INTERACTIONS BETWEEN *Zoophthora radicans* AND *Pandora blunckii* ISOLATES IN *Plutella xylostella* POPULATIONS

by

ARIEL WILBERT GUZMÁN FRANCO
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Division of Agricultural and Environmental Sciences
School of Biosciences
University of Nottingham
Sutton Bonington Campus
Loughborough
Leicestershire
LE1 5RD
UK

The University of Nottingham

ROTHAMSTED RESEARCH

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ABSTRACT

The interactions between isolates of the entomopathogenic fungi *Zoophthora radicans* and *Pandora blunckii* in *Plutella xylostella* populations were investigated. A series of experiments were carried out to detect major differences amongst isolates from each species, these included *in vitro* growth at different temperatures, *in vitro* intra and inter -specific interactions and genetic variability based on the internal transcribed spacer regions of the ribosomal DNA (rDNA-ITS). All the isolates showed different *in vitro* growth profiles at different temperatures (Chapter 2). Isolates with greater and smaller growth rates were selected for further experiments. The intra and inter-specific interactions *in vitro* were investigated using these selected isolates (Chapter 3). Overall, *P. blunckii* was more competitive than *Z. radicans*. The negative effect of some *P. blunckii* isolates on the *in vitro* growth of Z. radicans isolates was consistent over the two temperatures evaluated. This suggested that temperature did not have a significant effect on the outcomes. Particular isolates were selected based on their differential competitive abilities for further experiments. The genetic variability amongst isolates of *Z. radicans* (39 isolates) and *P. blunckii* (22 isolates) was examined (Chapter 4). ITS-RFLP analysis using the ITS rDNA region did not show differences amongst *P. blunckii* isolates, but large differences were found amongst the *Z. radicans* isolates. Sequence information of the ITS rDNA regions of *Z. radicans* isolates from different RFLP groups confirmed intra-specific genetic variability. The sequence information obtained allowed to develop species-specific primers for the detection of both pathogens using conventional PCR. The species-specific primers developed for *Z. radicans* could be optimized for use in quantitative real-time PCR, but not the *P. blunckii* species-specific primers (Chapter 6). The interactions between four selected isolates infecting *P. xylostella* larvae were investigated under different conditions (Petri dishes and on Chinese cabbage plants) and dose proportions (Chapter 5). The species with a numerical disadvantage in dose at inoculum was more likely to be excluded regardless of virulence, which was previously estimated for each isolate in Chapter 5. Both species were able to co-exist in individual larva via scramble competition, but a

reduction in fitness was found because both species produced fewer conidia or resting spores than when they were inoculated singly. The interactions during the infection process were also determined (Chapter 6). The outcomes were based on the quantities of fungal DNA determined using real-time PCR of *Z. radicans* either single or dual-inoculated with *P. blunckii* at different days post-inoculation. During the infection process, *Z. radicans* growth was stimulated within the host as a response to the presence of *P. blunckii* at day 3 and 4 of the infection (parasitic phase). However, at day 5 (saprophytic phase), *P. blunckii* out-competed *Z. radicans*. The production of resting spores by some isolates of *Z. radicans* seems to be an efficient strategy to survive direct competition against a more virulent species. The negative effect of the interactions between these two fungal pathogens found in this study, suggest that the use of both pathogens simultaneously in the field for the microbial control of *P. xylostella* should be reconsidered. Although mortality of the host is obtained, the persistence and survival of both pathogens is negatively affected.

CHAPTER 1. GENERAL INTRODUCTION

1.1 INTERACTIONS BETWEEN BIOLOGICAL CONTROL AGENTS

The factors influencing a successful outcome in biological control are normally complex (Rosenheim *et al.*, 1995). The development of a general theory for biological control is even more complex because of the differences between the organisms to be controlled e.g. insects, weeds, plant pathogens and parasites. Normally the research is carried out in isolated systems and in a one to one relationship between the biological control agent and the pest (Rosenheim *et al.*, 1995). It is common that an herbivore can be exploited by more than one natural enemy; these will interact with each other and with the herbivore (Furlong and Pell, 2005). When two different organisms sharing a host (or prey), and also a trophic interaction is involved between these two organisms (for example one feeds on another), this is considered as an intraguild trophic interaction, where a guild includes all organisms sharing a common food resource (Rosenheim *et al.*, 1995; Dixon, 2000; Brodeur and Rosenheim, 2000). There are also situations where two organisms compete for the same host, but are not involved in a trophic interaction (Rosenheim *et al.*, 1995).

Competition is an interaction between individuals, that share a common resource that is limited, and as a consequence, a reduction in survivorship, growth or reproduction for at least some of the competing individuals is obtained (Begon *et al.*, 1996). Competition between individuals can be classified as intra-specific or inter-specific. In the former, the competition is between individuals of the same species and the latter one between individuals of different species (Begon *et al.*, 1996). The outcomes of inter-specific competition are described briefly.

Competition can involve a direct or indirect interaction between species. In the first case, known as interference competition, both species interact directly (often physically) when one species attempts to prevent the other species from using the same resource in a specific space. This direct interaction can also be

caused by the production of chemicals that are toxic to other species. In indirect competitive interactions, called exploitation competition. one species responds to the amount of the resource left by other species (Begon et al. 1996).

When competition results in the exclusion of one species by another. it is known as competitive exclusion. When both species share resources and space. and normally at least one of them suffers reductions in survival and or fecundity, it is known as co-existence (Begon *et al.*, 1996). There are situations where the competition will affect both species negatively (for example when cannibalisms is involved and results in a reciprocal predation). this is called mutual antagonism (Begon *et al.*, 1996).

The outcome of competition not only depends on the ability of each species to compete, but also factors such as the initial density of the competitors. The species with the largest initial density will exclude the other species and if they have similar densities the outcome is unpredictable. Timing is also important, the species that first arrives will have competitive advantage over the late arriving species (Begon *et al.*, 1996). The environment also has an influence on the outcomes of competition, temperature being one of the most important (e.g. Furlong and Pell, 2005; Fargues and Bon, 2004; Inglis *et al.*, 1997).

From the biological control point of view, without considering the effect on the competitors themselves and only the effect on the prey (pest), the outcomes from interaction between two biological control agents (e.g. predator, parasitoid or pathogen) may be synergistic, when the combination results in a higher mortality of the pest than the individual mortalities combined, additive, when the total mortality of the pest is the sum of the individual mortalities combined, therefore no interaction is assumed or antagonistic, where the total mortality of the pest is less than the additive, or at least less than the mortality of one of the interacting biological control agents (Roy and Pell, 2000).

The interactions between two species or biological control agents sharing the same host can be separated at the individual host level or host population level.

and the result of the interactions at the individual level will have an effect at the population level (Furlong and Pell, 2005).

1.1.1 Interactions between parasitoids

Interactions between parasitoids can be competitive and may or may not involve a trophic interacion. For example, if a parasitoid oviposits in a healthy host (primary parasitoid), and then a second parasitoid (secondary parasitoid) oviposits in the already parasitised host a trophic interaction occurs because the secondary parasitoid consumes the primary parasitoid (Rosenheim *et al.*, 1995; Shi *et al.*, 2004)

When the interactions do not include a trophic interaction in parasitoids, this involves direct contests between individuals either defending their host, or trying to take over possession of a host (Pérez-Lachaud *et al.*, 2002). The aggressiveness of these contests can vary from contests where no signs of physical damage to the contestants was observed (e.g. Petersen and Hardy, 1996; Field and Calbert, 1999), to contests where severe fighting takes place as a result of an attack, leading in most of the cases to the death of one of the competitors (e.g. Pérez-Lachaud *et al.*, 2002).

In some examples, parasitoids can co-exist, but may result in less efficient pest population control, as discussed by Pérez-Lachaud *et al.* (2002). It has been suggested as well that parasitoids may coexist if they have different biological attributes. For example, a non-competitive parasitoid may survive because it avoids competition by leaving a patch containing the strong competitor (Hassell, 2000). However, as suggested by Pérez-Lachaud *et al.* (2002), if parasitoids sharing the same host cannot co-exist, the use of combinations of parasitoid species for biological control of this pest should be reconsidered.

1.1.2 Interactions between predators

Interactions between predators are common, because most predators are generalists (Rosenheim *et al.*, 1995). Four categories of interactions have been

described: 1) no interaction, 2) one predator attacks/kills the other. 3) one predator affects the foraging behaviour of the other predator and 4) one predator modifies prey behaviour affecting susceptibility of the prey to other predators (Losey and Denno, 1998).

When two predators do not interact, the effect is additive, where the total control of the prey is the sum of the control carried out by each predator individually (Losey and Denno, 1998). When one predator kills or affects the foraging behaviour of the other predator, this might disrupt the biological control of the pest (Losey and Denno, 1998). Rosenheim *et al.*, (1993) evaluated the influence of the presence of three generalist hemipteran predators *Geocoris* spp., *Nabis* spp. and *Zelus renardi* (Kolenati) on a specialist predator, the green lacewing *Chrysoperla carnea* (Stephens) when controlling the aphid *Aphis gossypii*. They found that the generalist predators ate *C. carnea* larvae affecting the survival of the lacewings. The mortality caused by the generalist predators on the lacewing larvae was sufficient to reduce the control of the aphids by the lacewings.

There are other examples were a negative effect was found between predators but still good control were achieved. Jakobsen *et al.* (2004) found a negative interaction between the predators *Orius majusculus* (Reuter) and *Macrolophus caliginosus* (Wagner) when controlling thrips *Frankliniella occidentalis* (Pergande). *Orius majusculus* adults could feed on nymphs and even adults of *M. caliginosus* in the absence of prey, but *M. caliginosus* did not prey on *O. majusculus*. Although this was a negative interaction, thrips control was not affected. Similar results were also found by Colfer *et al.* (2003) when they evaluated the impact of the introduction of the predatory mite *Galendromus occidentalis* (Nesbitt) to control spider mite populations in the presence of the naturally occurring generalist predators *Geocoris* spp. and *Orius tristicolor* (White) and the herbivore *F. occidentalis*. The densities of *G. occidentalis* were drastically reduced by the presence of the generalist predators. However, this negative effect did not reduce the control of spider mite populations achieved because the generalist predators fed on the spider mite populations as well. The thrips *F. occidentalis* did not affect the *G. occidentalis* population. In

this case, as the authors identified, although the biological control of the spider mite populations was not affected by the negative interaction between the generalist predators and the predatory mite *G. occidentalis*, economically it would not be advisable to introduce a biological control agent in a system where their establishment is affected by naturally occurring generalist predators.

An example of one predator affecting prey behaviour and changing their susceptibility to other predators is given by Losey and Denno (1998). They investigated interactions between the foliar foraging beetle *Coccinella septempuctata* L. and the ground foraging carabid *Harpalus pennsylvanicus* DeGeer on pea aphid *Acyrthosiphon pisum* (Harris) populations. The results suggested a synergistic interaction between both predators when controlling the prey. The presence of *C. septempunctata* made the pea aphids fall to the ground where they were attacked by the ground foraging *H. pennsylvanicus*. The combination of both predators gave good control of the aphid.

1.1.3 Interactions between parasitoids and predators

There are two types of interaction between these two groups of biological control agents. Firstly, the predators can prey directly on parasitoids and secondly predators can prey on parasitised hosts (Rosenheim *et al.*, 1995). Snyder and Ives (2001) found that the generalist predator *Pterostichus melanarius* (Illiger) had a negative interaction with the parasitoid *Aphidius ervi* (Haliday) when controlling the aphid *Acyrthosiphon pisum.* The negative interaction is a result of the predator feeding on parasitised hosts or mummies. The predator reduced the aphid population in the short term, but this reduction was ephemeral, because the aphid population in a long term increased as a consequence of the reduction of the parasitoid population. A negative effect was also described between the introduced parasitoid *Psyllaephagus bliteus* Riek, and the generalist predator *Anthocoris nemoralis* (Fabricius) when controlling the psyllid *Glycaspis brimblecombei* Moore a pest of eucalyptus trees (Erbilgin *et al.*, 2004). When the parasitoid and the predator were combined, a high mortality of parasitised hosts was observed, which was

followed by an increase in the psyllid population. The authors suggested that the parasitoid would establish only when the predator population was very low or absent.

Normally, in a direct predator – parasitoid interaction, parasitoids are the intraguild prey (Nakashima and Senoo, 2003). If the parasitoids avoid patches where the predator is present, the likelihood of intraguild predation is reduced (e.g. Taylor *et al.*, 1998). Nakashima and Senoo (2003), found that the parasitoid *Aphidius ervi* can avoid patches where the predator *Coccinella septempunctata* were present previously, but this behaviour seems to be made only by parasitoids with previous oviposition experience. The *C. septempunctata* semiochemicals responsible for inducing this avoidance behaviour on the parasitoid *A. ervi* were later identified (Nakashima *et al.*, 2004).

1.1.4 Interactions between entomopathogens and other biological control agents

The interactions between entomopathogens and predators or parasitoids can take place inside or outside the host. The interactions inside the host are more likely to occur with parasitoids because some of them (endoparasitoids) spend part of their life cycle inside the host (Furlong and Pell, 2005). Also, parasitoids and predators can be affected by the entomopathogens by being infected directly or indirectly, while competing for the host (Furlong and Pell, 2005; Roy and Pell, 2000).

There are examples were the interactions between pathogens and parasitoids are negative for the parasitoid but improve transmission of the pathogen (e.g. Tillman *et al.*, 2004; Furlong and Pell, 1996).

Tillman *et al.* (2004) found that the three parasitoids *Cardiochiles nigriceps* Viereck, *Campolestis sonorensis* (Cameron) and *Microplitis croceipes* (Cresson) that attack *Heliothis virescens* (F.) larvae can also transmit ascoviruses. When the parasitoid's ovipositor penetrated the host's body it

became contaminated with virus and transmitted the virus during oviposition into another host. However, the virus had a negative effect on the parasitoid' survival. The parasitoid larva was only able to survive if it was introduced into the larva first and had at least 48 hours to develop before the virus was introduced. When the host became infected before the parasitoid larva reached the second instar it was unable to survive. The virus did not infect the parasitoid larva directly, but it was possible that the presence and multiplication of the virions in the insect host depleted resources thereby starving the parasitoid larva. Furlong and Pell (1996), evaluated the susceptibility of the two main parasitoids of *P. xylostella*, *Diadegma semiclausum* and *Cotesia plutellae* to the entomophthoralean fungus *Z. radicans*. Only *D. semiclausum* were susceptible to the fungi, which suggested a possible negative effect in the field between these two biological control agents. However, the facts that *D. semiclausum* was 70 and 133 fold less susceptible than the larvae and adult of *P. xylostella* respectively, and that I have been able to find no report of *D. semiclausum* infected by *Z. radicans* from the field, suggests that this negative effect is unlikely to be ecologically relevant. It is important to highlight the difference between physiological susceptibility and ecological susceptibility (Furlong and Pell, 1996; Roy and Pell, 2000), where although the susceptibility of a parasitoid or predator has been tested in the laboratory under optimal conditions for the pathogen (physiological susceptibility), it might not be the same under field conditions where suboptimal conditions can be found for the pathogen (ecological susceptibility). The fact that the susceptibility of predators and parasitoids to entomopathogenic fungi investigated in the laboratory may not be realistically applicable to the field has also been suggested by other researchers (e.g. Lacey *et al.*, 1997, Poprawski *et al.*, 1998).

In some interactions between predators and entomopathogenic fungi, intraguild predation has been observed. For example, it has been found that *Coccinella septempunctata* L. fed on cadavers of the aphid *Acyrthosiphon pisum* infected with the fungus *Pandora neopahidis* Remaudière and Hennebert, with the potential to reduce transmission of this fungus to healthy aphids (Pell *et al.*, 1997; Roy *et al.*, 1998). However, infected aphid cadavers damaged by the *C.*

septempunctata feeding activity, continued sporulating and caused similar mortality as undamaged cadavers (Roy *et al.*, 1998). The experiments carried out by Roy *et al.* (1998) were done in Petri dishes. When the same interactions were considered on bean plants, *C. septempunctata* did consume complete aphids infected by *P. neoaphidis.* It is suggested that the volatiles released by the plant as a result of the feeding activities of the aphid may have encouraged the ladybird to consume the cadavers. Under these conditions, the fact that *C. septempunctata* consumed complete aphids cadavers, suggests negative intraguild interaction for the fungus, because the possibility of secondary transmission is reduced (Roy *et al.*, 2003). However, this predator foraging behaviour may also be an advantage for the fungus because the predator was able to transmit infective conidia to healthy aphid (*A. pisum)* populations initiating infections in approximately 10 % of the population (Pell *et al.*, 1997)

The transmission of fungi to healthy aphids by a predator was also demonstrated by Pell and Vandenberg (2002). These authors evaluated the interactions between the entomopathogenic fungus *Paecilomyces fumosoroseus* (Wize) attacking the aphid *Diuraphis noxia* Kurdjumov and the predator *Hippodamia convergens* Guerin. Negative intraguild interactions between the predator and the fungus were observed. The predator did not eat significant numbers of infected cadavers, which was positive for the fungus because secondary inoculum was not removed from subsequent transmissions. The predator was able to transfer conidia to healthy aphid populations. However, under certain conditions such as long periods of high humidity and stress, a high percentage of *H. convergens* infected by *P. fumosoroseus* can be obtained (22%).

