B	C	D	E	F			
15	68.97	3	11.56		B	C	D	E	F			
40	72.40	3	11.56			C	D	E	F			
44	73.90	3	11.56			C	D	E	F			
50	75.57	3	11.56			C	D	E	F			
28	76.50	3	11.56			C	D	E	F			
21	78.97	3	11.56			C	D	E	F			
31	79.93	3	11.56			C	D	E	F			
20	80.67	3	11.56				C	D	E	F		
33	80.80	3	11.56			C	D	E	F			
12	80.83	3	11.56				C	D	E	F		
45	81.07	3	11.56					D	E	F		
16	82.10	3	11.56					D	E	F		
23	83.87	3	11.56						E	F		
35	84.97	3	11.56						E	F		
14	85.53	3	11.56							E	F	
315-99	86.63	3	11.56									F
26	88.07	3	11.56									F

Appendix 9

ANOVA One way and Tukey test (p>0,05) for percentage of mortality on *Plutella xylostella* by 50 strains of *Beauveria* application.

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	48109.55	50	962.19	6.79	<0.0001
Strain	48109.55	50	962.19	6.79	<0.0001
Total	55053.86	99			

Test:Tukey Alpha:=0.05 LSD:=51.85082

Error: 141.7206 df: 49

Strain	Means	n	S.E.										
36	17.70	2	8.42	A									
33	24.05	2	8.42	A	B								
910-05	25.00	2	8.42	A	B	C							
47	34.30	2	8.42	A	B	C	D						
31	39.75	2	8.42	A	B	C	D	E					
8	40.00	2	8.42	A	B	C	D	E					
21	40.00	2	8.42	A	B	C	D	E					
22	50.00	2	8.42	A	B	C	D	E	F				
1	50.00	2	8.42	A	B	C	D	E	F				
17	51.65	2	8.42	A	B	C	D	E	F				
6	52.15	2	8.42	A	B	C	D	E	F				
48	53.35	2	8.42	A	B	C	D	E	F				
11	56.65	2	8.42	A	B	C	D	E	F				
45	57.15	2	8.42	A	B	C	D	E	F				
44	60.70	2	8.42	A	B	C	D	E	F				
23	69.50	2	8.42	A	B	C	D	E	F				
44	73.35	2	8.42		B	C	D	E	F				
15	75.00	2	8.42		B	C	D	E	F				
42	75.50	2	8.42		B	C	D	E	F				
34	76.65	2	8.42			C	D	E	F				
5	78.55	2	8.42				D	E	F				
32	78.55	2	8.42				D	E	F				
3	80.00	2	8.42				D	E	F				
18	80.00	2	8.42					D	E	F			
49	80.00	1	11.90					D	E	F			
9	82.35	2	8.42				D	E	F				
35	83.00	2	8.42					D	E	F			
20	85.70	2	8.42					D	E	F			
27	85.85	2	8.42					D	E	F			
26	85.95	2	8.42					D	E	F			
14	85.95	2	8.42						D	E	F		
19	86.20	2	8.42						E	F			
20	86.65	2	8.42							E	F		
50	86.65	2	8.42						E	F			
4	86.70	2	8.42						E	F			
10	88.30	2	8.42						E	F			
38	89.30	2	8.42							E	F		
43	89.50	2	8.42						E	F			
16	89.80	2	8.42							E	F		
24	90.00	2	8.42						E	F			
28	92.80	2	8.42							F			
37	92.90	2	8.42							F			
7	93.10	2	8.42							F			
12	93.10	2	8.42								F		
40	93.30	2	8.42							F			
2	93.30	2	8.42						F				
46	93.35	2	8.42							F			
29	100.00	2	8.42							F			
315-05	100.00	1	11.90									F	
30	100.00	2	8.42							F			
41	100.00	2	8.42							F			

Appendix 10

Culture media used to parasexual and sexual recombination

- **Basal Media (BM):** (per litre of distilled water) 30 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 mg $FeSO_4.7\ H_2O$, and 20 g agar; and 0.2 ml trace element solution (per 100 ml of distilled water) composed of 5g citric acid, 5g $ZnSO_4.7H_2O$, 1g $Fe(NH_4)_2(SO_4)_2.6\ H_2O$, 0.25 g $CuSO_4.5\ H_2O$, 50 mg $MnSO_4.H_2O$, 50 mg H_3BO_4, and 50 mg $Na_2MoO_4.2\ H_2O$.

- **Minimal medium (MM):** (per litre of distilled water) basal medium (BM) + 2 g L^{-1} $NaNO_3$.

- **Water Agar Chlorate Medium (WAC):** (per litre of distilled water) medium containing 20g of agar, 2g of glucose, and 45g $KClO_3$.

- **Nitrite Medium (NE):** (per litre of distilled water) BM + 0.5 g L^{-1} $NaNO_2$ (0,86g).

- **Hypoxanthine Medium (Hx):** (per litre of distilled water) BM + 0.2 g L^{-1} hypoxanthine (2g)

- **Ammonium medium:** (per litre of distilled water) BM + 0.2 g L^{-1} ammonium tartrate. (1g)

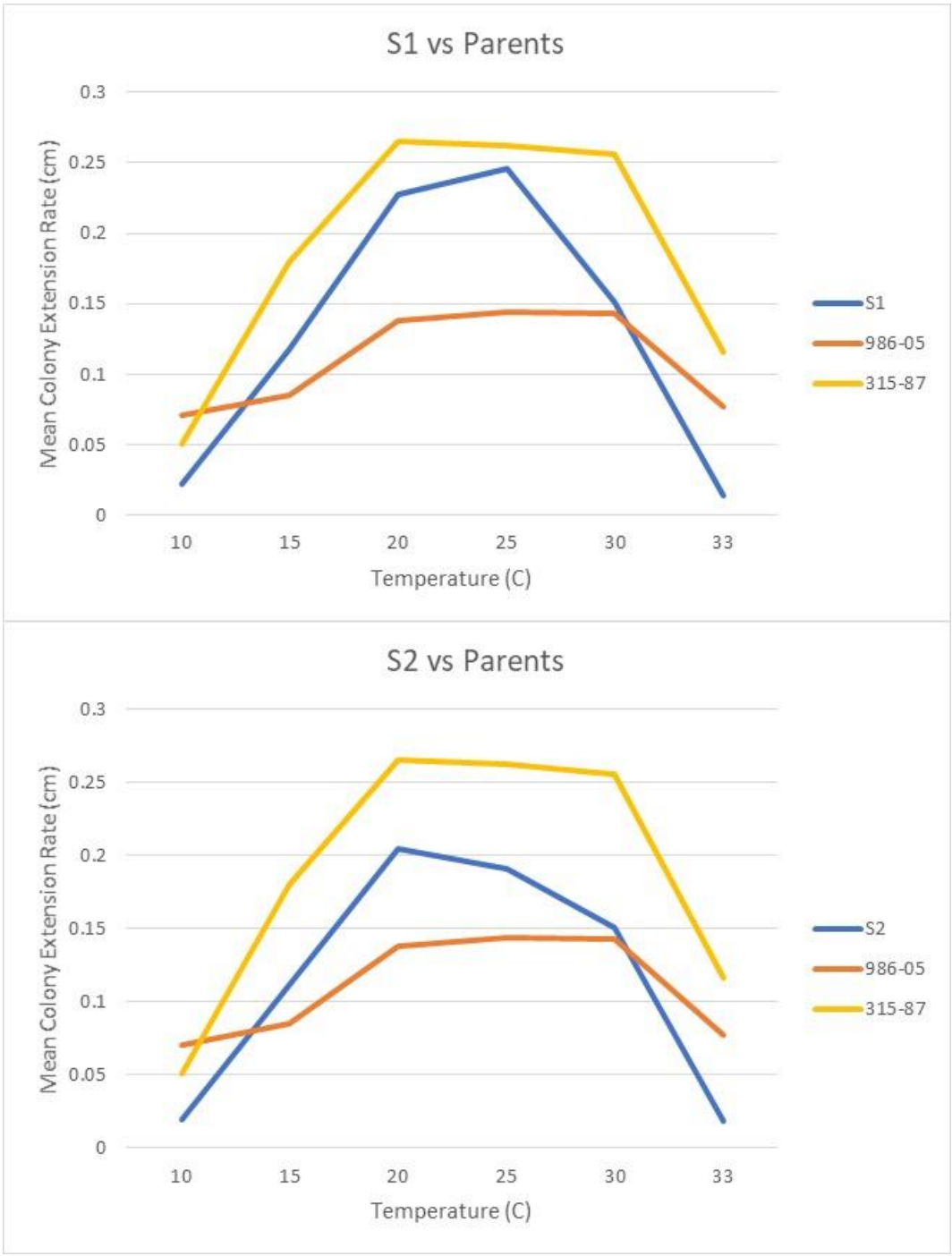
- **Oatmeal Agar:** (per litre of distilled water) 30 g oats (Quaker) + 15 g agar

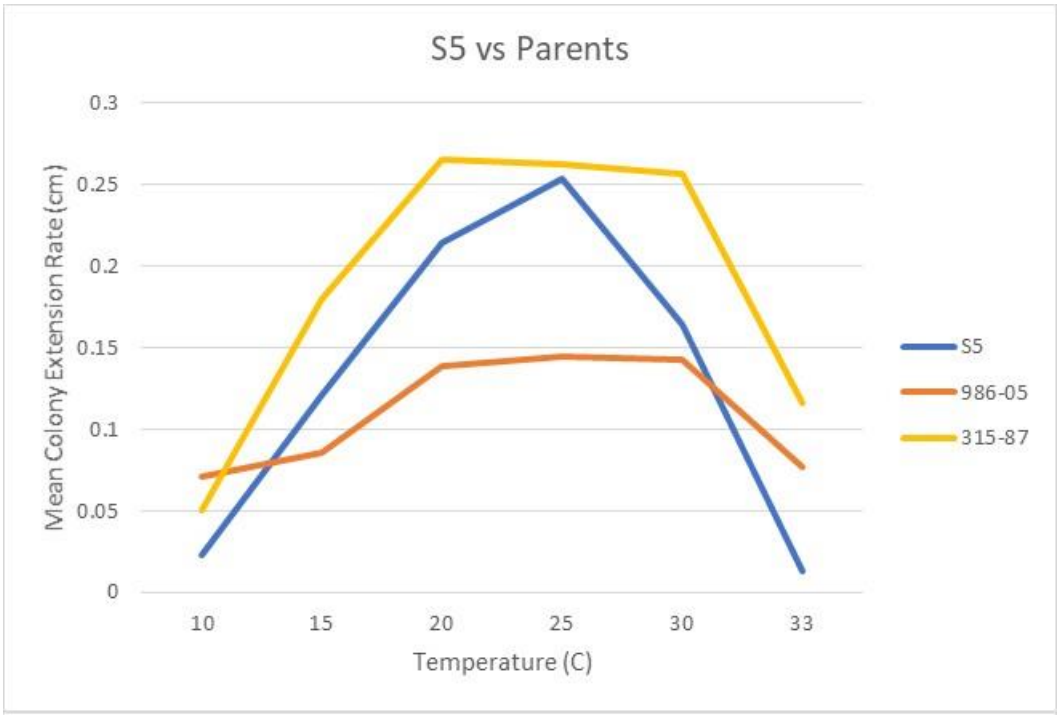
- **Malt extract 2% (MEA):** (per litre of distilled water) 1g MEA + 15 g agar