Furlong and Pell (1996) demonstrated that *D. semiclausum* enhanced the infection by *Z. radicans* in *P. xylostella* larvae. They found that *D. semiclausum* did not vector *Z. radicans* conidia, but still found more infected *P. xylostella* larvae in the presence of *D. semiclausum*. The parasitoid increased the movement of the *P. xylostella* larvae, increasing the chances of the larvae acquiring conidia.

1.1.5 Interactions between pathogens

Interactions between two or more pathogen species or genotypes are common, perhaps even the rule (Read and Taylor, 2001). These interactions have the potential to modify the population dynamics of particular insect–pathogen interaction (Thomas *et al.*, 2003). Normally, in a host-pathogen interaction only the presence of one pathogen is considered. For instance, the selection of pathogens to be used in microbial control is on the basis of the individual relationship between one pathogen and its host, and this can be based on the level of virulence (e.g. Pell *et al.*, 1993a; Butt and Goettel, 2000). The ability for transmission to other new hosts has also been considered as an important attribute, especially in those pathogens that do not produce large quantities of conidia, when pest control relies on self auto-dissemination (e.g. Furlong *et al.*, 1995; Vickers *et al.*, 2004). However, once the pathogen has been released in the field, possible interactions with other microorganisms are inevitable. In nature, infection caused my multiple pathogens or parasites is also very common (Cox, 2001).

The interactions between entomopathogens are complex and the outcomes are influenced by many factors such as temperature, sequence of infection, initial density of the competing pathogens, sex of the host, host range of the pathogens and relative virulence of the competitors. Some examples are described briefly.

Inglis *et al.* (1997) suggested the use of a combined application of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Gams and Rozsypal) to overcome the difficulties caused by extremes in temperature for the control of the grasshopper *Melanoplus sanguinipes* (Fabricius). Both pathogens were inoculated in the same proportion (10^5 conidia/insect of each pathogen). As both pathogens had the same opportunity to infect the host, temperature had a major role defining the outcome of the co-inoculation. *Metarhizium anisopliae* performed better than *B. bassiana* when the grasshoppers were incubated under a temperature regime simulating a hot day (temp max 35 °C), and *vice versa* under a regime simulating a cold day (temp

max 15 °C). The authors did not find any co-infected cadavers, suggesting that coexistence did not happen between these two pathogens, only competitive exclusion, and the winner of the competition was defined by the temperature where the inoculated hosts were incubated.

The effect of temperature on mixed infections using different isolates of *Paecylomyces fumosorosues* was also demonstrated by Fargues and Bon (2004). They found that isolates with different thermal profiles out-competed each other at their respective optimal temperatures. For example, at a variable temperature regime, between 20 and 25 °C, as well as constant 25 °C, both isolates co-existed in the host, possibly because all these temperatures were within the optimal temperature range for this species which is between 20 and 30 °C (Vidal *et al.*, 1997). In cooler and hotter temperatures only one isolate dominated, and this was related to the thermal preferences of that isolate, suggesting that temperature selects the most environmentally adapted isolate. The isolates were identified using molecular markers based on RFLP analysis of the ITS region and on random amplified polymorphic DNA (RAPD) in combination with the conidial size. (Fargues and Bon, 2004).

Thomas *et al.* (2003), dual inoculated adults of the locust *Schistocerca gregaria* (Forskål) using a virulent isolate of *M. anisopliae* var. *acridum* with either one of two avirulent isolates of *B. bassiana.* The speed of kill was used as a measure of virulence and sporulation as a measure of fitness. Only one dose, 10^3 conidia/insect, was used when single inoculations, and 10^3 conidia for each isolate per insect when dual inoculation. When single inoculated locusts were incubated at constant temperature (30 °C), *M. anisopliae* was more virulent (>80% mortality in about 8 days), than the *B. bassiana* isolates (*ca* 10% mortality in about 8 days). When dual inoculated, the results were similar to those obtained for *M. anisopliae* alone. When the *B. bassiana* isolates were inoculated four days before *M anisopliae*, no significant differences were observed in the final mortality compared to the mortality caused by *M. anisopliae* alone, but the sporulation of *M. anisopliae* was reduced. The same experiments, except sequential inoculations, were repeated under a simulated fever temperature regime (because locusts are active

behavioural thermoregulators), where the treated locusts were kept at 20 °C for 10 hours and gradually the temperature was increased up to 42 °C, and maintained at that temperature for 4 hours before reducing it again to 20 °C for 10 hours. This temperature regime was maintained for 25 days, and back to a constant 30 °C for another 20 days more. Overall, the mortality in all the treatments was greatly reduced. However, at the end of the 25 days, *M. anisoplaie* co-inoculated with one isolate one of *B. bassiana* caused significantly greater mortality (*ca.* 25 %) compared to the mortality caused with the other treatments (*ca.* 10%). When the temperature was returned to a constant 30 °C, mortality due to *M. anisopliae* increased again (ca. 70 %) when either single or dual inoculated with *B. bassiana.* However, the synergism obtained with isolate one of *B. bassiana* during the simulated fever temperature disappeared, and a decreased mortality due to *M. anisopliae* was obtained when co-inoculated with the other isolate of *B. bassiana*. The authors concluded that the presence of an avirulent (less virulent) pathogen (*B. bassiana*) affects the performance of a virulent pathogen (*M. anisopliae*), and that this is also influenced by temperature and order of infection. The lack of sporulation of some dual inoculated locusts (sequential inoculation) it is an important observation, because it represents a decrease in fungus fitness, even if the final host mortality was similar.

Koppenhöfer *et al.* (1995) evaluated interactions between the entomopathogenic nematodes *Steinernema carpocapsae* (Weiser) and *Steinernema glaseri* (Esteiner) in *Galleria mellonella* (L) larvae. *Steinernema glaseri* was more competitive than *S. carpocapsae* when both were inoculated simultaneously at the same concentration. Competitiveness was measured based on the number of progeny produced by each species in the host. Both species suffered a reduction in the number of progeny when dual inoculated. However, *S. carpocapsae* was more affected by the presence of *S. glaseri* than *vice versa*. The authors suggested that the faster development of *S. glaseri* might be responsible for this greater competitiveness compared to *S. carpocapsae*. However, both species were able to out-compete each other when they outnumbered their competitor. Under field conditions, it is possible that two or more entomopathogenic nematodes could coexist if the nematodes have

different foraging behaviour separating them spatially as well as by differential host susceptibilities (Koppenhöfer and Kaya, 1996).

When two pathogens with different biological attributes such as host specificity (specialist vs. generalist) interact within one host, it is very likely that the specialist uses the resource (host) more efficiently than the generalist, as described by Perlman and Jaenike (2001). These authors investigated interactions between the specialist nematode *Parasitylenchus nearcticus* Poinar and the generalist nematode *Howardula aoronymphium* Welch, both infecting the mushroom feeding fly *Drosophila recens* Wheeler. They found that the specialist nematode would competitively exclude the generalist nematode in individual hosts and at the population level. Despite these results, both nematodes were found in the field infecting *D. recens* and it was suggested that the generalist nematode persisted in the field by infecting alternate hosts.

The outcomes of interactions between some pathogens can sometimes be more complicated to assess, because more attributes have to be measured. For example Microsporidia, which are considered chronic pathogens and normally their effects result in prolonged developmental times and reduction in adult size, longevity, fecundity, mating and egg fertility (Bauer *et al.*, 1998).

Solter *et al.* (2002) evaluated the interactions between three microsporidian species *Nosema* sp., *Vairimorpha* sp. and *Endoreticulatus* sp. infecting *Lymantria dispar* L. larvae. Solter *et al.* (2002) found it difficult to determine whether any of the species were better competitors than the others. There were no differences in mortality between single and dual inoculated larvae, but in the simultaneous inoculation, the effect of the mixed infection was greater on male than on female hosts. Some evidence for competition was found when the larvae were dissected and tissues observed. *Nosema* was excluded from the silk glands by *Vairimorpha*, but the latter was excluded from fat tissue by *Nosema*. The species with the largest number of spores produced after a dual inoculation would be considered as the winner of the inter-specific competition, but this was not possible to quantify because spores produced by *Nosema* and *Vairimorpha* were identical morphologically.

Bauer *et al.*, (1998) using the same host (*L. dispar*), investigated the interactions between the microsporidium *Nosema* sp. and the Nuclear Polyhedrosis Virus (NPV), both infective to the larvae. They found that the larval susceptibility to the virus was not affected by *Nosema* sp. either inoculated simultaneously or after the viral inoculation. However, when the larvae were first inoculated with *Nosema* sp., the subsequent virus infection was more virulent, which suggested a synergism between these two pathogens. This, in essence, is considered positive from the pest control side. However, a negative effect was found for the NPV, because death was so fast that the production of viral units was reduced, even though the larvae produced both infective viral units and *Nosema* sp. spores. The occurrence of mixed infections of these pathogens was important for the natural enemy community of the pest (*L. dispar*), because if only virus was present in the field, the populations of *L. dispar* would be severely reduced affecting other natural enemies also. However, if mixed infections occured, the virus population was reduced enough to avoid induction of a viral epizootic.

Spatial structure may have a major role in the outcomes of interactions. As demonstrated by Massey *et al.* (2004) when they compared the performance of three different species of entomopathogenic bacteria, *Photorhabdus asymbiotica* (Fischer-Le), *Xenorhabdus nematophilus* (Poinar and Thomas) and *Photorhabdus luminescens* sp. *luminescens* (Poinar and Thomas) Boemare. Only the former two species were bacteriocin producers. Bacteriocins are antimicrobial toxins produced by bacteria that affect closely related isolates or bacteria species, but do not affect the species that is producing them. In *in vitro* experiments, they found that the two bacteria species that produced bacteriocins were able to exclude each other and the non-bacteriocin producer bacterial species, but that the non-bacteriocin bacteria species were not able to exclude the other species. However, when they carried out *in vivo* experiments, using *G. mellonella* larvae as a host, the overall virulence of the two bacteriocin producer species in a mixed infection was less than the mortality caused by each species separately. Although this is not discussed by the authors, this might be as a result of negative interactions between both bacterial species, reducing their development *in vivo* and, therefore, their ability to kill

the host. The bacteriocin producer species excluded the non-bacteriocin producer from the infected larvae but both bacteriocin producers were unable to exclude each other. A possible explanation is that both species had a different spatial structure allowing different species to dominate in different patches within the host and not compete with each other (Massey *et al.*, 2004)

The production of bacteriocin by some types of *Escherichia coli*, has been studied to try to explain how different types of *E. coli* that produce or do not produce colicin toxin (a type of bacteriocin) can co-exist and maintain biodiversity (Kerr *et al.*, 2002; Durret and Levin, 1997). If there are three types of *E. coli,* a colicin producer (which can kill other species but at the same time is resistant to colicin), a non-producer (is sensitive to colicin) and a resistant type (does not produce colicin but is resistant to it), their interaction and maintenance of diversity depends on the type of habitat they occupy. In a homogenous system (such as liquid medium in a flask), the colicin producer dominates and out-competes the others. However, on an agar plate, where interactions and dispersal might take place locally in very small places within the plate, the three types of *E. coli* can co-exist. They were not absolutely spatially isolated and they interacted at the boundaries of the colonies, where killing and competition took place. This suggested that the interactions and dispersion must be local (such as small patches within a Petri dish) for co-existence to occur (Kerr *et al.*, 2002).

It has been suggested that mixed infections lead to a selection for greater levels of virulence (e.g. Nowak and May, 1994, van Baalen and Sabelis, 1995; De Rode *et al.*, 2005). Parasites that slowly exploit the host will be out-competed by those that exploit the host faster (Read and Taylor, 2001).

De Roode *et al.* (2005) found that in mixed infections of mice by genetically different strains of the rodent malaria *Plasmodium chabaudi* (Landau), the more virulent strains out-competed the less virulent strains and the outcompeted strains, therefore, could not be transmitted to the mosquitoes, therefore reducing the transmission from mosquitoes to healthy mice. This suggested that the average virulence of a population of parasites can increase as

a result of within-host competition, or as a result of new virulent mutants out-competing the strains that were previously predominant in the host (De Roode *et al.*, 2005; Read and Taylor, 2001).

However there is also evidence to suggest that mixed infections could lead to reduced virulence, as discussed by Read and Taylor (2001). For example, Schjørring and Koella (2003) suggested, that the selection of greater levels of virulence applies where the parasites have only lethal effects on the host, but if the parasites have sub-lethal effects, such as reducing host growth rate, this in turn also reduces the parasite's growth and fecundity, and therefore the virulence.

Encouraging mixed infections that have negative effects on the fitness of a pathogen has been suggested as a positive outcome in human health research. The inter-specific competition between different types of pathogens in mosquitoes has been shown to reduce malaria infections in humans. When the fungus *B. bassiana* was used to control mosquito (*Anopheles stephensi* Liston) populations, not only were high mortality rates achieved but the fungus also affected the survival and maturation of the rodent pathogen *P. chabaudi* within the mosquitoes that survived the infection by *B. bassiana*. This suggests that an even greater reduction in the potential of transmission of this disease may be obtained (Blanford *et al.*, 2005).

Systems such as agricultural crops contain different species occupying the same habitat. It is clear that many of these species will interact and, therefore, affect each others development. Crucifers are important components of the human diet (Shelton, 2004). In 2005, 3,223,671 ha of cabbages and 893,993 ha of broccoli were harvested worldwide (FAO, 2005). The most important pest of these crops is the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Wright, 2004). It is considered the most universally distributed of all lepidoptera (Kfir, 1998). *Plutella xylostella* has different biological control agents, and all may interact with each other. Two of the most important natural enemies of *P. xylostella* are the entomophthoralean fungi *Zoophthora radicans* (Brefeld) Batko and *Pandora blunckii* (Bose & Mehta) (Pell *et al.*, 2001).

1.2 THE DIAMONDBACK MOTH *Plutella xylostella*

Worldwide, the diamondback moth (DBM) *Plutella xylostella* is considered the main insect pest of cruciferous crops (Talekar and Shelton, 1993; Verkerk and Wright, 1996), as well as one of the most widely distributed lepidopteran pests (Shelton, 2004; Kfir, 1998).

Some potential reasons for the pest status of *P. xylostella* have been suggested. It has a high reproductive potential, each female can lay about 200 eggs (Sarfraz and Keddie, 2005), a life cycle that, depending on temperature can be as short as 15 days at 24 °C (Liu *et al.*, 2002). This short life-cycle at high temperatures allows this pest to have as many as 20 generations in a year (Sarfraz and Keddie, 2005). The combination of these biological attributes and the cultivation of crucifers throughout the year in some regions, eliminating host free periods for the insect, provide an ideal environment for *P. xylostella* (Shelton, 2004).

Plutella xylostella has four developmental stages, egg, larva with four instars, pupa and adult, detailed descriptions of each of these developmental stages have been reported (e.g. Harcourt, 1957; Talekar and Shelton, 1993). As mentioned earlier, the developmental rates under field conditions are mainly affected by temperature. *Plutella xylostella* can develop under a wide range of temperatures, from 8 to 32 °C (Liu *et al.*, 2002), which contributes to their worldwide distribution. A possible relationship between temperature and geographical origin of different *P. xylostella* strains has been suggested (Liu *et al.*, 2002). However, Shirai (2000) did not find a thermal separation among nine populations of *P. xylostella* from tropical and temperate regions of Asia. A possible explanation could be that *P. xylostella* is considered an active flyer and may migrate long distances. It would be difficult, therefore, to obtain thermal relationships in such a highly mobile species (Shirai, 2000). Genetic differences (isoenzymes electrophoresis) and differences in biological attributes (oviposition) have been found amongst different *P. xylostella* populations from different parts of the world (Pichon *et al.*, 2004). However, no geographical relationship was found (Pichon *et al.*, 2004).