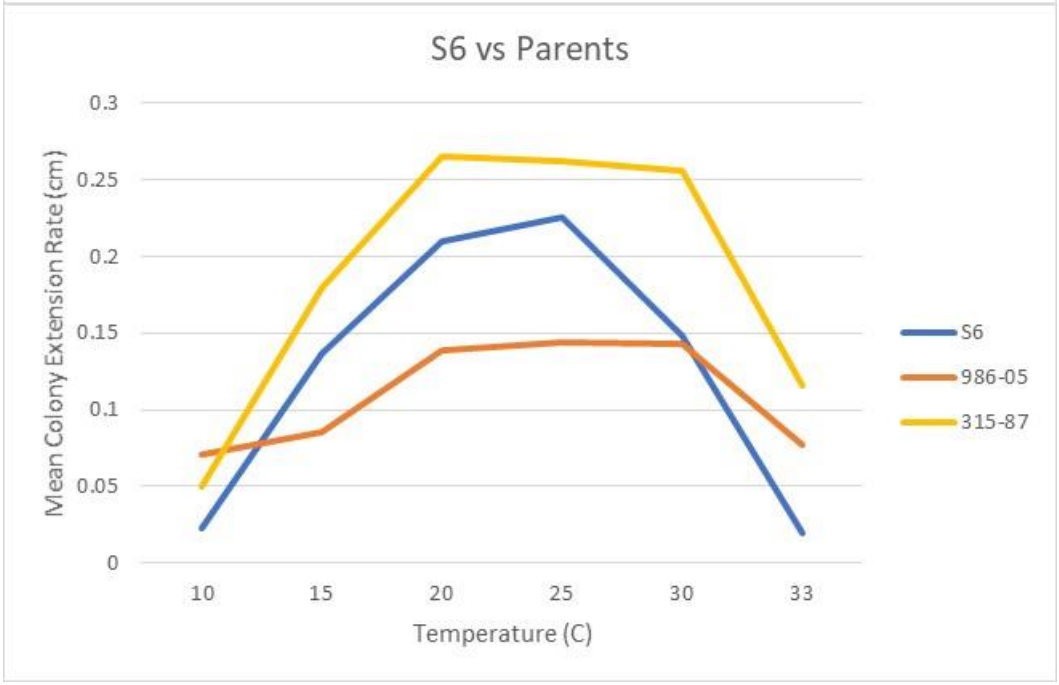
- **Czapek dox agar:** (per litre of distilled water) 45.4 g + 15 g agar

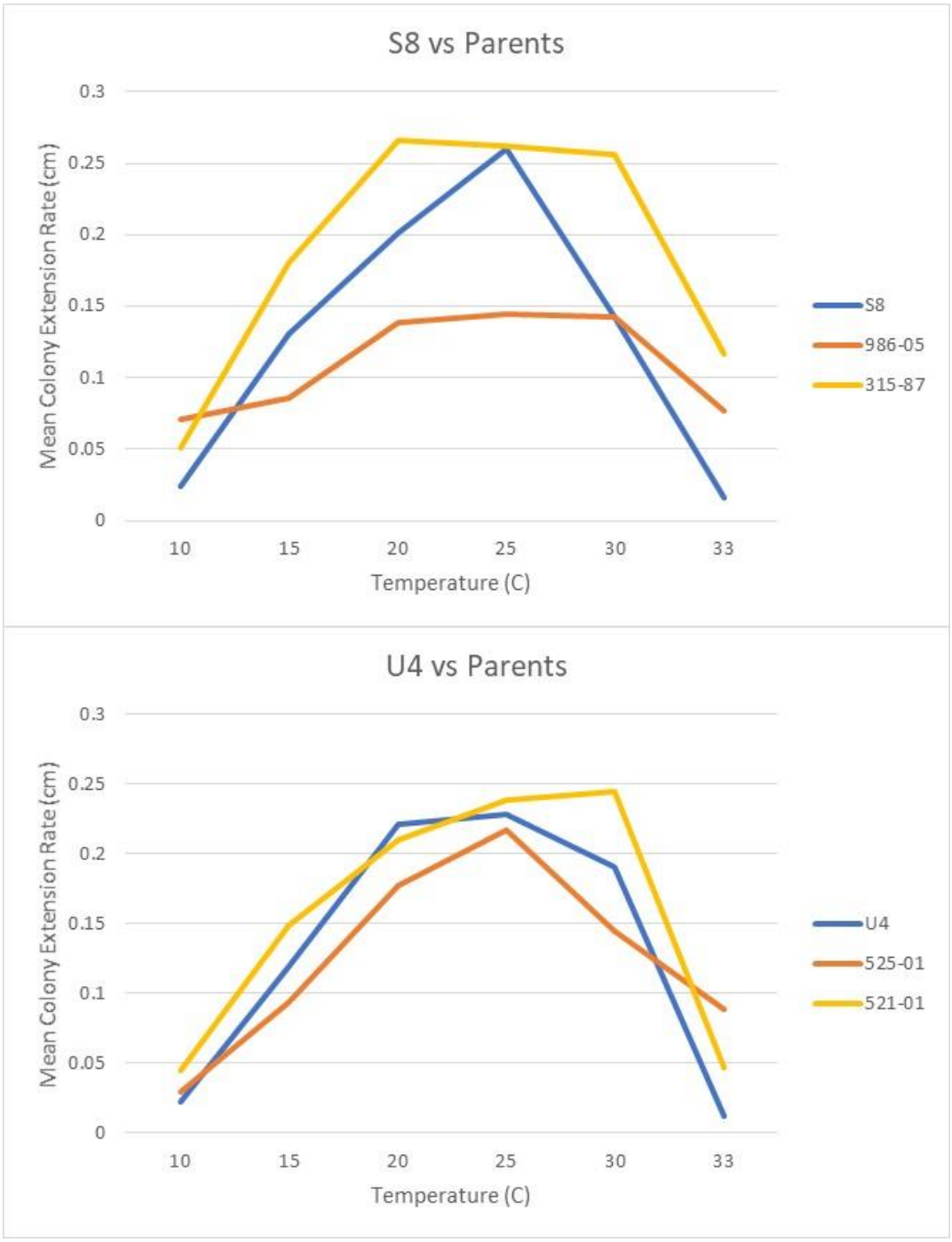
Appendix 11

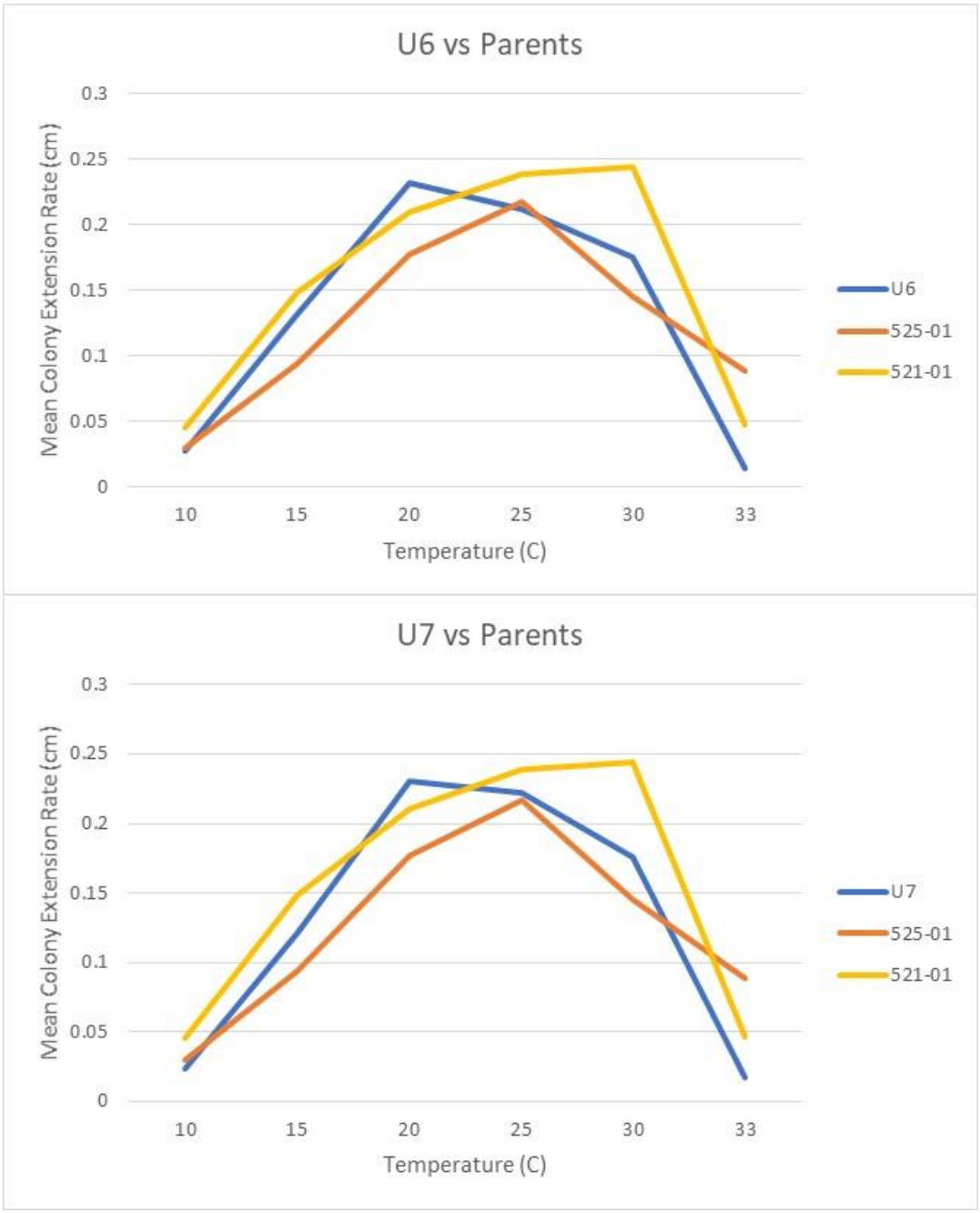
Colony extension growth for 15 hybrids of *Beauveria* from three combinations: S (49 x 29), U (42 x 41) and; X (42 x 29). Comparison between hybrids and parental strains radial growth.

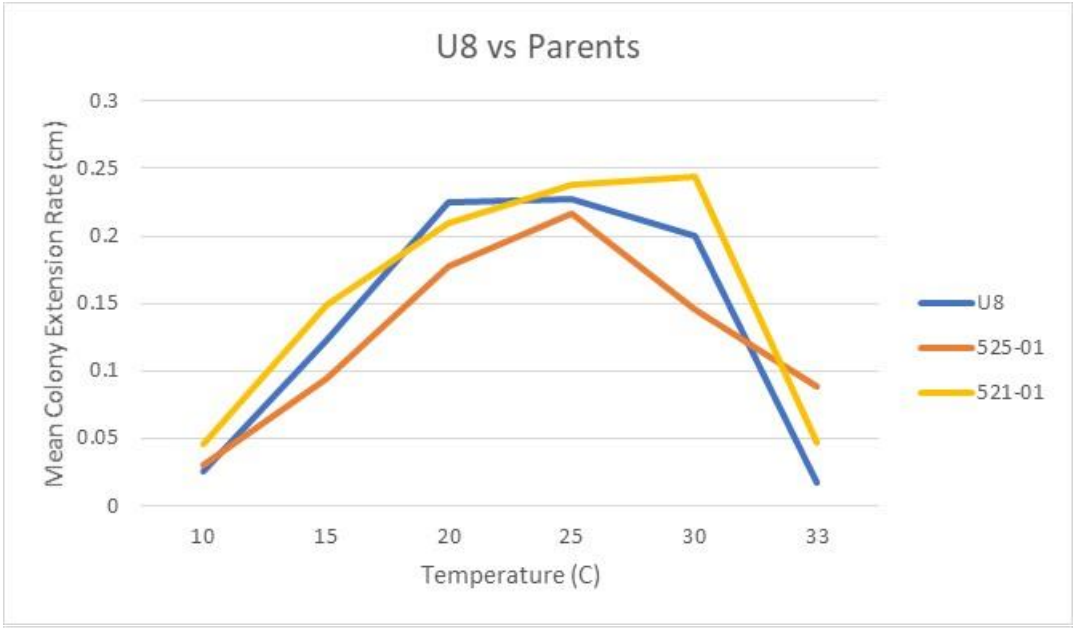


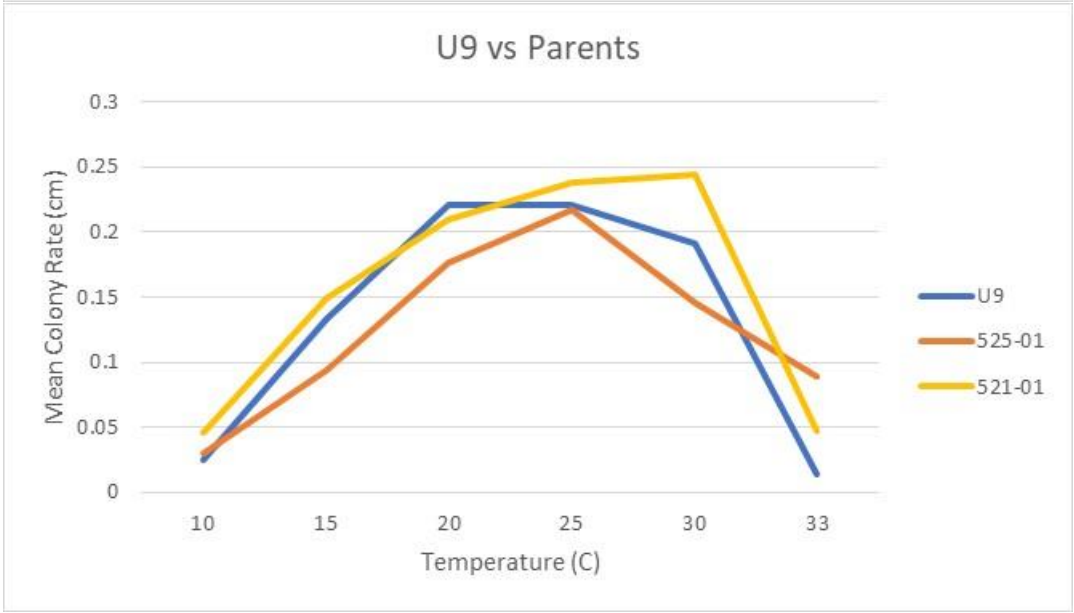

S5 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
S5
986-05
315-87

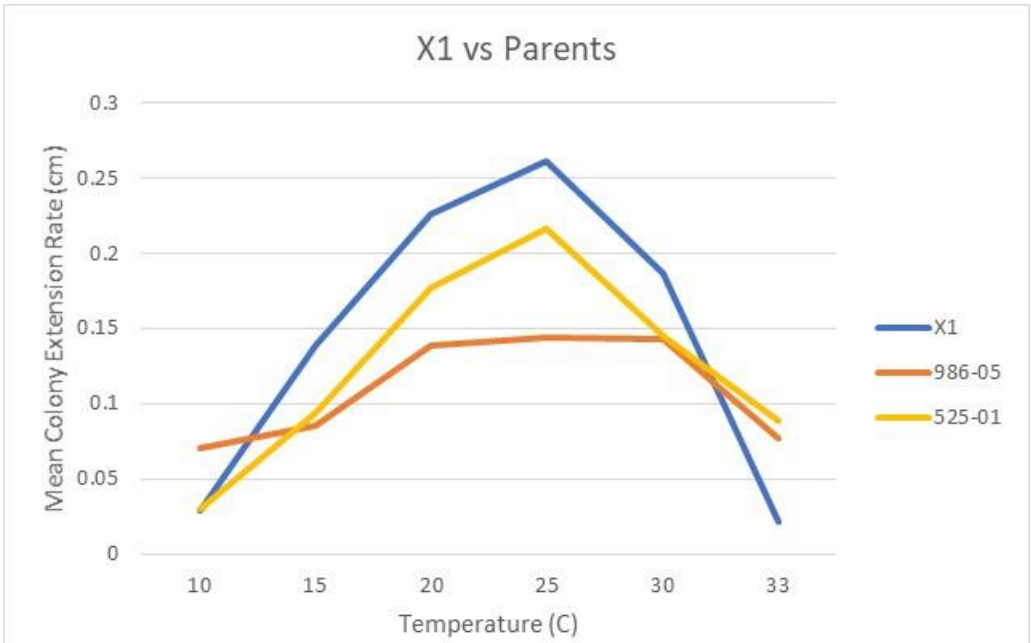

S6 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
S6
986-05
315-87

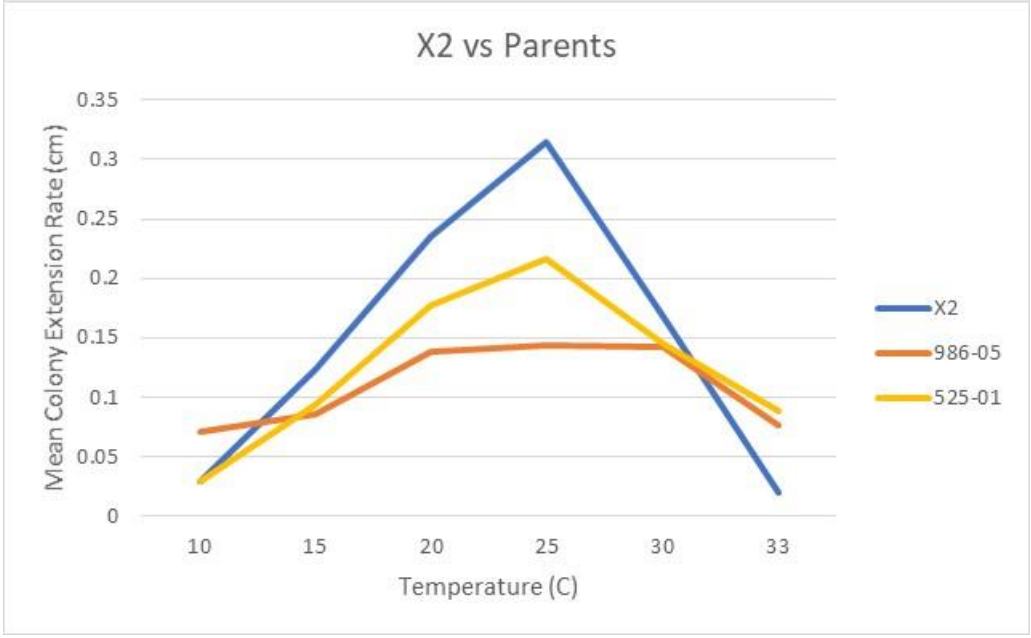

S8 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
S8
986-05
315-87
U4 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
U4
525-01
521-01

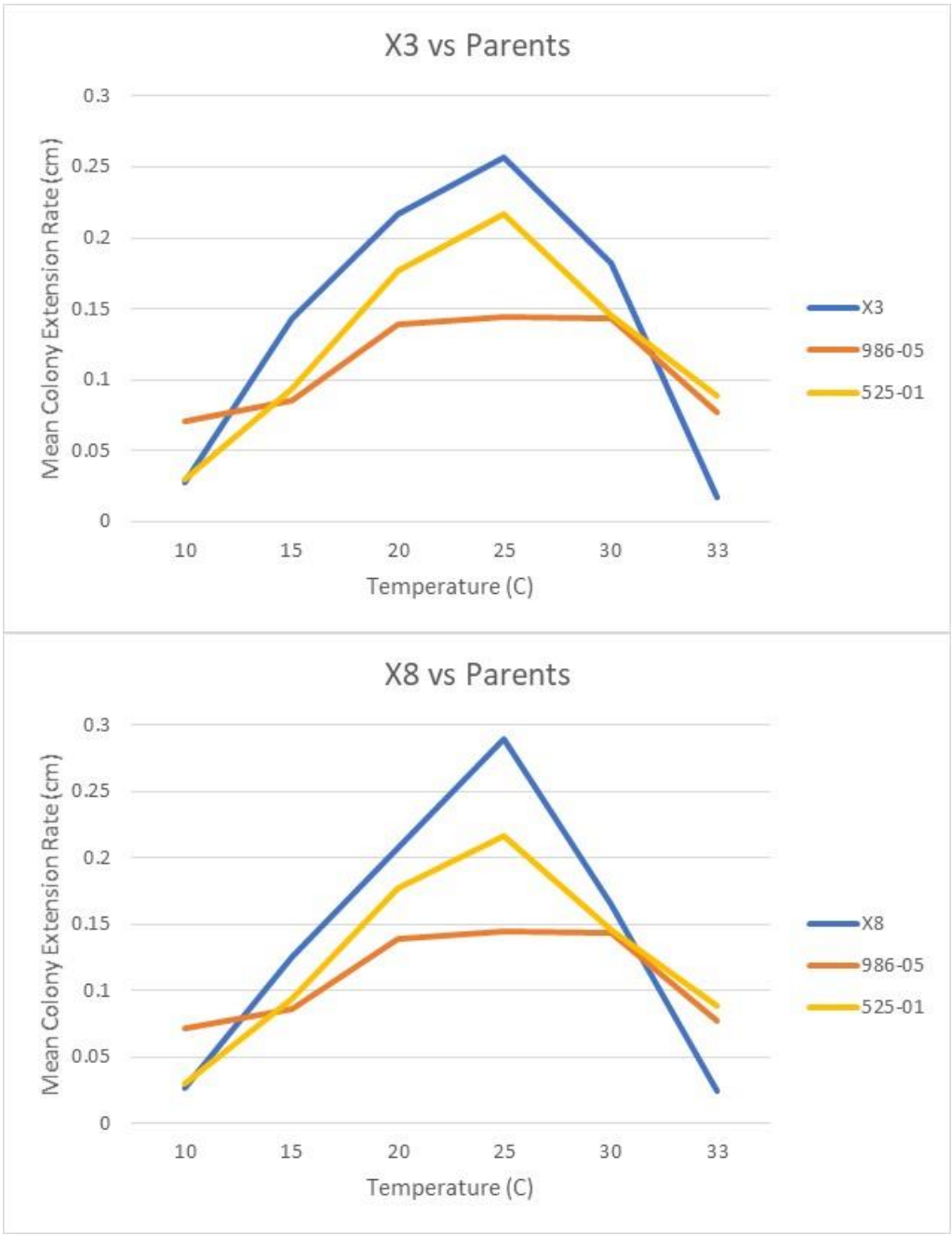

U6 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
U6
525-01
521-01
U7 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
U7
525-01
521-01