1.3 CONTROL OF *Plutella xylostella*

The use of chemical insecticides is still the main control strategy currently used against *P. xylostella* (CPC, 2005). In many countries, especially developing countries, chemical insecticides are available at low cost and in some countries pesticides are subsidized by the local government. As a consequence, farmers over-use chemical insecticides and have a complete dependence on them (CPC, 2005). The intensive use of chemical insecticides and the fact that this insect is multivoltine encourages the rapid development of insecticide resistance (CPC, 2005). As a consequence, *P. xylostella* is considered amongst the 20 most resistant arthropods (Mota-Sanchez *et al.*, 2002). It was the first insect pest in the world to develop resistance to DDT, and in many countries *P. xylostella* has become resistant to every synthetic insecticide including the bacterial insecticide *Bacillus thuringiensis* Berliner (Shelton *et al.*, 1993; Talekar and Shelton, 1993).

There is a movement throughout the world to reduce the use of pesticides. Consumer pressure demands products with no insect damage but at the same time with no insecticide contamination (Talekar and Shelton, 1993; Endersby and Morgan, 1991; Sarfraz and Keddie, 2005). To achieve this and still produce crucifers of an acceptable quality other alternatives must be considered. There are two possible ways to reduce the use of synthetic insecticides; the first is to eliminate these products, replacing them with alternative control methods and the second is to reduce the number of applications, by spraying only when necessary rather than on a regular prophylactic basis (Endersby and Morgan, 1991). The most practical strategy would be a combination of both, i.e. Integrated Pest Management (IPM). There are many alternative control methods such as the use of natural products (e.g. botanical insecticides), repellents, cultural control (e.g. physical barriers), intercropping, sprinkler irrigation, trap cropping, rotation, host plant resistance, sex pheromones and biological control (Endersby and Morgan, 1991; Talekar and Shelton, 1993). Biological control is an important strategy that can be incorporated into an IPM program, especially when only one insect species is

dominant as is the case for *P. xylostella* on cruciferous crops (Sarfraz and Keddie, 2005).

1.3.1 Biological control of *Plutella xylostella*

Biological control is the use of parasitoid, predator, pathogen, antagonist or competitor populations to suppress a pest population; this may be as a direct result of man intervention or naturally (Van Driesche and Bellows, 1996). Generally speaking, there are three biological control strategies to use natural enemies including entomopathogens: augmentation, introduction and conservation (Van Driesche and Bellows, 1996; Harper, 1987).

Augmentation- If a natural enemy is present in a system, but does not provide a pest population reduction, their numbers must be increased (Van Driesche and Bellows, 1996; Harper, 1987). In theory, augmentation has better chances than for example introduction, to be successful because larger amounts of an already existing natural enemy are released, and this natural enemy should be well adapted to its environment (Harper, 1987). Augmentation may be inoculative or inundative (Harper, 1987). Inoculative, when small amounts of the natural enemy are released and may results in the permanent establishment of the natural enemy and reducing the pest population (Harper, 1987). This method is considered as self-maintaining and therefore less expensive (Van Driesche and Bellows, 1996). Inundative introductions refers to the introduction of large amounts of the natural enemy to achieve relatively rapid control of the pest and is not expected an establishment of the natural enemy (Harper, 1987).

Introduction- This is the introduction of natural enemy into a system where it was not found before (Harper, 1987). When a new exotic pest is found in a system, conservation is not the appropriate method of control, because there are not sufficient or appropriate natural enemies. Therefore, the introduction of natural enemies found in the place of origin of the exotic pest is essential for a successful control (Van Driesche and Bellows, 1996). Again, there can be introduction by inoculative and inundative releases (Van Driesche and Bellows, 1996), and this they are often repeated to ensure establishment.

Conservation- These are examples of reductions of insect populations by the effect of natural enemies without man intervention, for example natural epizootics caused by entomopathogens (Harper, 1987). Therefore, it is assumed that species of natural enemies already exist locally with potential to suppress insect populations (Van Driesche and Bellows, 1996). Biological control by conservation tries to identify and avoid those negative influences that may affect natural enemies detrimentally (Van Driesche and Bellows, 1996). One of the main factors affecting natural enemies is the application of chemical insecticides, for example the use of fungicides to control plant pathogens can also affect entomopathogenic fungi (Van Driesche and Bellows, 1996; Harper, 1987). The application of selective pesticides and decreasing the application rate has been suggested as possible methods to minimize the negative effect of pesticides on natural enemies (Harper, 1987). The maintenance or creation of physical refuges has been suggested as an alternative method of conservation (Van Driesche and Bellows, 1996).

Plutella xylostella has many natural enemies including pathogens (Wright, 2004) which can be augmented for biological control. CPC (2005) has reported 53 species of parasitoids from different orders, and 26 species of predators attacking *P. xylostella*. Two main parasitoid genera, *Diadegma* and *Cotesia* are reported as primary parasitoids and most effective in controlling *P. xylostella* (Wright, 2004; Azidah *et al.*, 2000).

In the genus *Cotesia*, the main species used in biological control is *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae). There is extensive research carried out using this species (e.g. Talekar and Yang, 1993; Chilcutt and Tabashnik, 1997; Verkerk and Wright, 1997; Mitchell *et al.*, 1997a; Potting *et al.*, 1999). This species is a solitary endoparasitoid attacking mainly second and third instar larvae of *P. xylostella* (Wright, 2004).

There are different species reported from the genus *Diadegma* (Hymenoptera: Ichnemonidae) such as *Diadegma semiclausum* (Hellén), *D. fenestrale* (Homgren), *D. mollipla* (Holmgren), *D. insulare* (Cresson), *D. leontininae* (Brèthes), *D. novaezealandiae* (Azidah, Fitton and Quicke) and *D. rapi*

(Cameron) (Delvare, 2004). The majority of the research supporting biological control has been focused on *D. semiclausum* (e.g. Yang *et al.*, 1993; Yang *et al.*, 1994; Wang *et al.*, 2004; Noda *et al.*, 2000; Lavandero *et al.*, 2005) and *D. insulare* (e.g. Idris and Grafius, 1993; Mitchell *et al.*, 1997b; Hill and Foster, 2000; Xu *et al.*, 2004). These species are solitary endoparasitoids attacking mainly second and third instars larvae.

The use of predators for the control of *P. xylostella* has received little attention by comparison with parasitoids (Suenaga and Hamamura, 2001; Verkerk and Wright, 1996). Furthermore, there are few studies pointing to the possibility of using predators as a practical control strategy for *P. xylostella* (e.g. Suenaga and Hamamura, 1998; Suenaga and Hamamura, 2001). Suenaga and Hamamura (2001) found many species of carabid beetles in cabbage plots and boundary fields. The beetles *Chlaenius micans* (F.), *Campalita chinense* (Kirby) and *Chlaenius posticalis* Motschulsky preferred the cabbage plots to the field boundary. Some relationship between beetle abundance and reduction of *P. xylostella* population was observed, but as the authors suggested, this relationship not necessarily indicates that the carabid beetles are capable of controlling pests. Further studies are required to determine if the carabids have an influence on pest populations such as density manipulation or predator exclusion experiments (Suenaga and Hamamura, 2001). The possibility for these beetles to be used as biological control agents has also been evaluated in laboratory conditions. *Chlaenius micans* and *C. posticalis* had a high *P. xylostella* consumption rate and were able to climb plants suggesting their potential for the control of *P. xylostella* (Suenaga and Hamamura, 1998).

1.3.2 Microbial control of *Plutella xylostella*

Microbial control is the use of microorganisms (entomopathogens) for biological control of pest insects (Tanada and Kaya, 1993). There are some representatives from each major group of entomopathogens that are pathogenic to *P. xylostella*.

The most successful microbial insecticide currently is the bacterium *Bacillus thuringiensis* (Berliner), and is considered the main microbial insecticide used against *P. xylostella* (Cherry *et al.*, 2004a). Different commercial products containing different subspecies of *B. thuringiensis* highly effective against *P. xylostella* have been developed. There are 33 products registered specifically for the control of *P. xylostella* and other lepidopteran insects which contain *B. thuringiensis* from the subspecies *kurstaki;* five products containing *B. thuringiensis* from the subspecies *aizawai,* effective against other lepidopteran insects such as *Spodoptera* sp. but also effective against *P. xylostella*; two products that are effective against coleopteran and lepidopteran insects including *P. xylostella*, and contain mixtures of both subspecies (*kurstaki* and *aizawai*). There are also two products where the delta-endotoxin, which is a protein produced by *B. thuringiensis* that has insecticidal properties, were encapsulated inside the bacterium *Pseudomonas fluorescens* (Trevisan) Migula. These products contain the subspecies *kurstaki* and are mainly for *P. xylostella* control, but can also be effective against other lepidopteran insects. Finally, there are two products where the delta-endotoxin of *B. thuringiensis* subspecies *aizawai* were encapsulated in *P. fluorescens*, these products were mainly for other lepidopteran insects such as *Spodoptera* sp. but highly effective against *P. xylostella* as well (Copping, 2001).

The other major group of entomopathogens used against *P. xylostella* are the virus, and mainly from the family Baculoviridae (Cherry *et al.*, 2004a). The main characteristic of this family is the existence of bacilliform virions that have nucleocapsids singly or multiply enveloped (Evans and Shapiro, 1997). Included in this family, there are two representatives, the Nuclear Polyhedrosis Virus (NPV) and the Granulosis Virus (GV) (Evans and Shapiro, 1997). There are five commercial products where *P. xylostella* is in their list of target insects; two containing a NPV isolated from *Autographa californica* (AcNPV), and three containing a NPV isolated from *Mamestra brassicae* (MbNPV) (Copping, 2001). There are other NPV commercial products such as *Anagrapha falcifera* NPV, that although *P. xylostella* is not in its list of target pests (Copping, 2001), it has been found that *P. xylostella* is susceptible to it (e.g. Farrar and Shapiro, 2005). There are two viruses that have been proved

highly virulent against *P. xylostella*, although there are no commercial products available, research has been carried out on them. The first one is a GV isolated from *P. xylostella* (PxGV) (e.g. Grzywacz *et al.*, 2004; Cherry *et al.*, 2004b; Kadir *et al.*, 1999a,b). The second is a NPV isolated from *P. xylostella* (PxNPV) by Kariuki and McIntosh (1999) (e.g. Farrar and Shapiro, 2005; Farrar *et al.*, 2004).

Nematodes are not considered a significant natural enemy of *P. xylostella* for development in biological control (e.g. Sarfraz *et al.*, 2005). However, some research has been carried out evaluating species such as *Steinernema carpocapsae* Weiser (e.g. Schroer and Ehlers, 2005; Mason *et al.*, 1999).

There are many entomopathogenic fungi with potential for the control of *P. xylostella.* In the Ascomycota, there are different species proved as pathogenic against *P. xylostella*, for example *Beauveria bassiana* (Valsamo-Buillemin) (e.g. Selman *et al.*, 1997; Vandenberg *et al.*, 1998; Shelton *et al.*, 1998; Yoon *et al*, 1999), *Metarhizium anisopliae* (Metsch.) Sorokin (e.g. Amiri *et al.*, 1999), *Paecilomyces fumosoroseus* (Wise) Brown and Smith (e.g. Altre *et al.*, 1999). Although none of the species have been found causing natural epizootics in *P. xylostella* (Wilding, 1986, Stavely *et al.*, 2004), they have received considerable attention for the development of commercial products because they can be easily mass produced (Goettel and Inglis, 1997; Cherry *et al.*, 2004a), obtaining a large number of infective conidia (Cherry *et al.*, 2004a). The conidia can be applied in the field mixed with water and using the same equipment as chemical insecticides, which would be easily accepted by farmers, and made them ideal candidates either for inundative introduction or augmentation. For example the *B. bassiana* isolates ATCC 74040, Bb 147, GHA and Stanes have 16 commercial products registered in total (Copping, 2001). Although only the product Bio-Power® containing the isolate Stanes, has *P. xylostella* in its list of target pests (Copping, 2001), research has been carried out with the isolate GHA for the control of *P. xylostella* (e.g. Vandenberg *et al.*, 1998; Shelton *et al.*, 1998).

The other major group within the entomopathogenic fungi infecting *P. xylostella* are the Entomophthorales. Only two species has been found infecting *P. xylostella* populations naturally, *Zoophthora radicans* and *Pandora blunckii* (e.g. Riethmacher and Kranz, 1994; Velasco *et al.*, 2000; Tomiyama and Aoki, 1982). *Zoophthora radicans* has also been found infecting natural population of other insect species (e.g. Galaini-Wraight *et al.*, 1991; Mcguire *et al.*, 1987; Vandenberg and Soper, 1987). Although there are no commercial products from these pathogens, extensive research has been carried out to develop *Z. radicans* for the use in biological control of *P. xylostella* (e.g. Pell *et al.*, 1993a, b; Pell and Wilding, 1994; Furlong *et al.*, 1995; Furlong and Pell, 1997; Vickers *et al.*, 2004). The lack of commercial development of this type of fungi is due to the difficulty of mass production (Pell *et al.*, 2001). Some Entomophthorales can be grown on standard media, but other requires some supplements or even complex media for their *in vitro* mass production (Pell *et al.*, 2001). The conidia, which would eventually be used as infective units are sticky, which makes them difficult to harvest or suspend in liquid, and they are large, which makes them difficult to use in conventional spraying equipment (Pell *et al.*, 2001).

Recently, Hua and Feng (2005) succesfully evaluated broomcorn millet grain as a substrate for the mass production of *Z. radicans* suggesting potential for its use in the future. They found that large quantities of primary conidia (an average of 14.9 (±0.9) x 10^4 conidia) could be produced from each millet grain. Although this was smaller than the amount produced by a *P. xylostella* cadaver (28.7 (±8.9) x 10^4 conidia), each grain continued to sporulate for 7 days, compared to a *P. xylostella* cadaver that only sporulated 3 days. The infectivity of the conidia produced on the millet grains and from *P. xylostella* cadavers were similar. This method seems to be less expensive and laborious than previous methods, and has been succesfully tested for *P. neoaphidis* as well (Hua and Feng, 2003). Mass production and application has been attemped with structures other than the conidia. For example hyphal stages (e.g. Li *et al.*, 1993; Nolan, 1988), and resting spores (e.g. Latge *et al.*, 1977; Hajek and Webb, 1999; Kogan and Hajek, 2000). The difficulty of mass production, does not allow them to be used by inundative biological control methods, but their

ability of discharge conidia actively, makes them ideal for inoculative methods. For example, different experimental methods have been developed for *Z. radicans* application. One is the autodissemination approach, where male *P. xylostella* moths were attracted to sex pheromone traps, inoculated with *Z. radicans* conidia, and then able to leave the trap to disperse the fungal inoculum amongst uninfected larvae in the field (Pell *et al.*, 1993b; Furlong *et al.*, 1995). Another method was the release of *P. xylostella* adults moths inoculated with *Z. radicans*. These were released in a large field cage containing cabbage plants previously infested with larvae, pupae and adults of *P. xylostella*, obtaining as much as 79% infection after 48 days of evaluation (Vickers *et al.*, 2004).

1.3.2.1 Biology of the Entomophthorales

The entomophthoralean fungi are included in the Phylum Zygomycota, class Zygomycetes, order Entomophthorales (Alexopoulus *et al.*, 1996). The main characteristic of the Zygomycetes is the production of thick-walled resting spores called zygospores, which is the result of the union of two gametangia. There are also other structures called azygospores that form parthenogenetically (Alexopoulus *et al.*, 1996). In general, zygospores are referred to as sexual spores and azygospores as asexual spores. However, karyogamy and meiosis in entomopthoralean resting spores are unknown and evidence of these events is inconclusive (Humber, 1981). No evidence of heterothallisms has been found in this group (Humber, 1981; Alexopoulus *et al.*, 1996), and no parasexuality (Humber, 1981). In strict terms, zygospores and azygospores refer only to the mode of development of the resting spores (Humber, 1981). Resting spores are the most important mechanisms for survival of these fungi during adverse conditions, allowing them to germinate later in the season under more favourable conditions such as a suitable climate and the presence of the primary host (Pell *et al.*, 2001; Papierok and Hajek, 1997). Asexual reproduction can take place by conidia in Entomophthorales (Alexopoulus *et al.*, 1996; Humber, 1981). Another characteristic of this group is the presence of coenocytic mycelium (no septa) (Alexopoulus *et al.*, 1996),

and the ability to discharge primary conidia actively (Pell *et al.*, 2001; Tanada and Kaya, 1993).

The entomophthoralean fungi have a characteristic life cycle, starting with the conidia produced on conidiophores that emerge in large numbers from a cadaver, especially through intersegmental membranes. These primary conidia are discharged actively by hydrostatic pressure and may land on a suitable host or other surfaces. If they land on a suitable host, the infection process starts again. If they land on a non-host surface the conidia produce secondary conidia which eventually are also discharged actively. The production of secondary conidia and even higher order conidia continues until the protoplasm is depleted and the conidia die (Pell *et al.*, 2001; Tanada and Kaya, 1993).

Some genera e.g. *Zoophthora* do not always produce actively discharged secondary conidia (Wilding, 1986). Instead they produce a capilliconidium, on a thin elongate conidiophore. During development, the cytoplasm within the conidiophore is evacuated into the capilliconidium (Humber, 1981; Alexopoulus *et al.*, 1996). The capilliconidium is sticky and will attach to the host when it passes close to the cadaver (Alexopoulus *et al.*, 1996; Pell *et al.*, 2001).

The first barrier that the conidia need to overcome during the infection process is the cuticle of the insect. Normally, conidia use mechanical and enzymatic mechanisms to penetrate the cuticle which may or may not include the development of specialised infection structures such as appressoria (Pell *et al.*, 2001).

Once inside the insect body, the fungus reproduces as protoplasts or hyphal bodies. The death of the host occurs mainly due to fungal proliferation (Alexopoulus *et al.*, 1996). The fungus uses the host's nutrient reserves killing by physiological starvation (Pell *et al.*, 2001). Once the fungus has completely invaded the insect body, depending on the environmental conditions, emerges from the cadaver producing conidiophores and eventually conidia (Tanada and Kaya, 1993). The cadavers produced by some entomphthoralean fungi are

fixed to the surface on which the host died by rhizoids. This retains the cadaver in the environment increasing the chance for the conidia to contact other hosts and start a new infection (Pell *et al.*, 2001).

Some entomopthoralean fungi also produce, at the end of the cycle, resting spores e.g *Zoophthora radicans* (e.g. Glare *et al.*, 1989; Yeo *et al.*, 2001). The factors responsible for resting spore formation are not well understood (Glare *et al.*, 1989; Hajek and St. Leger, 1994), and this attribute is highly variable even amongst isolates of the same species (Glare *et al.*, 1987). This makes it difficult to relate this process to particular environmental or biotic conditions. Determining the time of germination of resting spores under field conditions is also difficult. Once the resting spore has germinated, it produces primary conidia beginning the life cycle again (Pell *et al.*, 2001).

The ecology of mixed infection by two species of pathogens (inter-specific) or different strains of the same pathogen (intra-specific) can be complex. The outcome of these interactions depends on diverse biotic and abiotic factors. The ecology of mixed infections has been studied in diverse areas such as human medicine (e.g. De Roode *et al.*, 2005; Blanford *et al.*, 2005). However, there is little information about the interactions between fungi attacking insects (e.g Inglis *et al.*, 1997; Thomas *et al.*, 2003). Nothing has been done for entomophthoralean fungi. *Zoophthora radicans* and *P. blunckii* are two of the most important pathogens of *P. xylostella* (Pell *et al.*, 2001). These two fungal species can be found in the same habitat and infecting the same *P. xylostella* population (Reithmacher and Kranz, 1994, Velasco *et al.*, 2000). It is likely that these two species will interact and that their spatial and temporal distribution will be affected as a result of these interactions, eventually affecting the biology of *P. xylostella*.

1.4 AIMS

The aim of this research is to gain a better understanding of the types of interactions that may occur between *Z. radicans* and *P. blunckii* in *P. xylostella* populations. A large number of isolates of both species have been isolated from *P. xylostella* and were available for use in these experiments.

It was important to have initial information about all the isolates and be able to select a sub-set of isolates for the *in vivo* interactions experiments. The series of experiments carried out are defined in the following specific objectives:

1- Determine the effect of temperature on the radial growth of *Z. radicans* and *P. blunckii* isolates separately.

2- Determine the *in vitro* intra and inter-specific interactions between *Z. radicans, P. blunckii* and *Pandora* sp. isolates.

3- Assess the genetic variability of *Z. radicans* and *P. blunckii* isolates.

With the information obtained for all the isolates in experiments one to three, fewer isolates could be selected and used to investigate their further *in vivo* inter-specific interactions, defined by the following specific objectives:

4- Estimate the virulence of selected isolates of *Z. radicans* and *P. blunckii* against *P. xylostella* and their *in vivo* inter-specific interactions.

5- Develop and use of molecular detection techniques and quantification using real time PCR to quantify *Z. radicans* in *P. xylostella* larvae single or dual inoculated with *P. blunckii*.

CHAPTER 2. EFFECT OF TEMPERATURE ON RADIAL GROWTH OF *Zoophthora radicans* AND *Pandora blunckii*

ABSTRACT

The effect of four different temperatures (15, 20, 25 and 30 °C) on the *in vitro* growth of 19 isolates of *P. blunckii* and 14 isolates of *Z. radicans*, all isolates from *P. xylostella* larvae was investigated. Both species showed more growth at 20 and 25 °C than the other two temperatures. *Zoophthora radicans* showed greater growth than *P. blunckii* at 20 and 25 °C. The overall results showed large variability within each species amongst: all isolates regardless of geographical origin, isolates from different countries and amongst isolates from the same country or geographical origin (Mexico). No relationship was found between optimal growth temperature and geographical origin. This represents the first report of the relationship between temperature and the *in vitro* growth of *P. blunckii*. The ecological role of this large variability amongst isolates within each species is discussed. The results helped to select isolates for further experiments based on their *in vitro* growth at the different temperatures.

2.1 INTRODUCTION

Temperature, as with other abiotic factors, plays an important role in determining the fundamental niche of all fungi (Cooke and Whipps, 1993). The importance of temperature in the biology of entomopathogenic fungi is also well recognised (Tanada and Kaya, 1993; Benz, 1987). As a result, the characterisation of different fungal species and isolates according to their thermal preference has been suggested as a selection tool to find suitable isolates for use in microbial control programmes under specific climatic conditions (e.g. Dimbi *et al.*, 2004; Tefera and Pringle, 2003; Vidal *et al.*, 1997; Fargues *et al.*, 1992). However, variability amongst isolates of the same species make it difficult to determine a clear relationship between geographical origin and thermal preference (Fargues *et al.*, 1997; Moorhouse *et al.*, 1994).

Some attributes such as radial growth, sporulation, germination and virulence of fungal pathogens studied *in vitro* under a range of temperatures can help to select candidates for use in microbial control under specific climatic conditions. For example *M. anisopliae* var. *acridum* can cause infection in the grasshopper *Zonocerus variegatus* when insect body temperatures reach close to 40 °C (Blanford *et al.*, 2000), but *B. bassiana* cannot survive temperatures above 35 °C reducing dramatically the mycosis on *Melanoplus sanguinipes* nymphs (Inglis *et al.*, 1996). These data inform us about the type of climatic conditions under which each of these pathogens could have some impact in a pest population and also indicate their natural ecological niche.

There have been several studies of the effect of temperature on the biology and ecology of *Z. radicans* (Milner and Lutton, 1983; Glare *et al.*, 1987; Uziel and Shtienberg, 1993; Furlong and Pell, 1997; Griggs *et al.*, 1999) but there is very limited information for *P. blunckii* and nothing is known about its temperature optima (Riethmacher and Kranz, 1994; Tomiyama, 1982; Wilding, 1986). *Zoophthora radicans* isolates vary in their response to temperature and in other biological attributes e.g. virulence (Pell *et al.*, 1993a; Yeo *et al.*, 2001; Feng and Johnson, 1991; Milner and Lutton, 1983; Glare *et al.*, 1987). Temperature optima for *Z. radicans* growth *in vitro* have been reported as 25 °C (Milner and Lutton, 1983; Milner and Mahon, 1985; Glare *et al.*, 1987). Although there are no specific studies for *P. blunckii*, temperature optima for *Pandora neoaphidis*, another species in the same genus, are between 15 and 20 °C (Stacey *et al.*, 2003; Morgan *et al.*, 1995).

Both *Z. radicans* and *P. blunckii* can infect the same host population (*P. xylostella*), in the same fields at the same time (Riethmacher and Kranz, 1994). Differences in temperature optima may influence possible competitive interactions between these two species under field conditions. Understanding the behaviour of these fungi at different temperatures, particularly *P. blunckii* for which there are no reports, is a logical starting point for isolate characterisation based on adaptation to a particular environment. The aim of this experiment was to determine the effect of temperature on the *in vitro* growth of *Z. radicans* and *P. blunckii* from Mexico and elsewhere.

2.2 MATERIAL AND METHODS

2.2.1 Fungal isolates

Experiments were carried out with 19 isolates of *P. blunckii* and 14 isolates of *Z. radicans*. All isolates were isolated from *P. xylostella*. Many isolates were from the Rothamsted culture collection and some were from the USDA-ARSEF collection (Table 2.1).

2.2.1.1 Recovering fungal material from liquid nitrogen

The isolates from the USDA-ARSEF collection were requested from Richard Humber (USDA, Ithaca, N.Y., USA), and the isolates from the Rothamsted culture collection were retrieved from liquid nitrogen.

To recover the fungal material from liquid nitrogen, an individual sachet was transferred to the laboratory in a small Dewar flask containing liquid nitrogen and then transferred to a water bath at 37-40 °C for two minutes until it had reached room temperature. The sachet was cleaned in 95% alcohol and was opened under sterile conditions. The mycelium was removed and placed onto Sabouraud dextrose agar supplemented with egg yolk and milk (SEMA medium, Wilding and Brobyn, 1980) (2.2.1.2) in 90 mm triple vented Petri dishes. Each plate was labelled with the isolate reference code, subculture number and date, sealed with Parafilm M (Pechiney Plastic Packaging, Inc., Wisconsin) and incubated in darkness at 20 °C until required. After been retrieved from the liquid nitrogen, all the isolates were maintained sub-culturing them in new SEMA plates at 15 days intervals. For the experiments, the isolates were never sub-cultured more than three times before use.

2.2.1.2 Production of nutrient agar, SEMA

To prepare SEMA plates, a 500 ml Duran bottle with 20.8 g of Sabouraud Dextrose Agar (SDA, Oxoid, Hampshire, England) and 320 ml of distilled water, a 100 ml Duran bottle containing 34 ml of semi-skimmed milk, one empty 500 ml Duran bottle with lid, one Pyrex bowl (190 mm diameter, 90 mm

depth) covered with aluminium foil were sterilised in an autoclave at 115 °C for 20 minutes.

All sterile material was placed inside a laminar-flow cabinet previously cleaned with 95% alcohol. The molten SDA was cooled and maintained at 55 °C in a water bath until required. Two eggs, previously surface sterilised (soaked for at least one hour in a 1:100 acetone:alcohol solution) were cracked individually on the edge of the Pyrex bowl and the white separated from the yolk by decantation. The yolks were placed inside the empty 500 ml Duran bottle, shaken to break them and the milk added and shaken again to mix. The milk-yolk mixture was then added to the SDA and mixed again. The resulting SEMA was pipetted into 90 mm triple vented Petri dishes (24 ml per dish) and allowed to solidify. The amounts described here provided enough material for 16 plates. When more plates were needed the quantities were increased proportionally to achieve the amount required.

Table 2.1. *Pandora blunckii* and *Zoophthora radicans* isolates used in the growth at different temperatures experiment. All isolates originated from the diamondback moth, *Plutella xylostella*.

Species	Isolate reference	Country of origin
P. blunckii	NW 344	Mexico
”	NW 345	”
”	NW 346	”
”	NW 347	”
”	NW 348	”
”	NW 349	”
”	NW 350	”
”	NW 352	”
”	NW 380	”
”	NW 381	”
”	NW 383	”
”	NW 384	”
”	NW 385	”
”	ARSEF 6293	”
”	ARSEF 6306	”
”	ARSEF 6305	”
”	ARSEF 6310	”
”	ARSEF 6311	”
”	ARSEF 217	”

Table 2.1. Continued.

Species	Isolate reference	Country of origin
Z. radicans	NW 353	Mexico
”	NW 378	”
”	NW 379	”
”	NW 382	”
”	NW 386	”
”	NW 250	Malaysia
”	NW 325	Kenya
”	NW 168	Japan
”	NW 328	N. Zealand
”	NW 329	”
”	NW 364	Australia
”	NW 410	”
”	NW 182	Taiwan
”	ARSEF 2893	Philippines

2.2.2 Experimental design

The effect of temperature on the growth of each isolate was estimated by measuring the radial growth of replicate colonies at 15, 20, 25 and 30°C. Each replicate of each isolate at each temperature was produced using the same procedure. A 5 mm diameter plug was taken from the growing edge of a 14 day old colony and placed into the centre of a 9 cm triple vented Petri dish containing 24 ml of SEMA. Each dish was sealed with Parafilm “M” and incubated at the selected temperature. Radial growth was measured every two days for 22 days (11 measurement points) or until the colony had reached the edge of the plate. All Petri dishes with bacterial or fungal contamination at any stage of the experiment were excluded from the analysis.

A Latin Square design was used to assign temperatures to four incubators. An Alpha-Design was used to distribute the 34 isolates in seven blocks of five isolates amongst the four shelves of each incubator. A completely random design was used to allocate each Petri dish on a given shelf. The experiment was repeated two times on different dates, with three replicates for each isolate at each temperature and on each occasion. The temperatures that each of the incubators was running at was changed on the second occasion.

2.2.3 Statistical analysis

Prior to analysing the data using an ante-dependance analysis (Kenward, 1987), in the statistical package GenStat v. 6.0 an order structure was estimated using an ant-order test. The order structure describes the dependence that a given data point has with a number of preceding data points (order number), and is independent of all subsequent points. This procedure calculated the statistics that assisted in the selection of an appropriate order of ante-dependence structure (Kenward, 1987). The results of the ante-dependance test analysis indicate the times at which treatment effects occurred and also the overall treatment effects.

Using the same statistical analysis, the results were analysed at different levels, the overall difference between the two species, the differences among isolates of each species, differences among isolates of each species in relation to geographical origins and differences among Mexican isolates of each species. The possible interaction with temperature at all levels of comparison was also analysed.

2.3 RESULTS

The ant-order test showed that the set of data analysed had an ante-dependence structure of order six (Table 2.2). This means that all the data points were dependent on the six preceding measurements points and independent of all subsequent points. The ante-dependance analyses were done using this order structure.

Table 2.2. Comparison of ante-dependance structures with order 10 (maximum order). Row in bold shows order number selected and therefore used in all subsequent ante-dependance analysis. DF = degrees of freedom.

Order	Unadjusted Chi-square	Adjustment factor	Adjusted Chi-square	DF	Prob.
Order 0 v. order 10	14719.74	0.616	9064.61	55	<0.001
Order 1 v. order 10	825.97	0.604	499.01	45	<0.001
Order 2 v. order 10	188.51	0.591	111.44	36	<0.001
Order 3 v. order 10	102.76	0.577	59.27	28	<0.001
Order 4 v. order 10	68.18	0.561	38.23	21	0.012
Order 5 v. order 10	60.11	0.543	32.66	15	0.005
Order 6 v. order 10	**30.32**	**0.525**	**15.93**	**10**	**0.102**
Order 7 v. order 10	14.12	0.507	7.15	6	0.307
Order 8 v. order 10	5.85	0.489	2.86	3	0.414
Order 9 v. order 10	0.36	0.47	0.17	1	0.683

2.3.1 Differences between species and their interaction with temperature

Overall, there were significant differences in the growth profiles of the two fungal species averaged over the two temperatures (χ^2_{11}=393.9, P<0.001). However, the differences were not significant at all the measurement points. Significant differences were detected only during the first 8 measurement times (from day 4 to day 16), which corresponded mainly to the linear growth phase. After that, from day 18 to day 22, the growth profiles for the two species were not significantly different (Table 2.3). *Zoophthora radicans* showed greater growth than *P. blunckii.* The average radius per colony for *Z. radicans* at the end of the experiment was 30.6 mm and for *P. blunckii* was 26.4 mm (Fig 2.1). The differences between species had a significant interaction with temperature (χ^2_{33}=1246.2, P<0.001).