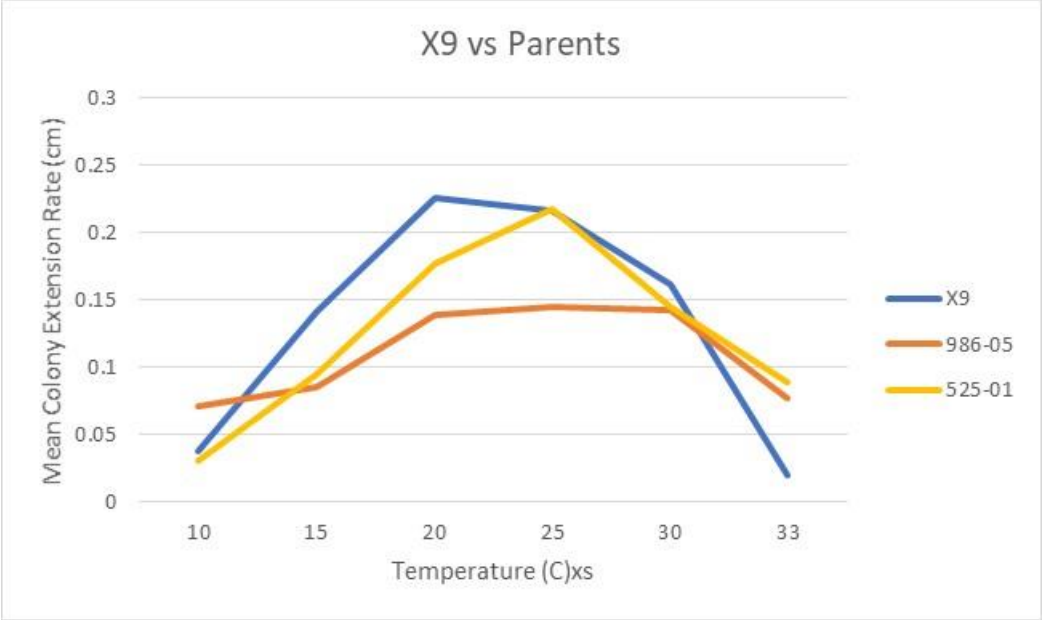

U8 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
U8
525-01
521-01


U9 vs Parents
Mean Colony Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
U9
525-01
521-01


X1 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
X1
986-05
525-01


X2 vs Parents
Mean Colony Extension Rate (cm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
X2
986-05
525-01


X3 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
X3
986-05
525-01
X8 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)
X8
986-05
525-01


X9 vs Parents
Mean Colony Extension Rate (cm)
0.3
0.25
0.2
0.15
0.1
0.05
0
10
15
20
25
30
33
Temperature (C)xs
X9
986-05
525-01

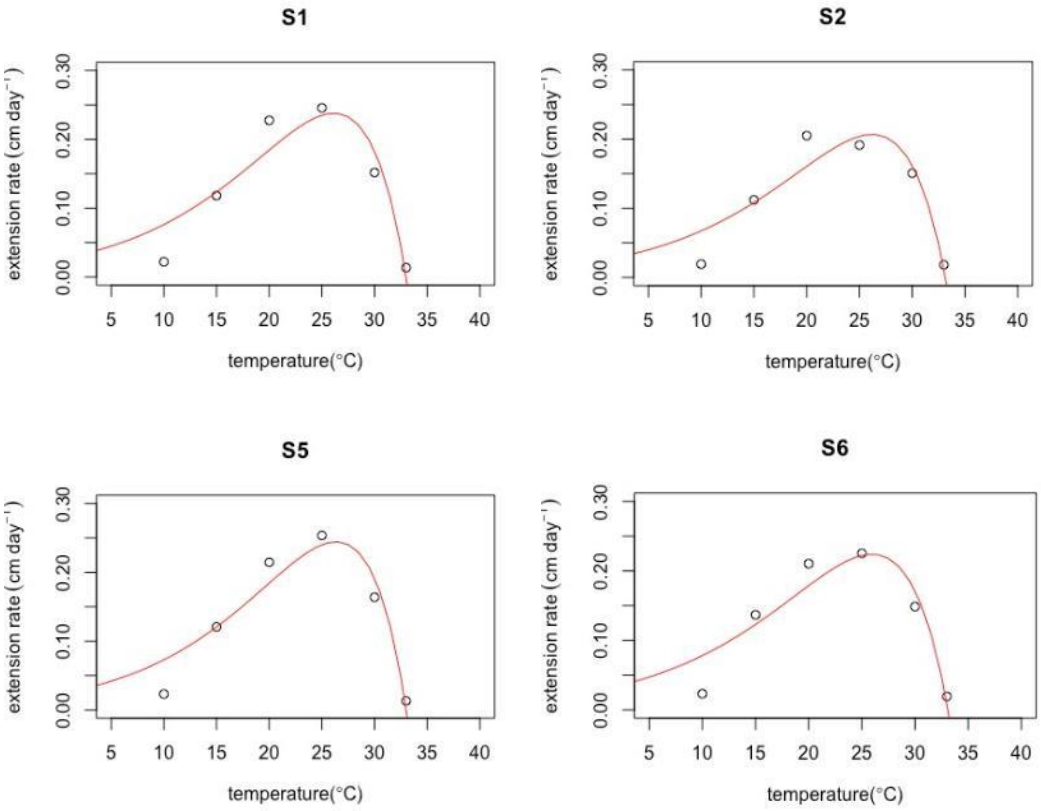
Appendix 12

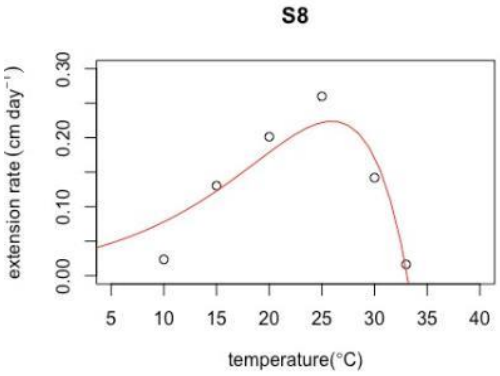
Fitted parameters, r2 and AIC values of Lactin-1 model fitted to percentage of extension radial growth at six temperatures for 15 hybrids of *Beauveria* from three combinations: S (49 x 29), U (42 x 41) and; X (42 x 29).

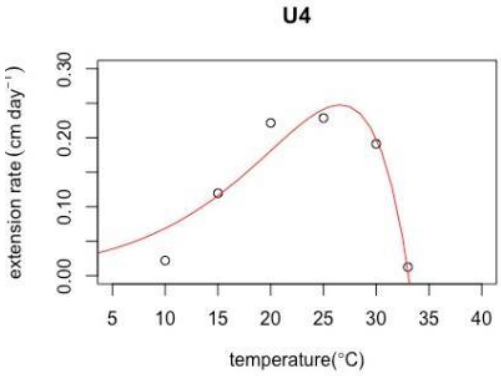
Hybrid	p	Tmax	Topt	Δ	r2	AIC
S1	0.14513	33.01092	26.15735	6.85357	0.88	-16.38171
S2	0.1432	33.1559	26.2068	6.9491	0.86	-18.00123
S5	0.14944	33.02508	26.36527	6.65981	0.91	-18.22999
S6	0.13915	33.11308	25.97014	7.14294	0.88	-17.7255
S8	0.14411	33.02296	26.12153	6.90143	0.88	-16.8377
U4	0.15349	33.08205	26.59409	6.48796	0.91	-18.79212
U6	0.14254	33.10372	26.12789	6.97583	0.88	-17.0226
U7	0.14659	33.1165	26.32891	6.78759	0.88	-17.30846
U8	0.15286	33.13752	26.62389	6.51363	0.92	-18.87544
U9	0.1487	33.11353	26.42103	6.6925	0.91	-18.54059
X1	0.14783	33.12732	26.39837	6.72895	0.92	-19.01024
X2	0.15017	33.01607	26.39212	6.62395	0.88	-14.49853
X3	0.1473	33.08838	26.33528	6.7531	0.92	-19.20038
X8	0.15043	33.09487	26.47922	6.61565	0.89	16.63339
X9	0.13462	33.15879	25.7834	7.37539	0.89	-18.513

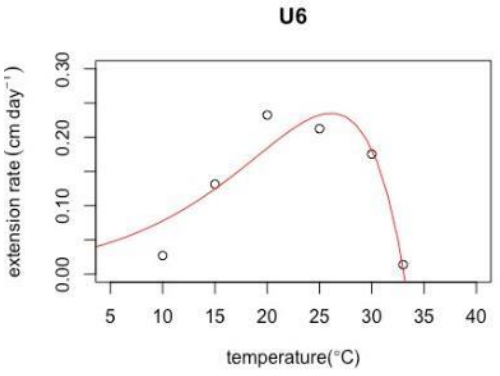
Appendix 13

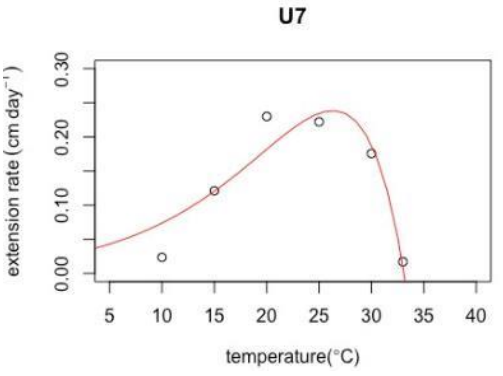
Lactin-1 non-linear model fitted to mean of radial extension growth plotted against six temperatures for 15 hybrid strains of *Beauveria* from three combinations: S (49 x 29), U (42 x 41) and; X (42 x 29).