The average colony radius for both species was larger at 20 and 25 °C than at 15 and 30 °C (Fig. 2.2). *Zoophthora radicans* had a larger average radius than *P. blunckii* at 20, 25 and 30 °C, but was similar to *P. blunckii* at 15°C (Fig. 2.2)

Table 2.3. Comparison between *Zoophthora radicans* and *Pandora blunckii* radial growth averaged over all isolates and temperatures. Test assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	0.118	1	0.731
4	24.778	1	<0.001
6	56.930	1	<0.001
8	81.504	1	<0.001
10	130.534	1	<0.001
12	58.571	1	<0.001
14	73.677	1	<0.001
16	6.655	1	0.01
18	2.864	1	0.091
20	0.168	1	0.682
22	6.496	1	0.011

Overall test using data from all the measurement times

Statistic=393.963, DF=11, Probability <0.001

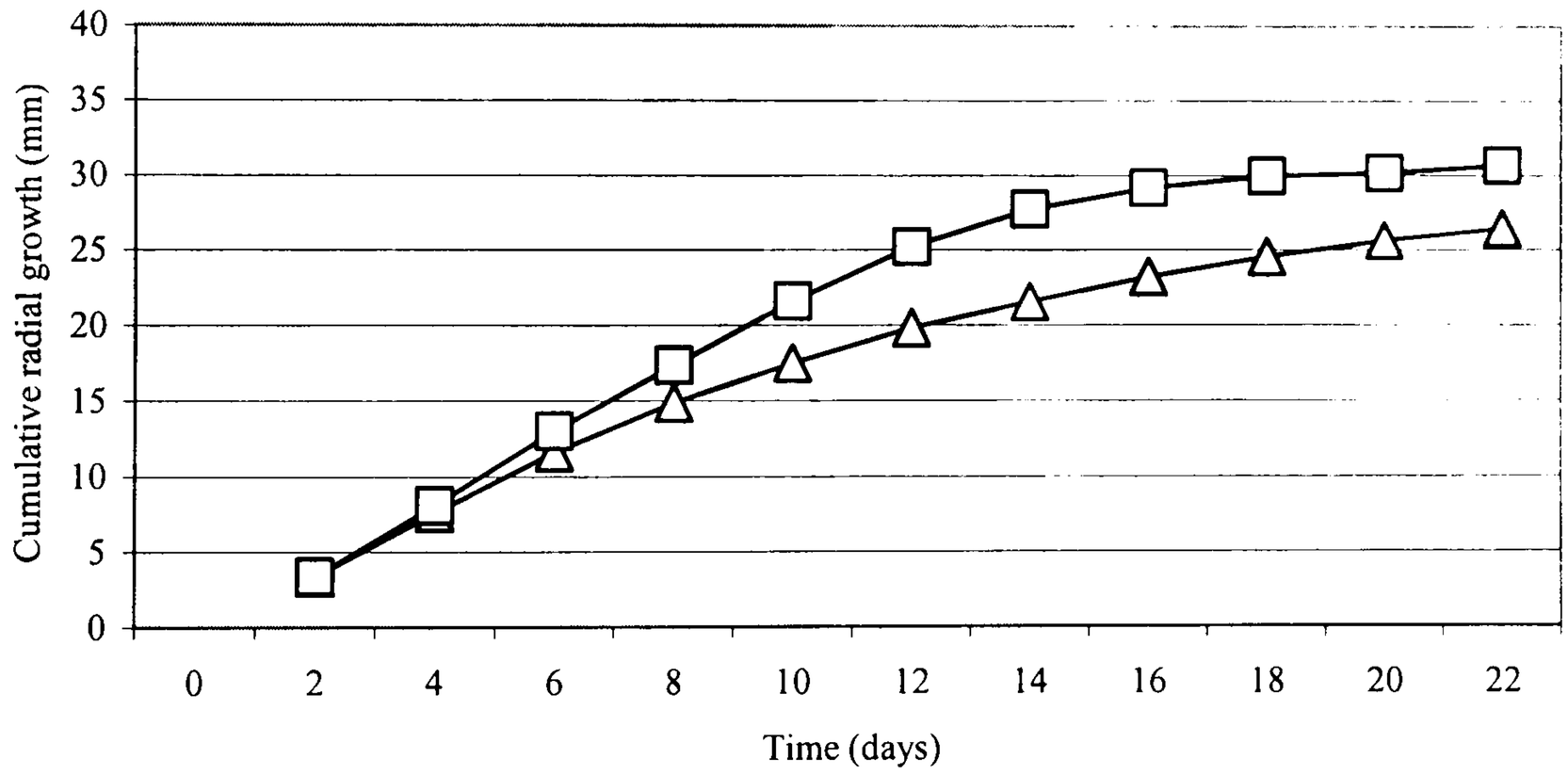


Figure 2.1. Cumulative radial growth of *Pandora blunckii* (△) and *Zoophthora radicans* (□) expressed in mm. Growth profiles for both species represent the mean values of all isolates over all temperatures.

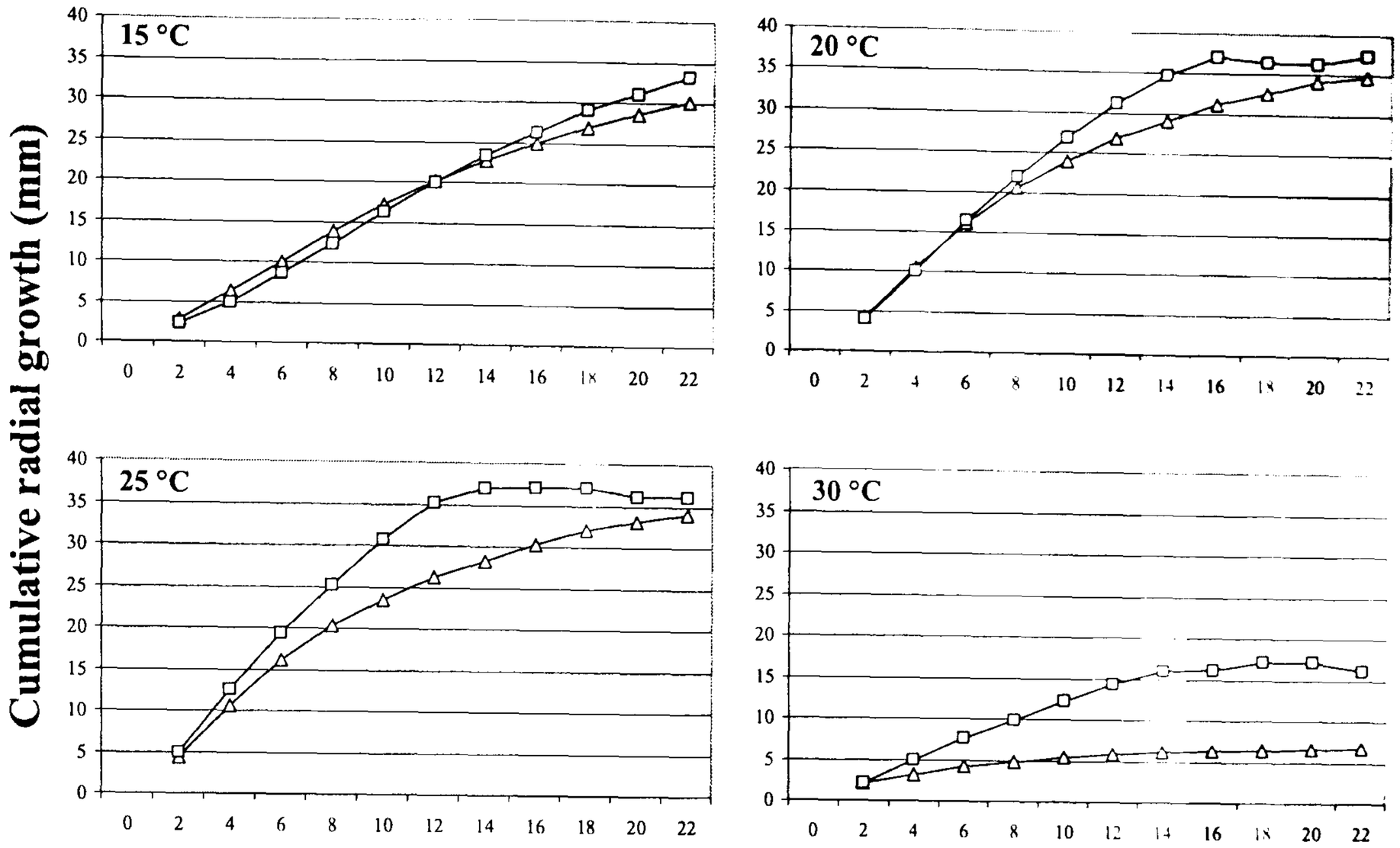


Figure 2.2. Cumulative radial growth of *Pandora blunckii* (△) and *Zoophthora radicans* (□) expressed in mm. Growth profiles for both species represent the mean values of all isolates at each temperature.

2.3.2 Differences amongst isolates of each species and their interaction with temperature

Significant differences in growth profile were found amongst the *P. blunckii* isolates (χ^2_{198}=596.2, P<0.001) and among the *Z. radicans* isolates (χ^2_{143}=814.3, P<0.001). The differences were detected at all measurement times, except at days 16 and 20 for *P. blunckii* isolates (Table 2.4 and 2.5 for *P. blunckii* and *Z. radicans* respectively). It was difficult to identify the behaviour of specific isolates at each temperature because their growth profiles were close. Only at 30 °C could some differences be identified. For example, the *P. blunckii* isolate ARSEF6293 had the greatest growth compared to the rest of the *P. blunckii* isolates at this temperature. The *Z. radicans* isolates ARSEF2893, NW410, NW325 and NW386 obtained a very similar growth, and together had the greatest growth compared to the other *Z. radicans* isolates at this temperature.

Table 2.4. Comparison among *Pandora blunckii* isolates averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	147.555	18	<0.001
4	114.039	18	<0.001
6	40.638	18	0.002
8	36.284	18	0.006
10	80.539	18	<0.001
12	39.116	18	0.003
14	42.010	18	0.001
16	28.312	18	0.057
18	29.722	18	0.04
20	24.152	18	0.15
22	47.231	18	<0.001

Overall test using data from all the measurement times

Statistic=596.285, DF=198, Probability <0.001

Table 2.5. Comparison among *Zoophthora radicans* isolates averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	215.39	13	<0.001
4	139.718	13	<0.001
6	95.519	13	<0.001
8	54.986	13	<0.001
10	52.203	13	<0.001
12	29.8	13	0.005
14	25.858	13	0.018
16	35.467	13	<0.001
18	50.318	13	<0.001
20	54.04	13	<0.001
22	84.776	13	<0.001

Overall test using data from all the measurement times

Statistic=814.394, DF=143, Probability <0.001

Differences in growth amongst *P. blunckii* isolates showed a significant interaction with temperature (χ^2_{588}=1071.8, P<0.001), but these differences were only from day 2 to day 10 corresponding mainly to the linear phase of

growth (Table 2.6). After that time there were no significant differences until days 20 and 22. Differences in growth amongst *Z. radicans* isolates also showed a significant interaction with temperature (χ^2_{386}=1478.144, P<0.001), which was significant at all the measurement points (Table 2.7).

Table 2.6. Comparison among *Pandora blunckii* isolates in interaction with temperature, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	185.606	54	<0.001
4	175.620	54	<0.001
6	125.649	54	<0.001
8	103.291	54	<0.001
10	112.626	54	<0.001
12	53.134	54	0.508
14	60.782	54	0.245
16	56.967	54	0.365
18	62.789	53	0.168
20	83.558	53	0.005
22	85.753	50	0.001

Overall test using data from all the measurement times

Statistic=1071.887, DF=588, Probability <0.001

Table 2.7. Comparison among *Zoophthora radicans* isolates in interaction with temperature, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	115.67	39	<0.001
4	168.271	39	<0.001
6	163.59	39	<0.001
8	112.7	39	<0.001
10	111.092	39	<0.001
12	149.34	39	<0.001
14	116.46	39	<0.001
16	105.755	37	<0.001
18	146.12	29	<0.001
20	107.196	24	<0.001
22	185.725	23	<0.001

Overall test using data from all the measurement times

Statistic=1478.144, DF=386, Probability <0.001

2.3.3 Differences amongst isolates from different geographical origins within each species and their interaction with temperature

For each fungus species, an analysis was carried out to detect differences in growth between groups of isolates from different countries. *Pandora blunckii* was only represented by isolates from two countries, Mexico and Germany; overall, there were significant differences in the growth profile detected between isolates from the two countries (χ^2_{11}=93.3, P<0.001). However, the significant differences were not consistent at all the measurements points (Table 2.8). The Mexican isolates were generally faster growing than the German isolate. This is exemplified by the larger average colony radius attained by the Mexican isolates compared to the German isolate especially from day 4 to day 14 which corresponded to the linear growth phase (Fig 2.3). The variability found between the two groups of *P. blunckii* isolates showed a significant interaction with temperature (χ^2_{33}=86.5, P<0.001) from day 2 to 10 (Table 2.9). The Mexican isolates showed significantly greater growth than the German isolate at all temperatures (Fig. 2.4), except at 30 °C where the German isolate showed the greatest growth (Figs. 2.5).

Table 2.8. Comparison among *Pandora blunckii* isolates from different countries averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	39.300	1	<0.001
4	19.792	1	<0.001
6	6.517	1	0.011
8	1.120	1	0.290
10	24.262	1	<0.001
12	3.974	1	0.046
14	4.410	1	0.036
16	0.148	1	0.700
18	4.755	1	0.029
20	0.732	1	0.392
22	0.605	1	0.437

Overall test using data from all the measurement times

Statistic=93.398, DF=11, Probability <0.001

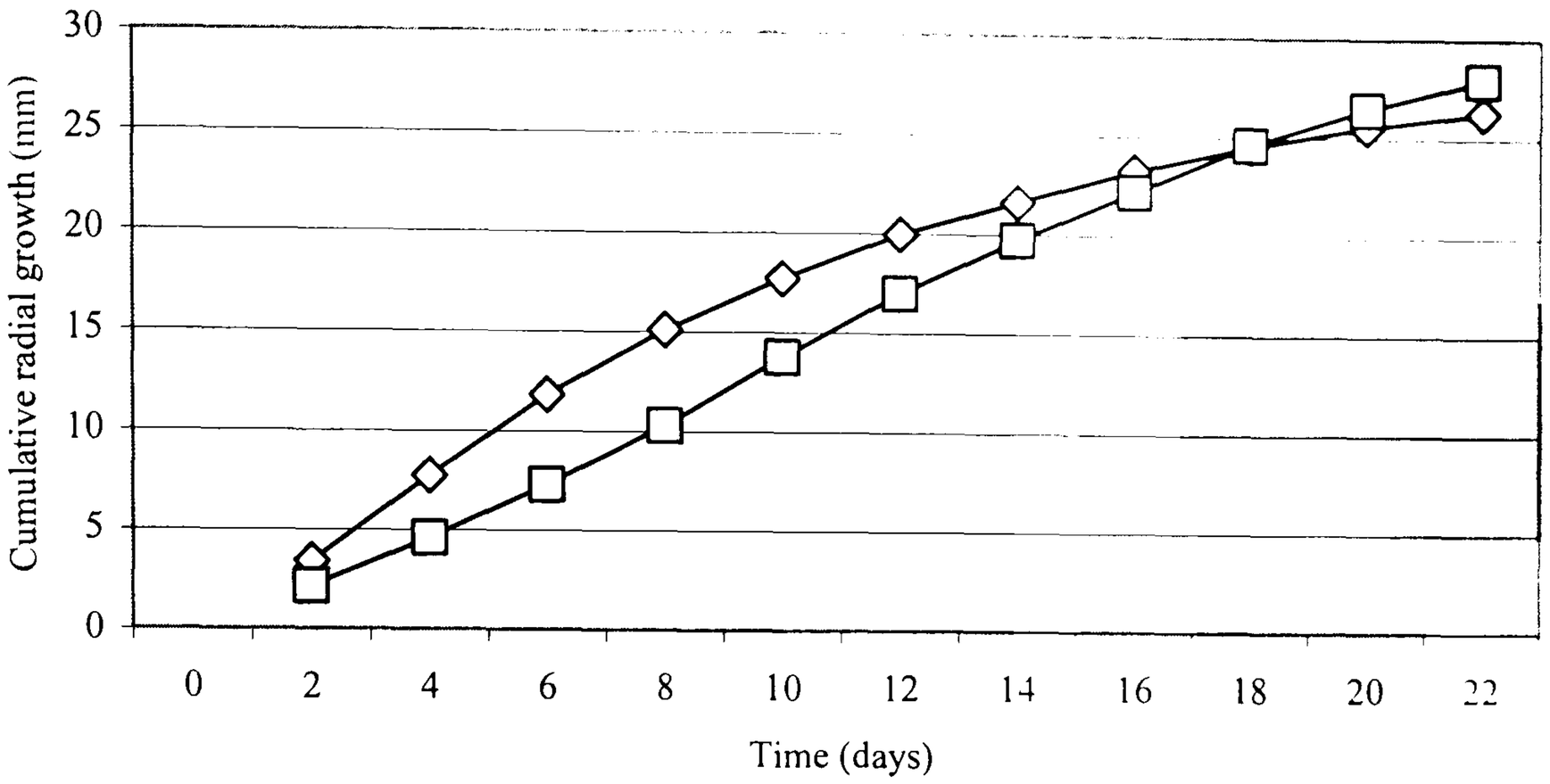


Figure 2.3. Cumulative radial growth of *Pandora blunckii* from Mexico (◇) and Germany (□) expressed in mm. Growth profiles for both countries represent the mean values of all isolates and over all temperatures.