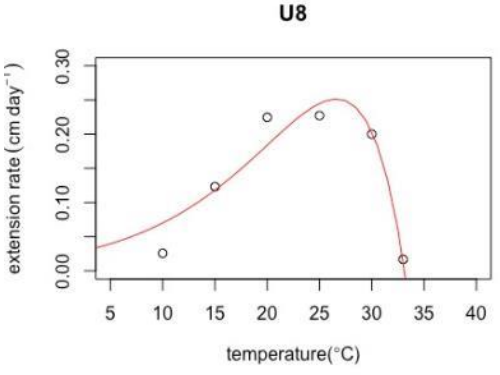


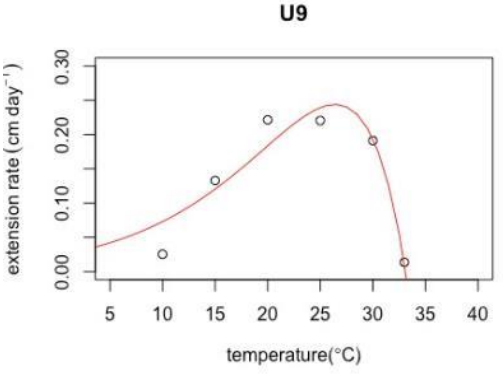

S8
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)

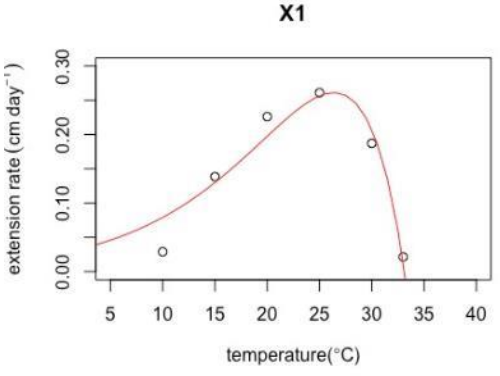

U4
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)

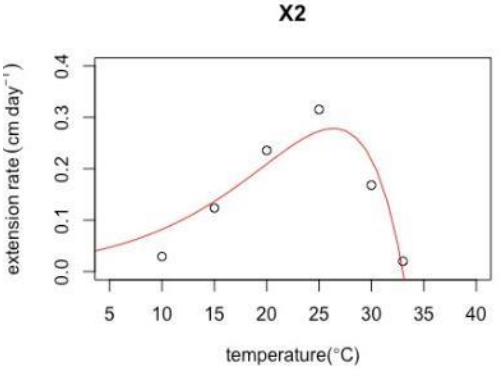

U6
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)

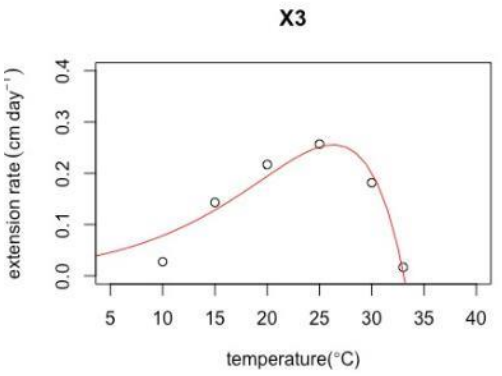

U7
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)

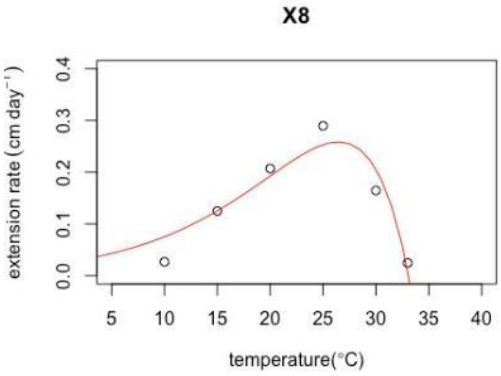

U8
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)

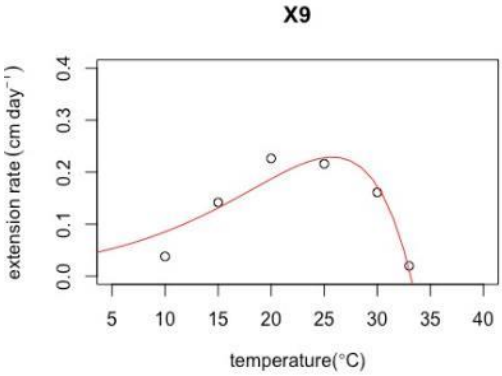

U9
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)


X1
extension rate (cm day⁻¹)
0.00
0.10
0.20
0.30
5
10
15
20
25
30
35
40
temperature(°C)


X2
extension rate (cm day⁻¹)
0.0
0.1
0.2
0.3
0.4
5
10
15
20
25
30
35
40
temperature(°C)

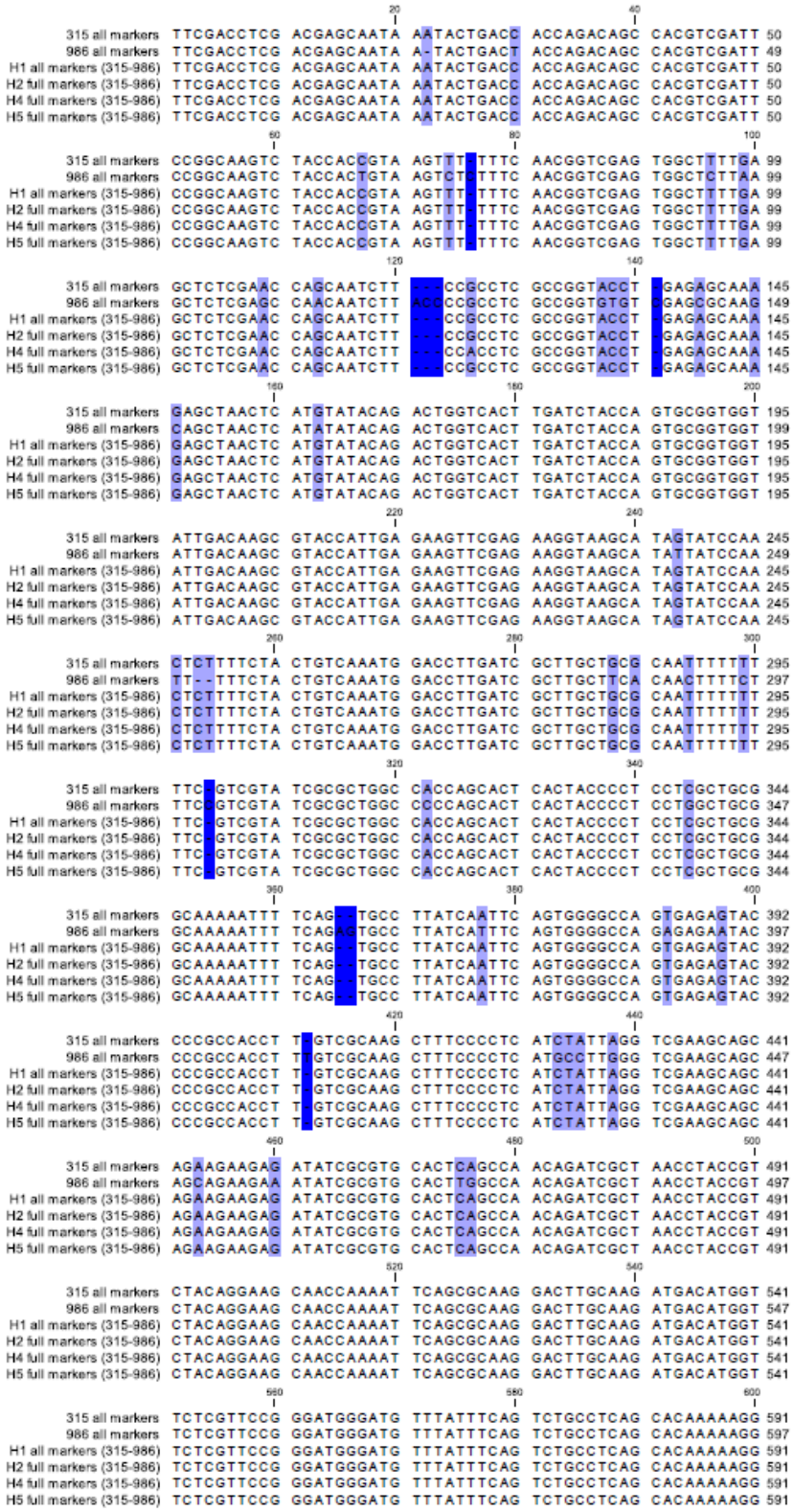

X3
extension rate (cm day⁻¹)
0.0
0.1
0.2
0.3
0.4
5
10
15
20
25
30
35
40
temperature(°C)

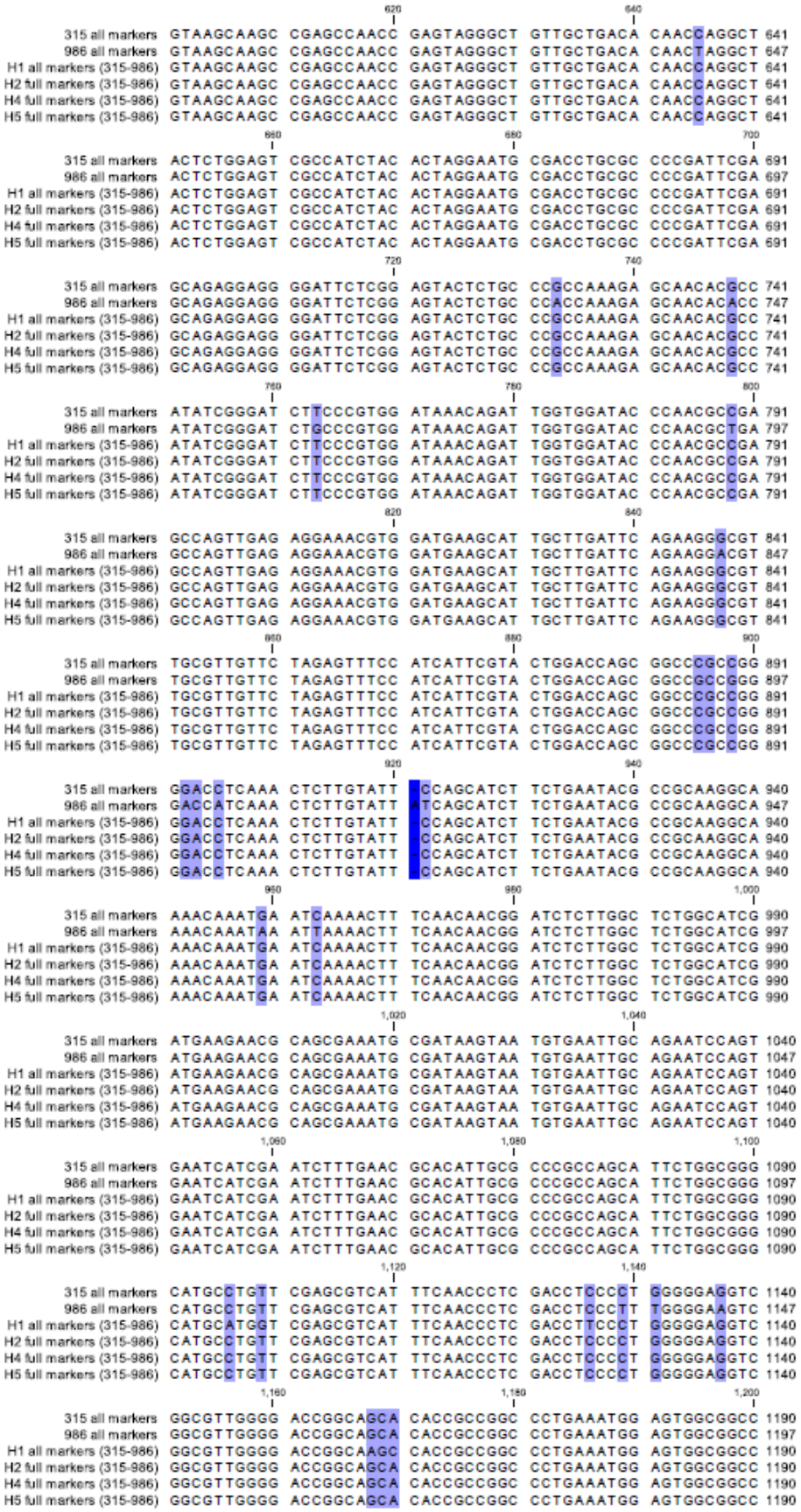

X8
extension rate (cm day⁻¹)
0.0
0.1
0.2
0.3
0.4
5
10
15
20
25
30
35
40
temperature(°C)

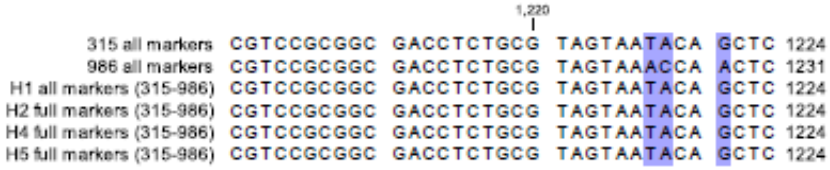

X9
extension rate (cm day⁻¹)
0.0
0.1
0.2
0.3
0.4
5
10
15
20
25
30
35
40
temperature(°C)

Appendix 14

All marker alignment for the four hybrids from the combination S (49 x 29) and their respective parental strains. In the graphic letter H correspond to the combination S, 315 is strain 49 and 986 is strain 29.

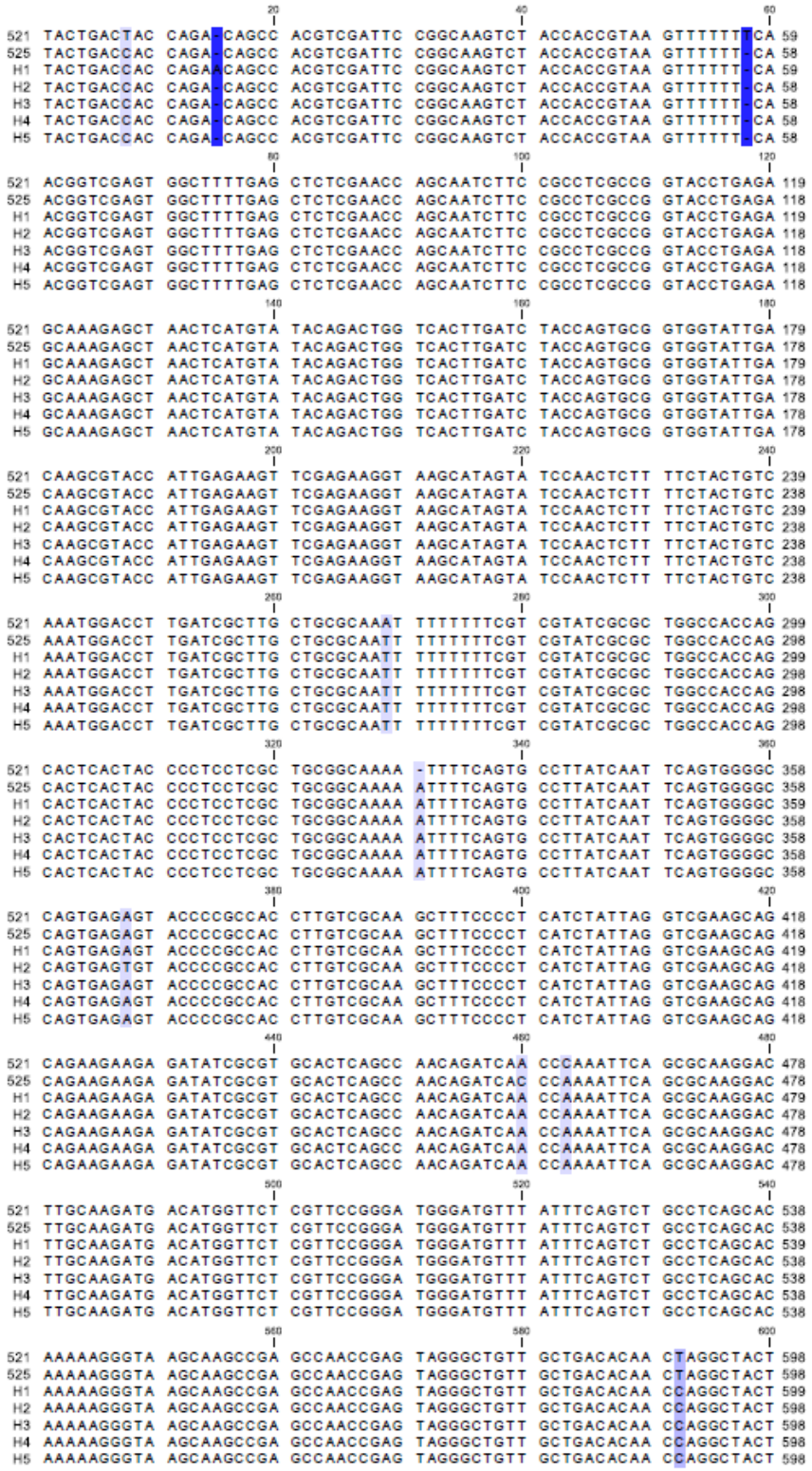


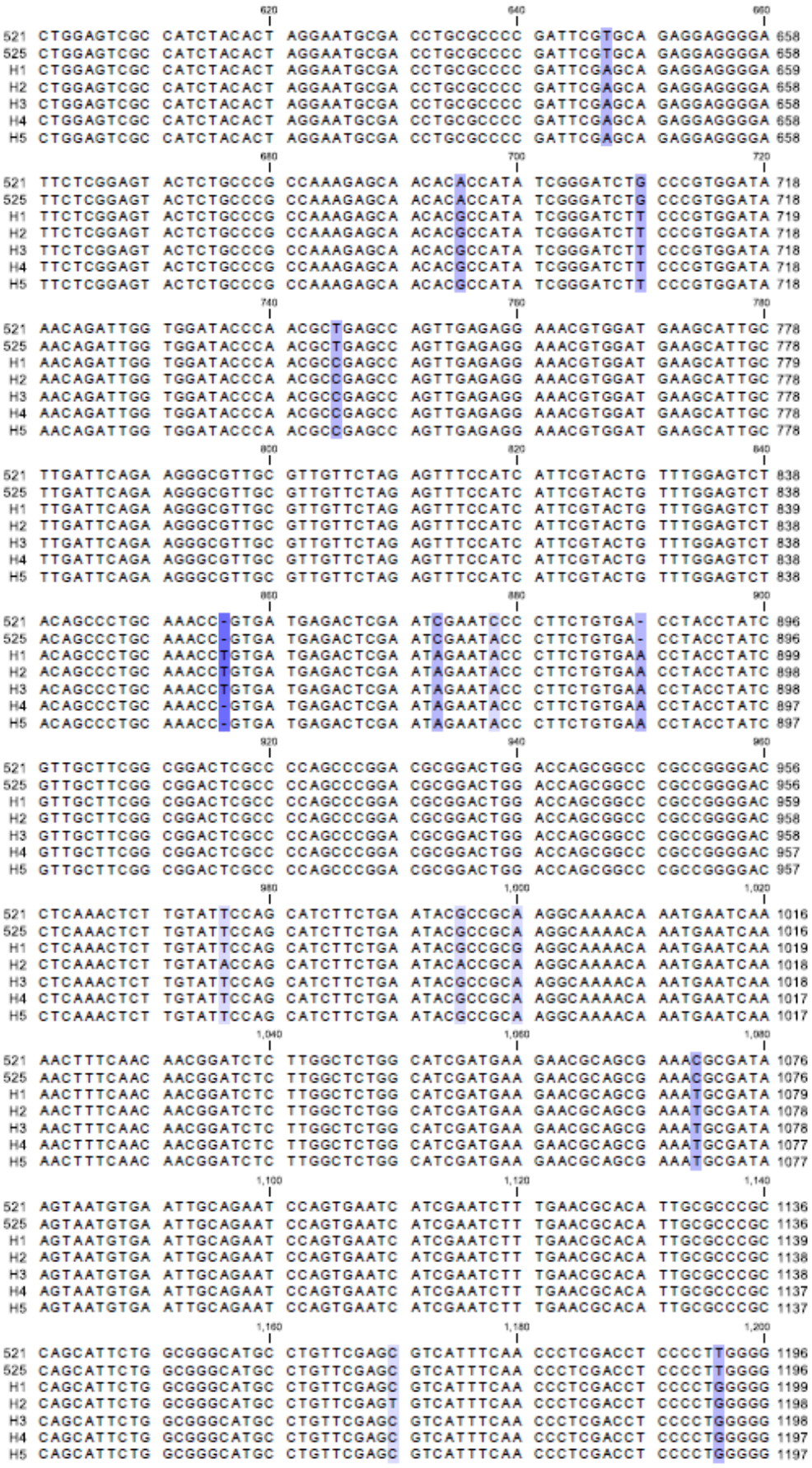

620 640
315 all markers GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACCAGGCT 641
986 all markers GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACTAGGCT 647
H1 all markers (315-986) GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACCAGGCT 641
H2 full markers (315-986) GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACCAGGCT 641
H4 full markers (315-986) GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACCAGGCT 641
H5 full markers (315-986) GTAAGCAAGC CGAGCCAACC GAGTAGGGCT GTTGCTGACA CAACCAGGCT 641
660 680 700
315 all markers ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 691
986 all markers ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 697
H1 all markers (315-986) ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 691
H2 full markers (315-986) ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 691
H4 full markers (315-986) ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 691
H5 full markers (315-986) ACTCTGGAGT CGCCATCTAC ACTAGGAATG CGACCTGCGC CCCGATTCGA 691
720 740
315 all markers GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCGCCAAAGA GCAACACGCC 741
986 all markers GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCACCAAAGA GCAACACACC 747
H1 all markers (315-986) GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCGCCAAAGA GCAACACGCC 741
H2 full markers (315-986) GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCGCCAAAGA GCAACACGCC 741
H4 full markers (315-986) GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCGCCAAAGA GCAACACGCC 741
H5 full markers (315-986) GCAGAGGAGG GGATTCTCGG AGTACTCTGC CCGCCAAAGA GCAACACGCC 741
760 780 800
315 all markers ATATCGGGAT CTTCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCCGA 791
986 all markers ATATCGGGAT CTGCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCTGA 797
H1 all markers (315-986) ATATCGGGAT CTTCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCCGA 791
H2 full markers (315-986) ATATCGGGAT CTTCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCCGA 791
H4 full markers (315-986) ATATCGGGAT CTTCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCCGA 791
H5 full markers (315-986) ATATCGGGAT CTTCCCGTGG ATAAACAGAT TGGTGGATAC CCAACGCCGA 791
820 840
315 all markers GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGGCGT 841
986 all markers GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGACGT 847
H1 all markers (315-986) GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGGCGT 841
H2 full markers (315-986) GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGGCGT 841
H4 full markers (315-986) GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGGCGT 841
H5 full markers (315-986) GCCAGTTGAG AGGAAACGTG GATGAAGCAT TGCTTGATTC AGAAGGGCGT 841
860 880 900
315 all markers TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCCGCCGG 891
986 all markers TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCGCCGGG 897
H1 all markers (315-986) TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCCGCCGG 891
H2 full markers (315-986) TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCCGCCGG 891
H4 full markers (315-986) TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCCGCCGG 891
H5 full markers (315-986) TGCGTTGTTC TAGAGTTTCC ATCATTCGTA CTGGACCAGC GGCCCGCCGG 891
920 940
315 all markers GGACCTCAAA CTCTTGTATT -CCAGCATCT TCTGAATACG CCGCAAGGCA 940
986 all markers GACCATCAAA CTCTTGTATT ATCAGCATCT TCTGAATACG CCGCAAGGCA 947
H1 all markers (315-986) GGACCTCAAA CTCTTGTATT -CCAGCATCT TCTGAATACG CCGCAAGGCA 940
H2 full markers (315-986) GGACCTCAAA CTCTTGTATT -CCAGCATCT TCTGAATACG CCGCAAGGCA 940
H4 full markers (315-986) GGACCTCAAA CTCTTGTATT -CCAGCATCT TCTGAATACG CCGCAAGGCA 940
H5 full markers (315-986) GGACCTCAAA CTCTTGTATT -CCAGCATCT TCTGAATACG CCGCAAGGCA 940
960 980 1,000
315 all markers AAACAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 990
986 all markers AAACAAATAA ATTAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 997
H1 all markers (315-986) AAACAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 990
H2 full markers (315-986) AAACAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 990
H4 full markers (315-986) AAACAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 990
H5 full markers (315-986) AAACAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGC TCTGGCATCG 990
1,020 1,040
315 all markers ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1040
986 all markers ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1047
H1 all markers (315-986) ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1040
H2 full markers (315-986) ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1040
H4 full markers (315-986) ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1040
H5 full markers (315-986) ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATCCAGT 1040
1,060 1,080 1,100
315 all markers GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1090
986 all markers GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1097
H1 all markers (315-986) GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1090
H2 full markers (315-986) GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1090
H4 full markers (315-986) GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1090
H5 full markers (315-986) GAATCATCGA ATCTTTGAAC GCACATTGCG CCCGCCAGCA TTCTGGCGGG 1090
1,120 1,140
315 all markers CATGCCTGTT CGAGCGTCAT TTCAACCCTC GACCTCCCCT GGGGGAGGTC 1140
986 all markers CATGCCTGTT CGAGCGTCAT TTCAACCCTC GACCTCCCTT TGGGGAAGTC 1147
H1 all markers (315-986) CATGCATGGT CGAGCGTCAT TTCAACCCTC GACCTTCCCT GGGGGAGGTC 1140
H2 full markers (315-986) CATGCCTGTT CGAGCGTCAT TTCAACCCTC GACCTCCCCT GGGGGAGGTC 1140
H4 full markers (315-986) CATGCCTGTT CGAGCGTCAT TTCAACCCTC GACCTCCCCT GGGGGAGGTC 1140
H5 full markers (315-986) CATGCCTGTT CGAGCGTCAT TTCAACCCTC GACCTCCCCT GGGGGAGGTC 1140
1,160 1,180 1,200
315 all markers GGCGTTGGGG ACCGGCAGCA CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1190
986 all markers GGCGTTGGGG ACCGGCAGCA CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1197
H1 all markers (315-986) GGCGTTGGGG ACCGGCAAGC CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1190
H2 full markers (315-986) GGCGTTGGGG ACCGGCAGCA CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1190
H4 full markers (315-986) GGCGTTGGGG ACCGGCAGCA CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1190
H5 full markers (315-986) GGCGTTGGGG ACCGGCAGCA CACCGCCGGC CCTGAAATGG AGTGGCGGCC 1190

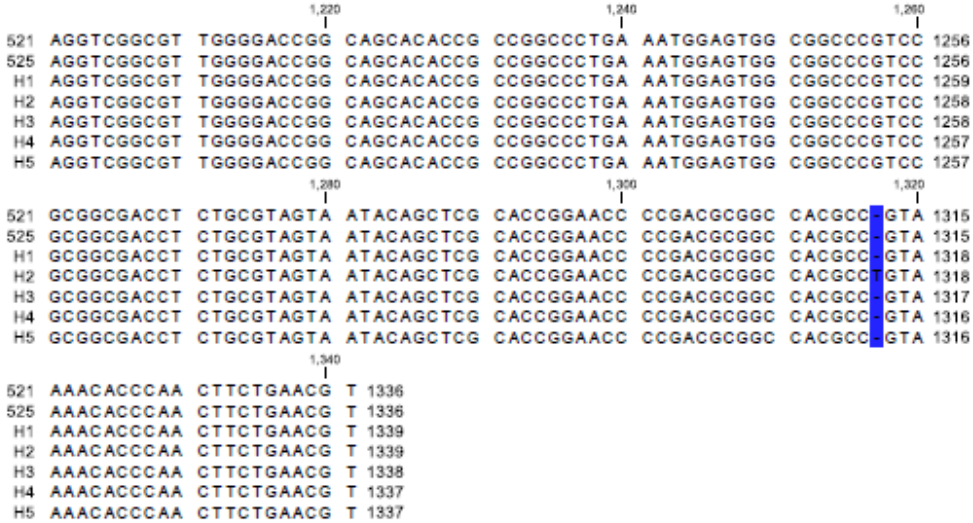

1,220
315 all markers CGTCCGCGGC GACCTCTGCG TAGTAATACA GCTC 1224
986 all markers CGTCCGCGGC GACCTCTGCG TAGTAAACCA ACTC 1231
H1 all markers (315-986) CGTCCGCGGC GACCTCTGCG TAGTAATACA GCTC 1224
H2 full markers (315-986) CGTCCGCGGC GACCTCTGCG TAGTAATACA GCTC 1224
H4 full markers (315-986) CGTCCGCGGC GACCTCTGCG TAGTAATACA GCTC 1224
H5 full markers (315-986) CGTCCGCGGC GACCTCTGCG TAGTAATACA GCTC 1224

Appendix 15

All marker alignment for the four hybrids from the combination U (42 x 41) and their respective parental strains. In the graphic letter H correspond to the combination U, 521 is strain 41 and 525 is strain 42.