Table 2.9. Comparison among *Pandora blunckii* isolates from different countries in interaction with temperature assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	18.542	3	<0.001
4	22.634	3	<0.001
6	7.792	3	0.051
8	9.490	3	0.023
10	15.545	3	0.001
12	1.991	3	0.574
14	2.539	3	0.468
16	0.605	3	0.895
18	0.599	3	0.897
20	8.764	3	0.033
22	4.330	3	0.228

Overall test using data from all the measurement times

Statistic=86.553, DF=33, Probability <0.001

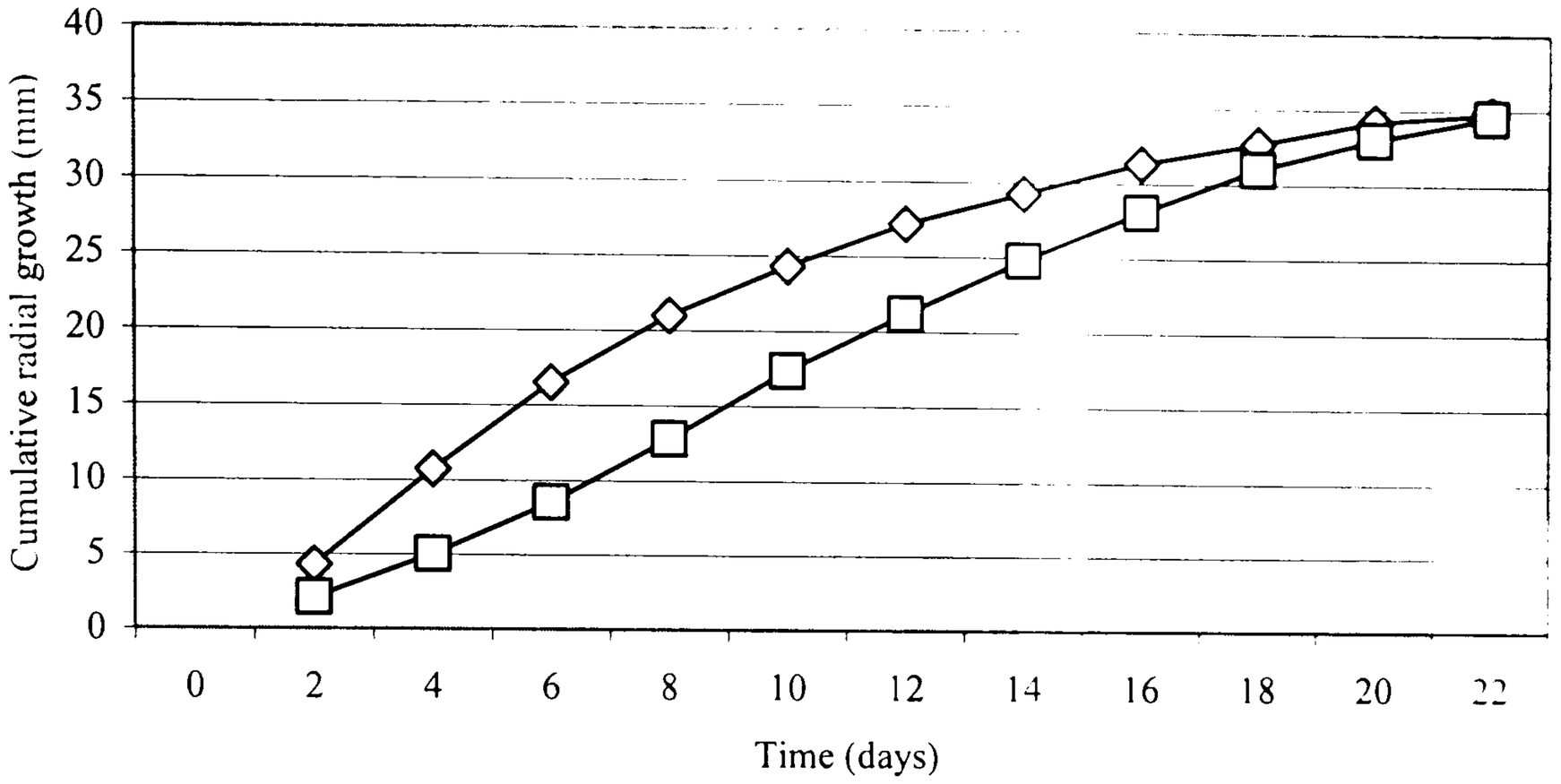


Figure 2.4. Cumulative radial growth of *Pandora blunckii* from Mexico (◇) and Germany (□) expressed in mm at 20 °C. The graph is an example of the greater cumulative radial growth obtained by the Mexican isolates compared to the German isolate.

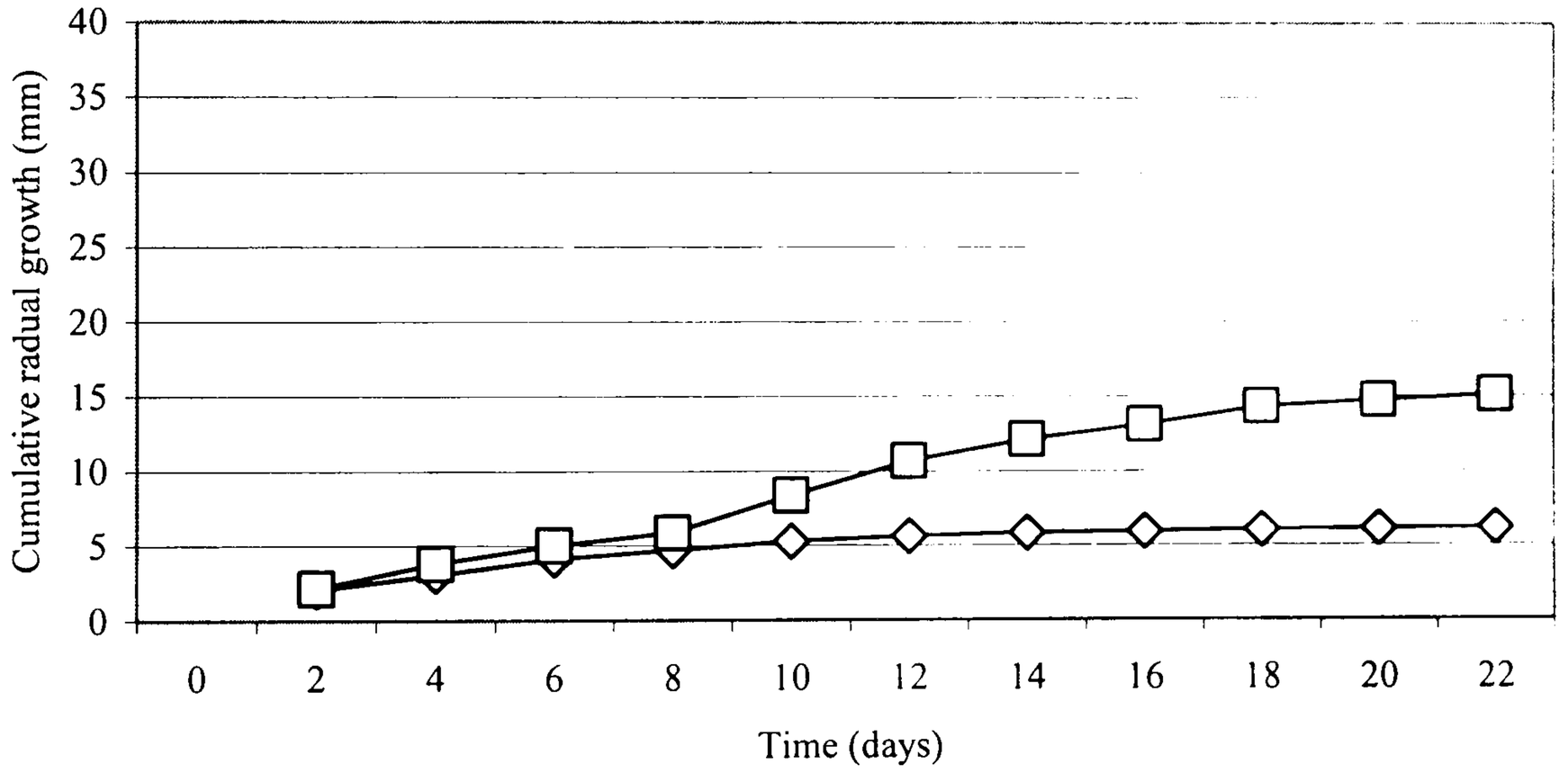


Figure 2.5. Cumulative radial growth of *Pandora blunckii* from Mexico (◇) and Germany (□) expressed in mm at 30 °C. The graph shows the greater cumulative radial growth obtained by the German isolate compared to the Mexican isolates.

The *Zoophthora radicans* isolates came from eight different countries, and significant differences were detected among isolates from different countries (χ^2_{77}=461.5, P<0.001). These differences were detected at all measurement points except day 16 (Table 2.10).

Table 2.10. Comparison among *Zoophthora radicans* isolates from different countries averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	138.073	7	<0.001
4	56.448	7	<0.001
6	40.447	7	<0.001
8	32.561	7	<0.001
10	37.789	7	<0.001
12	22.991	7	0.002
14	17.388	7	0.015
16	13.362	7	0.064
18	25.346	7	<0.001
20	41.652	7	<0.001
22	46.476	7	<0.001

Overall test using data from all the measurement times

Statistic=461.530, DF=77, Probability <0.001

A clear separation among the different countries can be observed from day 4 to day 16; the fastest isolates were from the Philippines, followed by Kenya, Taiwan, Mexico, Australia, New Zealand, Japan and finally Malaysia (Fig. 2.6). The variability found among the 8 groups of *Z. radicans* showed a significant interaction with temperature at all the measurement times except day 16 (χ^2_{211}=720.1, P<0.001) (Table 2.11).

Table 2.11. Comparison among *Zoophthora radicans* isolates from different countries in interaction with temperature assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	68.080	21	<0.001
4	89.655	21	<0.001
6	65.342	21	<0.001
8	59.332	21	<0.001
10	55.619	21	<0.001
12	78.359	21	<0.001
14	74.571	21	<0.001
16	23.622	20	0.259
18	95.767	16	<0.001
20	56.892	14	<0.001
22	57.520	14	<0.001

Overall test using data from all the measurement times

Statistic=720.199, DF=211, Probability <0.001

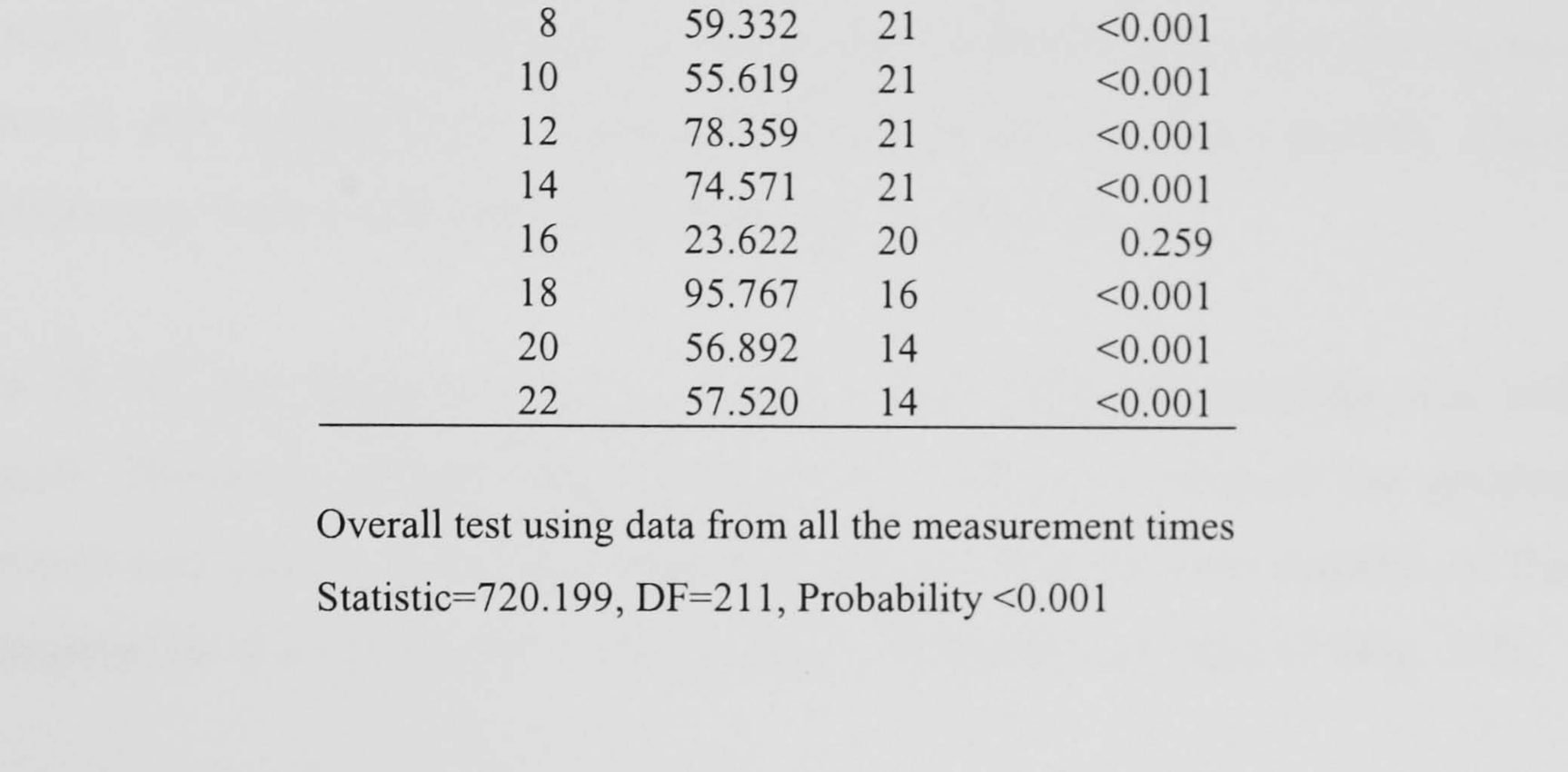


Figure 2.6. Cumulative radial growth of *Zoophthora radicans* from different countries expressed in mm. Growth profiles represents the mean values of all isolates from each country and over all temperatures. Australia (◇), Japan (□), Kenya (△), Malaysia (×), Mexico (∗), New Zealand (○), Philippines (+), Taiwan (_).

At 15 °C, isolates from Philippines showed the greatest growth and the isolate from Japan the least growth. Although significant differences were found among the countries the actual separation between the growth lines was narrow. Apart from isolates from Philippines and Japan, isolates from the other countries seemed to have a similar behaviour (Fig. 2.7).

At 20 °C the separation among isolates from different countries was even smaller. However, isolates from Taiwan and Philippines showed the greatest growth and isolates from Japan and Malaysia had the least growth. These differences were particularly clear until day 14 (Fig. 2.7).

At 25 °C, the separation among isolates from different countries was still small. However, isolates from Kenya and Philippines showed the greatest growth and isolates from Japan and Malaysia showed the least growth. At this temperature, the differences could be observed clearly until day 12 (Fig. 2.7).

There were definite and clear differences among isolates from different countries at 30 °C. Isolates from Kenya and Philippines showed the greatest growth and isolates from Malaysia and New Zealand the least. Isolates from Malaysia did not grow at all at 30 °C (Fig. 2.7).