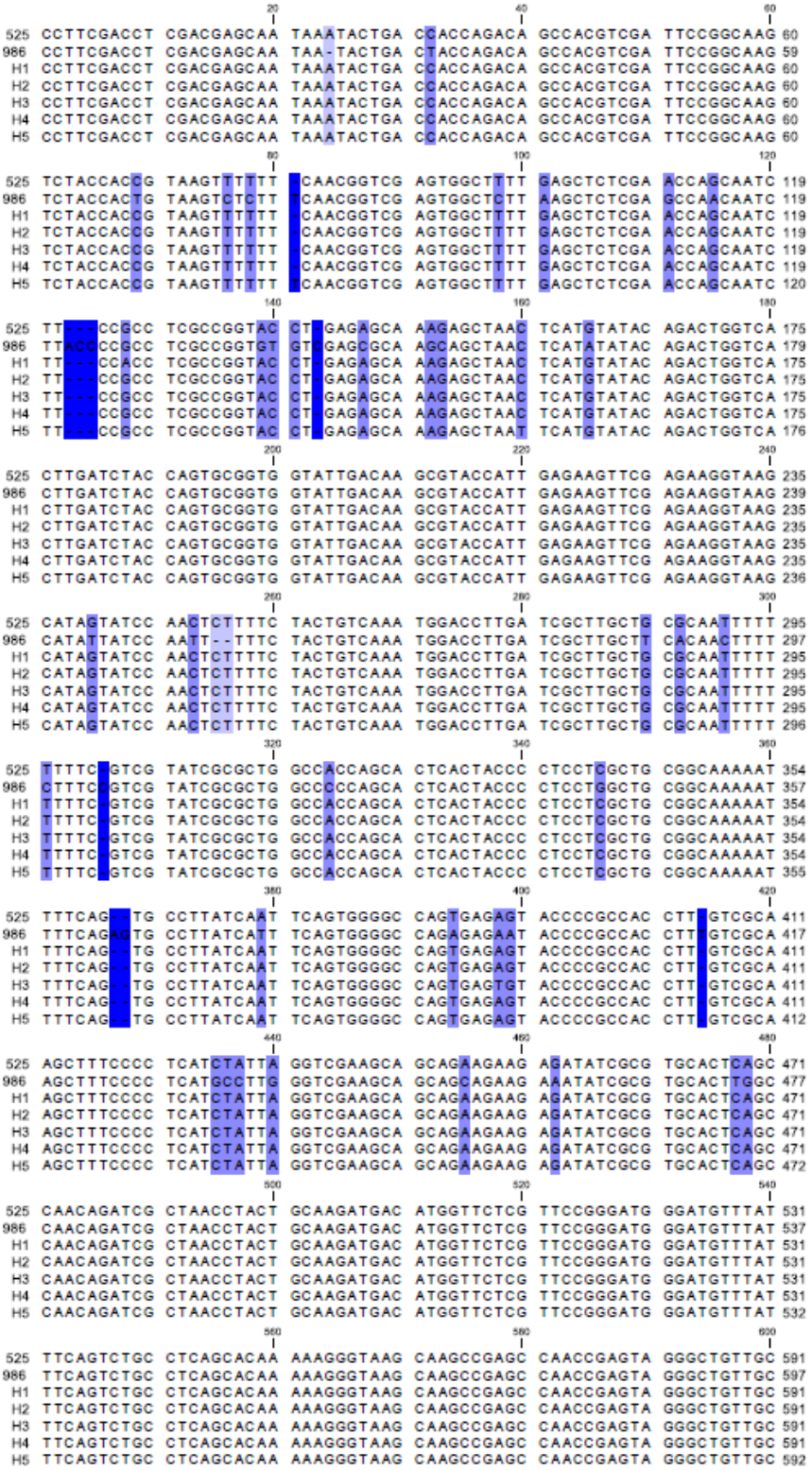


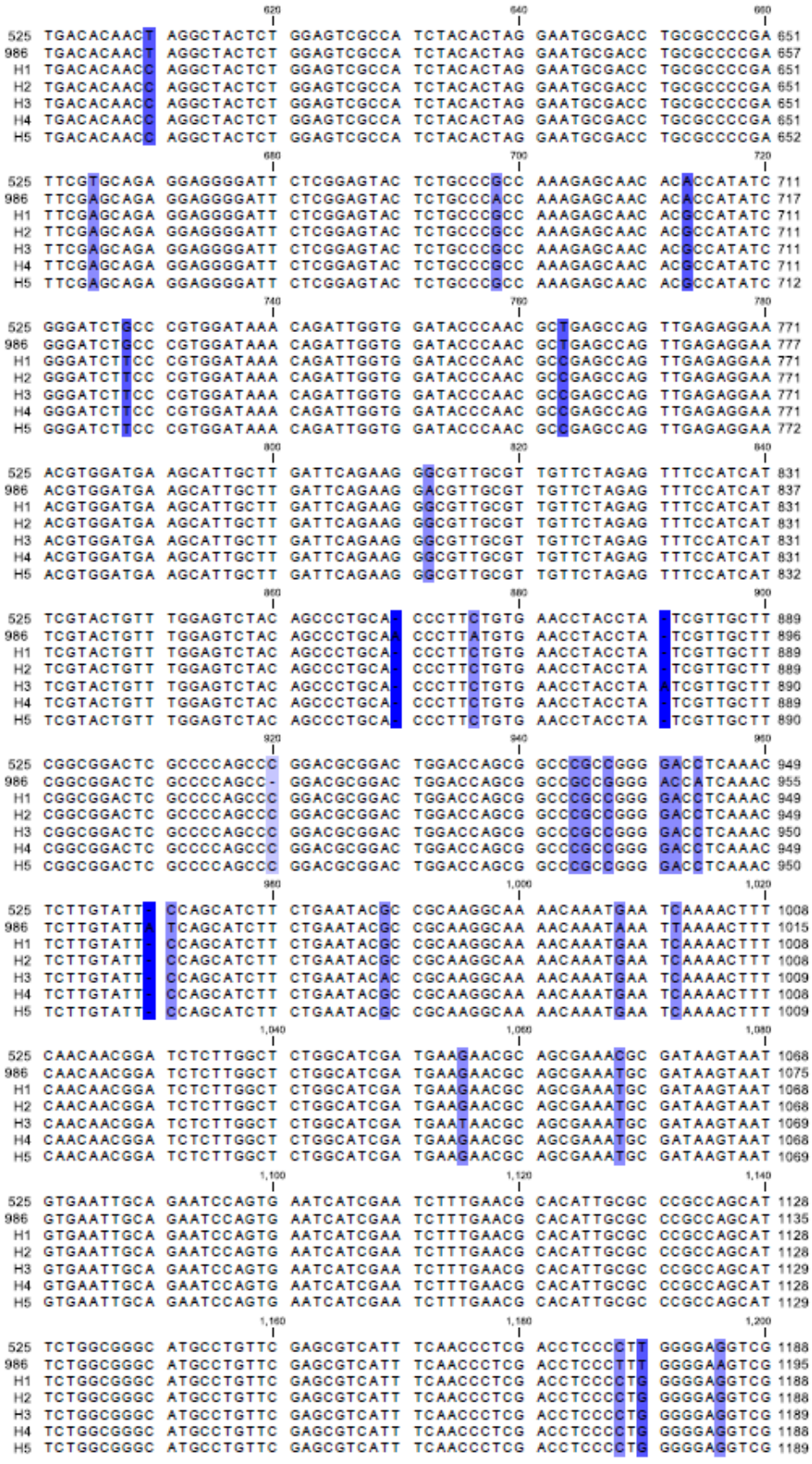

620 640 660
521 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGTGCA GAGGAGGGGA 658
525 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGTGCA GAGGAGGGGA 658
H1 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGAGCA GAGGAGGGGA 659
H2 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGAGCA GAGGAGGGGA 658
H3 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGAGCA GAGGAGGGGA 658
H4 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGAGCA GAGGAGGGGA 658
H5 CTGGAGTCGC CATCTACACT AGGAATGCGA CCTGCGCCCC GATTCGAGCA GAGGAGGGGA 658
680 700 720
521 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACACCATA TCGGGATCTG CCCGTGGATA 718
525 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACACCATA TCGGGATCTG CCCGTGGATA 718
H1 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACGCCATA TCGGGATCTT CCCGTGGATA 719
H2 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACGCCATA TCGGGATCTT CCCGTGGATA 718
H3 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACGCCATA TCGGGATCTT CCCGTGGATA 718
H4 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACGCCATA TCGGGATCTT CCCGTGGATA 718
H5 TTCTCGGAGT ACTCTGCCCG CCAAAGAGCA ACACGCCATA TCGGGATCTT CCCGTGGATA 718
740 760 780
521 AACAGATTGG TGGATACCCA ACGCTGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
525 AACAGATTGG TGGATACCCA ACGCTGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
H1 AACAGATTGG TGGATACCCA ACGCCGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 779
H2 AACAGATTGG TGGATACCCA ACGCCGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
H3 AACAGATTGG TGGATACCCA ACGCCGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
H4 AACAGATTGG TGGATACCCA ACGCCGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
H5 AACAGATTGG TGGATACCCA ACGCCGAGCC AGTTGAGAGG AAACGTGGAT GAAGCATTGC 778
800 820 840
521 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
525 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
H1 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 839
H2 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
H3 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
H4 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
H5 TTGATTCAGA AGGGCGTTGC GTTGTTCTAG AGTTTCCATC ATTCGTACTG TTTGGAGTCT 838
860 880 900
521 ACAGCCCTGC AAACC-GTGA TGAGACTCGA ATCGAATCCC CTTCTGTGA- CCTACCTATC 896
525 ACAGCCCTGC AAACC-GTGA TGAGACTCGA ATCGAATACC CTTCTGTGA- CCTACCTATC 896
H1 ACAGCCCTGC AAACCTGTGA TGAGACTCGA ATAGAATACC CTTCTGTGAA CCTACCTATC 899
H2 ACAGCCCTGC AAACCTGTGA TGAGACTCGA ATAGAATACC CTTCTGTGAA CCTACCTATC 898
H3 ACAGCCCTGC AAACCTGTGA TGAGACTCGA ATAGAATACC CTTCTGTGAA CCTACCTATC 898
H4 ACAGCCCTGC AAACC-GTGA TGAGACTCGA ATAGAATACC CTTCTGTGAA CCTACCTATC 897
H5 ACAGCCCTGC AAACC-GTGA TGAGACTCGA ATAGAATACC CTTCTGTGAA CCTACCTATC 897
920 940 960
521 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 956
525 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 956
H1 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 959
H2 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 958
H3 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 958
H4 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 957
H5 GTTGCTTCGG CGGACTCGCC CCAGCCCGGA CGCGGACTGG ACCAGCGGCC CGCCGGGGAC 957
980 1,000 1,020
521 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCA AGGCAAAACA AATGAATCAA 1016
525 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCA AGGCAAAACA AATGAATCAA 1016
H1 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCG AGGCAAAACA AATGAATCAA 1019
H2 CTCAAACTCT TGTATACCAG CATCTTCTGA ATACACCGCA AGGCAAAACA AATGAATCAA 1018
H3 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCA AGGCAAAACA AATGAATCAA 1018
H4 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCA AGGCAAAACA AATGAATCAA 1017
H5 CTCAAACTCT TGTATTCCAG CATCTTCTGA ATACGCCGCA AGGCAAAACA AATGAATCAA 1017
1,040 1,060 1,080
521 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAACGCGATA 1076
525 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAACGCGATA 1076
H1 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 1079
H2 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 1078
H3 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 1078
H4 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 1077
H5 AACTTTCAAC AACGGATCTC TTGGCTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 1077
1,100 1,120 1,140
521 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1136
525 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1136
H1 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1139
H2 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1138
H3 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1138
H4 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1137
H5 AGTAATGTGA ATTGCAGAAT CCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC 1137
1,160 1,180 1,200
521 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTTGGGG 1196
525 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTTGGGG 1196
H1 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTGGGGG 1199
H2 CAGCATTCTG GCGGGCATGC CTGTTCGAGT GTCATTTCAA CCCTCGACCT CCCCTGGGGG 1198
H3 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTGGGGG 1198
H4 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTGGGGG 1197
H5 CAGCATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCGACCT CCCCTGGGGG 1197

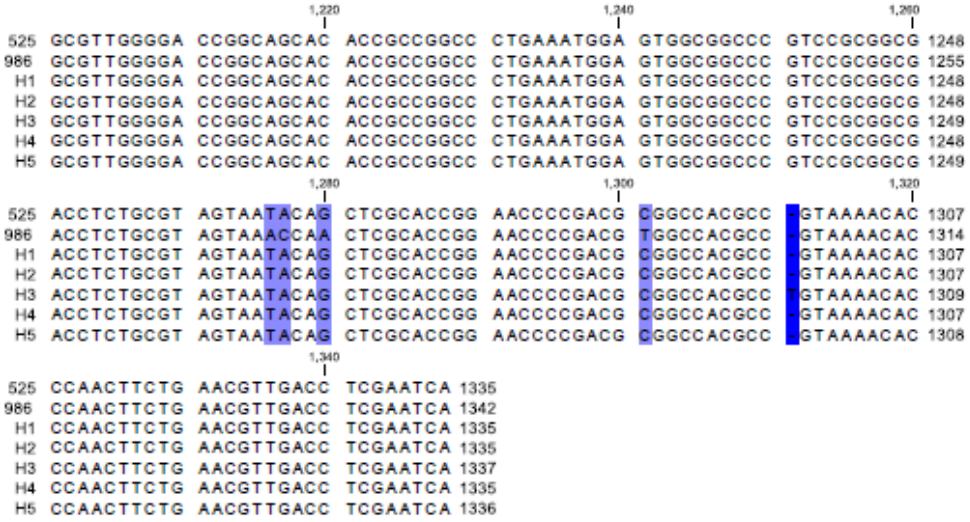

1,220
1,240
1,260
521 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1256
525 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1256
H1 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1259
H2 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1258
H3 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1258
H4 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1257
H5 AGGTCGGCGT TGGGGACCGG CAGCACACCG CCGGCCCTGA AATGGAGTGG CGGCCCGTCC 1257
1,280
1,300
1,320
521 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1315
525 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1315
H1 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1318
H2 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCCTGTA 1318
H3 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1317
H4 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1316
H5 GCGGCGACCT CTGCGTAGTA ATACAGCTCG CACCGGAACC CCGACGCGGC CACGCC-GTA 1316
1,340
521 AAACACCCAA CTTCTGAACG T 1336
525 AAACACCCAA CTTCTGAACG T 1336
H1 AAACACCCAA CTTCTGAACG T 1339
H2 AAACACCCAA CTTCTGAACG T 1339
H3 AAACACCCAA CTTCTGAACG T 1338
H4 AAACACCCAA CTTCTGAACG T 1337
H5 AAACACCCAA CTTCTGAACG T 1337

Appendix 16

All marker alignment for the four hybrids from the combination X (42 x 29) and their respective parental strains. In the graphic letter H correspond to the combination X, 525 is strain 42 and 986 is strain 29.






1,220
1,240
1,260
525 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1248
986 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1255
H1 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1248
H2 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1248
H3 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1249
H4 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1248
H5 GCGTTGGGGA CCGGCAGCAC ACCGCCGGCC CTGAAATGGA GTGGCGGCCC GTCCGCGGCG 1249
1,280
1,300
1,320
525 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC -GTAAAACAC 1307
986 ACCTCTGCGT AGTAAACCAA CTCGCACCGG AACCCCGACG TGGCCACGCC -GTAAAACAC 1314
H1 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC -GTAAAACAC 1307
H2 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC -GTAAAACAC 1307
H3 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC TGTAAAACAC 1309
H4 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC -GTAAAACAC 1307
H5 ACCTCTGCGT AGTAATACAG CTCGCACCGG AACCCCGACG CGGCCACGCC -GTAAAACAC 1308
1,340
525 CCAACTTCTG AACGTTGACC TCGAATCA 1335
986 CCAACTTCTG AACGTTGACC TCGAATCA 1342
H1 CCAACTTCTG AACGTTGACC TCGAATCA 1335
H2 CCAACTTCTG AACGTTGACC TCGAATCA 1335
H3 CCAACTTCTG AACGTTGACC TCGAATCA 1337
H4 CCAACTTCTG AACGTTGACC TCGAATCA 1335
H5 CCAACTTCTG AACGTTGACC TCGAATCA 1336