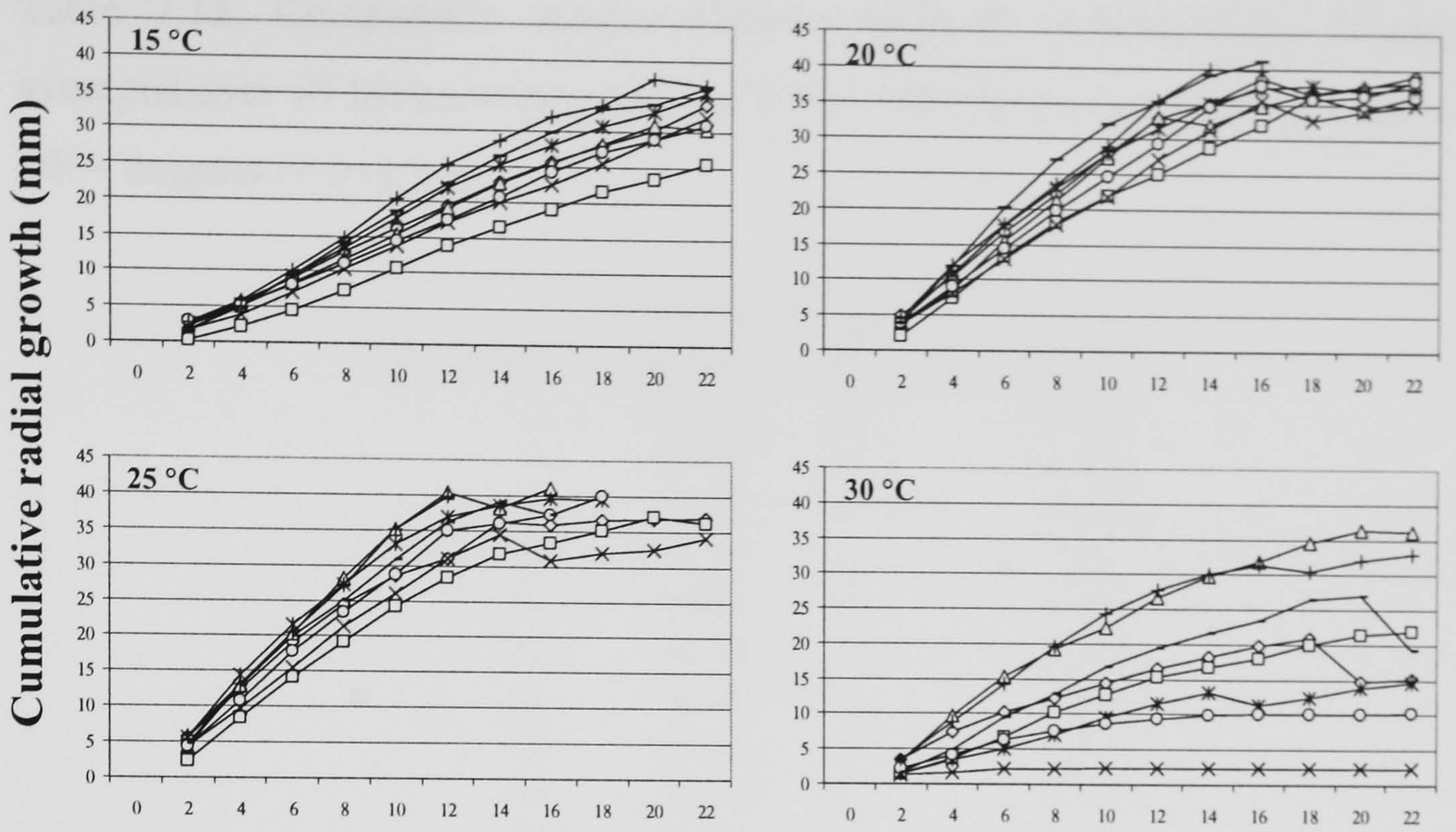


Figure 2.7. Cumulative radial growth of *Zoophthora radicans* from different countries expressed in mm at each temperature. Growth profiles represent the mean values of all isolates from each country. Australia (◇), Japan (□), Kenya (△), Malaysia (×), Mexico (∗), New Zealand (○), Philippines (+), Taiwan (_).

2.3.4 Differences amongst isolates from Mexico within each species and their interaction with temperature

A large number of isolates of both species were available from Mexico and so within species variability from a single geographic region could be compared. Overall, significant differences were detected amongst *P. blunckii* isolates from Mexico (χ^2_{187}=486.3, P<0.001), at all measurement times except day 20 (Table 2.12). Isolate ARSEF6293 showed the greatest growth and ARSEF6306 the least growth *in vitro*. The remaining Mexican isolates were in a compact group and it was difficult to separate them (Fig. 2.8).

Table 2.12. Comparison among *Pandora blunckii* isolates from Mexico averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	102.294	17	<0.001
4	92.565	17	<0.001
6	33.992	17	0.008
8	33.083	17	0.011
10	54.923	17	<0.001
12	33.766	17	0.009
14	36.613	17	0.004
16	28.902	17	0.035
18	28.373	17	0.041
20	22.068	17	0.182
22	41.225	17	<0.001

Overall test using data from all the measurement times

Statistic=486.345, DF=187, Probability <0.001

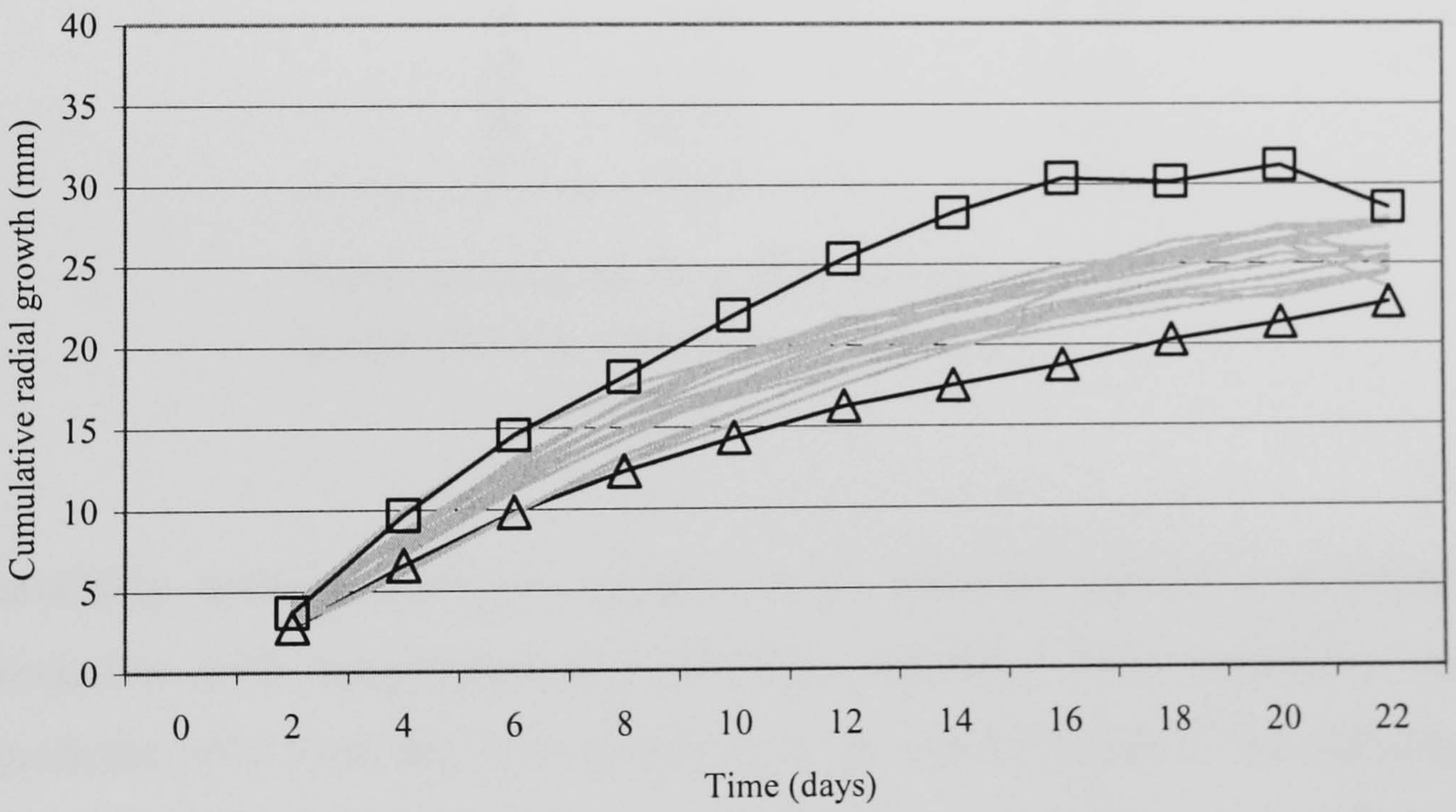


Figure 2.8. Cumulative radial growth of *Pandora blunckii* isolates from Mexico expressed in mm. Growth profiles represents the mean values over all temperatures. Only the cumulative radial growth of isolates ARSEF6293 (□) and ARSEF6306 (△) are indicated in bold.

Overall, there were also significant differences among the Mexican isolates of Z. *radicans* (χ^2_{44}=232.1, P<0.001). However, in this species significant differences were only detected from day 2 to 8 and from day 18 to 22 (Table 2.13). Until day 14, isolate NW386 showed the greatest growth overall, followed by NW382 and then NW353. Isolates with the least growth were NW378 and NW379 (Fig. 2.9).

Table 2.13. Comparison among *Zoophthora radicans* isolates from Mexico averaged over all temperatures, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	25.735	4	<0.001
4	59.727	4	<0.001
6	27.211	4	<0.001
8	10.513	4	0.033
10	5.25	4	0.263
12	4.875	4	0.3
14	7.064	4	0.133
16	7.479	4	0.113
18	24.322	4	<0.001
20	12.339	4	0.015
22	41.71	4	<0.001

Overall test using data from all the measurement times

Statistic=232.170, DF=44, Probability <0.001

Variability amongst Mexican isolates of *P. blunckii* showed a significant interaction with temperature (χ^2_{555}=939.9, P<0.001). This variability was significant only from day 2 to 10 and days 20 and 22 (Table 2.14). Although this demonstrates that the behaviour of each isolates changes according to temperature, because all the isolates were very similar, it is difficult to separate isolates with different behaviour. However, at 30 °C, some isolates could be separated more obviously from the rest; the greatest growth was achieved by isolate ARSEF6293, followed by isolates NW347 and NW346 (Figs. 2.10).

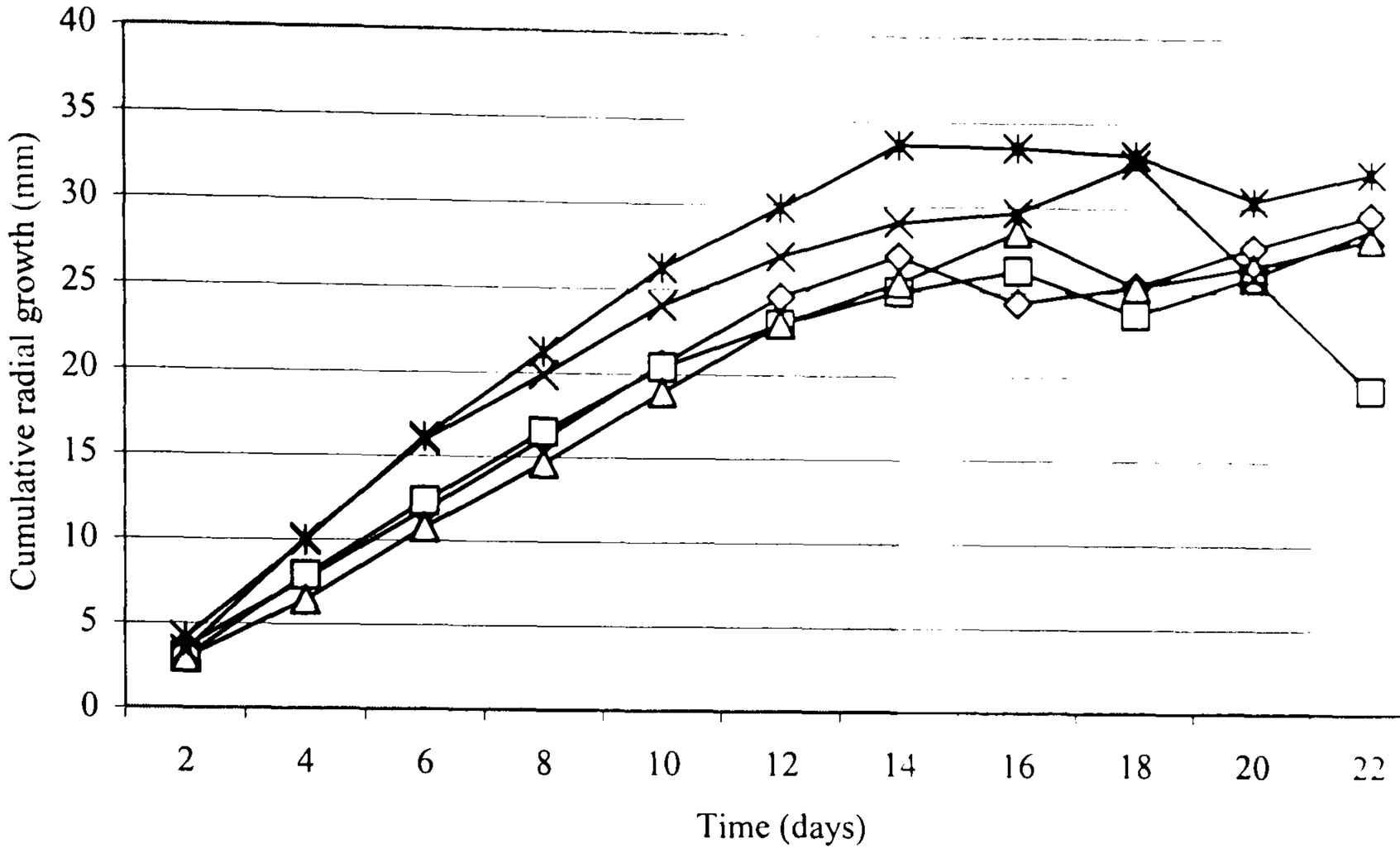


Figure 2.9. Cumulative radial growth of *Zoophthora radicans* isolates from Mexico expressed in mm. Growth profiles represents the mean values over all temperatures. NW353 (◇), NW378 (□), NW379 (△), NW382 (×) and NW386 (∗).

Table 2.14. Comparison among *Pandora blunckii* isolates from Mexico in interaction with temperature, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	156.412	51	<0.001
4	144.846	51	<0.001
6	107.861	51	<0.001
8	91.535	51	<0.001
10	95.627	51	<0.001
12	50.283	51	0.502
14	53.871	51	0.365
16	56.431	51	0.279
18	60.567	50	0.146
20	74.448	50	0.014
22	74.443	47	0.007

Overall test using data from all the measurement times

Statistic=939.956, DF=555, Probability <0.001

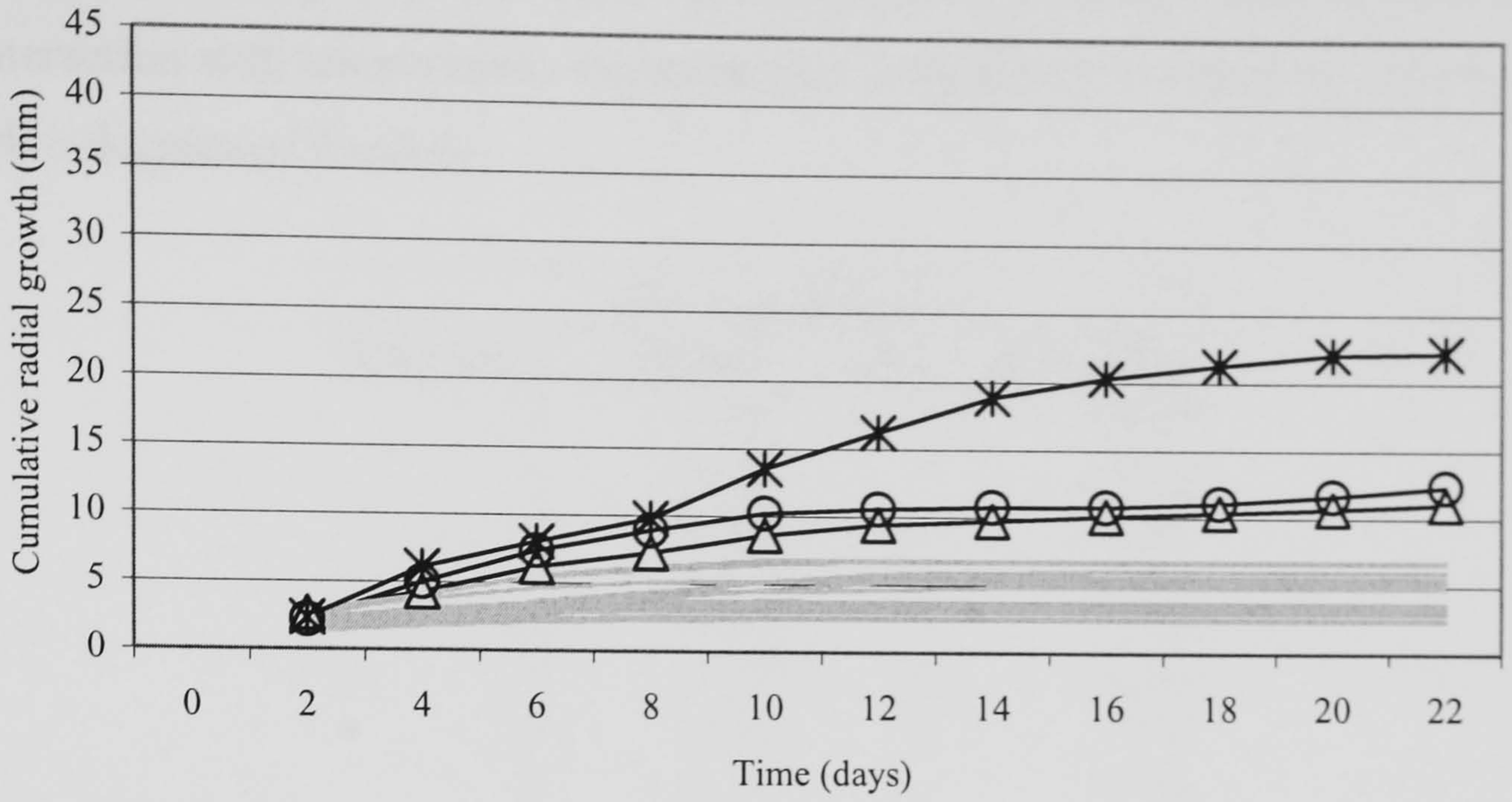


Figure 2.10. Cumulative radial growth of *Pandora blunckii* isolates from Mexico expressed in mm at 30 ºC. Only the cumulative radial growth of isolates ARSEF6293 (∗), NW347 (○) and NW346 (△) are indicated in bold.

A significant interaction with temperature was also found for the Mexican isolates of *Z. radicans* (χ^2_{114}=420.3, P<0.001). The variability in their response to temperature amongst the Mexican Z. *radicans* isolates was greater compared to *P. blunckii* isolates because the differences were detected at all the measurement times except day 14 (Table 2.15). The differences amongst Mexican isolates of *Z. radicans* could be easily observed at 15 and 30 °C (Fig 2.11).

At 15 °C isolate NW382 showed the greatest growth, followed by NW386 then NW378 and, with the least growth, isolates NW353 and NW379 (Fig. 2.11). At 20 and 25 °C it was difficult to make a clear separation among the isolates as their growth behaviour was very similar (Fig. 2.11). At 30 °C isolate NW386 showed the greatest growth, isolates NW353, NW379 and NW382 were very similar with a medium range of growth and finally isolate NW378 with no growth at all (Fig. 2.11).

Table 2.15. Comparison among *Zoophthora radicans* isolates from Mexico in interaction with temperature, assuming ante-dependance structure of order 6. DF = degrees of freedom.

Test at each time			
Time (days)	**Statistic**	**DF**	**Probability**
2	34.145	12	<0.001
4	61.017	12	<0.001
6	65.276	12	<0.001
8	29.641	12	0.003
10	25.686	12	0.012
12	33.882	12	<0.001
14	12.688	12	0.392
16	42.516	11	<0.001
18	57.963	8	<0.001
20	37.884	6	<0.001
22	17.618	5	0.003

Overall test using data from all the measurement times

Statistic=420.383, DF=114, Probability <0.001

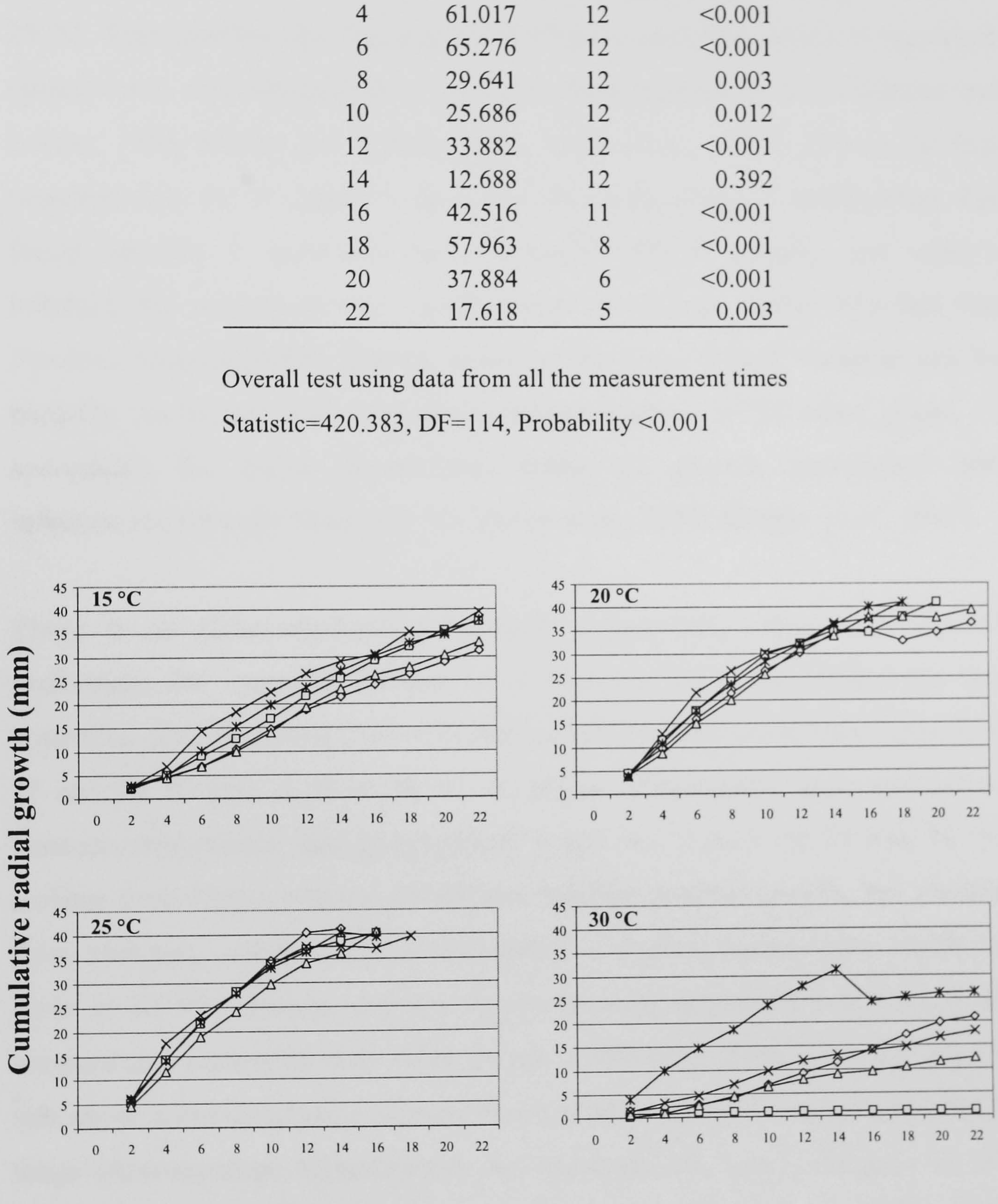


Figure 2.11. Cumulative radial growth of *Zoophthora radicans* isolates from Mexico expressed in mm at each temperature. NW353 (◇), NW378 (□), NW379 (△), NW382 (×) and NW386 (∗).

2.4 DISCUSSION

Overall the results suggest that *Z. radicans* had faster growth than *P. blunckii*. Both species grew best at 20 and 25 °C. *Zoophthora radicans* had greater growth at 25 than at 20 °C which suggests that this species prefers slightly higher temperatures than *P. blunckii,* which had greater growth at 20 °C than at 25 °C. This confirms the findings of previous researchers where temperature optima for *Z. radicans* growth *in vitro* have been reported as 25 °C (Milner and Lutton, 1983; Milner and Mahon, 1985; Glare *et al.*, 1987). This is the first recorded data for *P. blunckii*. In a previous field study, a relationship was found between *P. xylostella* larvae infected with *P. blunckii* and relative humidity but not temperature (Riethmacher and Kranz, 1994). The fact that *Pandora blunckii* prefers slightly lower temperatures than *Z. radicans* can be partially confirmed with studies on another species in the same genus, *P. neoaphidis,* for which temperature optima for growth, germination and infection are between 15 and 20 °C (Stacey *et al.*, 2003; Morgan *et al.*, 1995).

There is no clear relationship between geographical origin and thermal preference. For example, isolates of *Z. radicans* from the Philippines and Australia, countries associated with tropical environments, had better growth at 15 and 20 °C than at 25 or 30 °C. At higher temperatures some correlation between temperature and geographical origin was found. At 25 and 30 °C isolates from Kenya and the Philippines had the greatest growth, but isolates from Malaysia which is also considered as a tropical region were unable to grow at 30 °C. Mexican isolates always showed intermediate growth at all temperatures compared with other isolates. Although, there are some reports that show some correlation between thermal preferences of entomopathogenic fungi (Ascomycetes: Hypocreales) and geographical origin (Fargues *et al.*, 1992; Vidal *et al.*, 1997; Thomas and Jenkins, 1997; Dimbi *et al.*, 2004), there are also reports where there is no correlation between origin and thermal preferences (e.g. Moorhouse *et al.*, 1994, Yeo *et al.*, 2003).

The inconsistency in finding a direct correlation between geographical origin and thermal preference could be because some of these studies were carried out

with a very limited number of isolates and because. as reported previously. even isolates coming from the same geographical area could show a highly variable response to temperature (Bidochka *et al.*. 2001: Milner and Mahon. 1985; Tefera and Pringle, 2003). The potential risk when trying to obtain correlation between geographical origin and thermal response using a few or single isolates, is the great difficulty to knowing *a priori* how representative any particular isolate may be of the natural population, and the lack of knowledge about the presence and sources of variation in natural populations of fungi (Cooke and Rayner, 1984)

Variability was found amongst the Mexican isolates. They came from the same geographical area, the same host and still had different responses to temperature. The result obtained with the Mexican isolates confirms previous work demonstrating variability among isolates from the same geographical area (Bidochka *et al.*, 2001; Milner and Mahon, 1985; Tefera and Pringle. 2003).

One difference amongst some of the isolates evaluated in this experiment was the season of the year in which they were collected which may influence the actual environmental conditions under which infection of the host was occurring. When a good correlation is found in experiments using only a "representative" number of isolates from a certain area, the results could be "by chance". Unless information about the specific climatic conditions at the time when an isolate is collected is available, it is not possible to infer a relationship. As observed by other researchers, the effect of local variability in climatic and microclimatic conditions in any particular region will influence the temperature profile of the isolates coming from that area (Yeo *et al.*, 2003). Therefore. it is probably more important to know the climatic conditions at the actual time when the isolate was collected than to correlate the isolate behaviour with the general climatic conditions of the place. With respect to selecting isolates for microbial control it is important to select isolates in relation to the temperatures under which the host is normally found as this will also contribute to ensuring successful infection by the fungal pathogen (Yeo *et al.*, 2003).

The fungi are the major group of haploid eukaryotes (Deacon, 1997). This includes the Entomophthorales (Humber, 1981). Haploid organisms can express all their genes and, therefore, are exposed to mutations. As a result they can have different types of nuclei in the cytoplasm of the hyphae. The most interesting feature about this is that the ratio of nuclear types can vary according to environmental conditions. This attribute is called heterokaryosis which basically refers to fungi with genetically different nuclear types (the nuclei that suffered a mutation and the non-mutated nuclei (wild type)) in the cytoplasm. It allows the fungi to accumulate mutations, hide them from selection pressure among the wild type nuclei and eventually allow the colony to alter the nuclear ratio in response to the prevailing conditions (Deacon, 1997). This phenomenon is an important attribute which allows fungi to adapt to changes in environmental conditions by changing the nuclear type ratio; it may also be one of the reasons why, from the same geographical area, isolates with different biological attributes can be isolated.

Because both *Z. radicans* and *P. blunckii* are present in the same environment, the interaction between them will definitely take place and so it is important to evaluate the type of potential interactions that could take place between them, and their relationship with temperature as one of the most important abiotic factors affecting these interactions.

The effect of abiotic and biotic factors such as temperature, light, water and the presence of other organisms in the same environment will have a great impact on the population structure of *Z. radicans* and *P. blunckii*. The most important feature of natural populations is genetically based variation within them and the variability shown by the isolates of these species in their response to temperature suggests a large adaptability to the environment as has been recorded for other species (Cooke and Rayner, 1984; Vidal *et al.*, 1997). This same variability will have a strong influence on the outcomes of potential interactions between these two species and even between isolates of the same species. By selecting isolates with different attributes and forcing interaction between them, valuable information can be obtained about the mechanisms that these species use to survive.

CHAPTER 3. *IN VITRO* INTRA AND INTER-SPECIFIC INTERACTIONS BETWEEN *Zoophthora radicans, Pandora blunckii* AND *Pandora* SP. ISOLATES

ABSTRACT

The *in vitro* intra and inter-specific interactions between *Z. radicans*, *P. blunckii* and *Pandora* sp. isolates were investigated at two different temperatures, 20 and 25 °C. The results were obtained by comparing the size of a given isolate when grown in the presence of another isolate either from the same or different species. Overall, *Z. radicans* and *P. blunckii* isolates achieved the least growth in the presence of the *Pandora* sp. The results also showed that *P. blunckii* was more competitive than *Z. radicans*. The outcomes of these interactions can be classified as a result of primary resource capture. Inter and intra-specific mycelial interactions were also investigated. During the inter-specific mycelial interactions, no evidence of antagonism was found and the final outcome of all inter-specific interactions can be classified as "deadlock". Mycelial incompatibility was found amongst isolates within each species (intra-specific interactions). Temperature did not have a large effect on the interactions. Although the outcomes may not represent entirely what was happening within the original host during an *in vivo* inter-specific interaction, the results gave enough information to select isolates for further *in vivo* interaction experiments using the original host, *P. xylostella*.

3.1 INTRODUCTION

3.1.1 Inter-specific interactions

The study of inter-specific competition has long been one of ecology's most interesting subjects (Schoener, 1983). Interaction studies are important to understand the fungal community and also give valuable information on how to exploit specific activities of fungi used in biological control of pests and diseases (Rayner and Webber, 1984). Intraguild predation can result from

microbial interactions if they are using the same resource or food. Overall, the definitions for the major types of interactions are the same as those described for other organisms (e.g. Begon *et al.*, 1996). However, there are a set of definitions more related to fungal interactions and these are described here (e.g. Cooke and Rayner, 1994; Boddy, 2000).

An interaction occurs between two organisms when one of them interferes with the performance of another (Cooke and Rayner, 1984; Rayner and Webber, 1984). The necessity to have a classification for the types of interactions that could occur between organisms is important. There are many such classifications but they are specific to particular groups which can cause confusion or not take into account interactions that are specific to fungi and entomopathogenic fungi particularly. Because there are many classifications it is important to understand how the interactions relate to the life strategies of mycelial fungi (Rayner and Webber, 1984).

One classification proposed by Cooke and Rayner (1984) is based on mycelial interactions and is divided into three main types; Competitive, Neutralistic and Mutualistic interactions. This is based on the interaction being detrimental for one or both of the competitors, having no negative or positive effect, or being beneficial for both organisms respectively.

3.1.1.1 Competition

Competition has been defined as the negative effect that one fungus has on another by consuming or controlling access to a limited resource (Shearer, 1995). Sometimes we consider that one species is more competitive than another when both species have a resource available but one becomes more abundant than the other. However, as Shearer (1995) states, numerical dominance does not necessarily mean greater competitiveness.

Fungal competition can be broadly separated into two types, competition by exploitation and competition by interference (Rayner and Webber, 1984; Schoener, 1983; Boddy, 2000). The first refers to when one competitor uses the

resources and deprives others of them. Interference is when individuals harm competitors by fighting or producing toxins (Schoener, 1983; Boddy, 2000). These interactions have parallels with interactions between other organisms (Chapter 1). However, this separation cannot be applied to all fungal interactions in the way it has been applied to fungi that decompose leaves and woody litter or fungi which colonize solid organic resources. Competition for food is very closely related to competition for space in these situations (Boddy, 2000).

Fungal competition for organic resources can also be divided into primary resource capture and combat. Primary resource capture is dependent only on the time of arrival and use of the available resources without any direct confrontation with other fungi. Success in primary resource capture is based on factors such as good dispersal, rapid spore germination, rapid mycelial extension, the ability to use the resources by production of suitable enzymes and tolerance to adverse conditions (Boddy, 2000; Rayner and Webber, 1984; Marin *et al.*, 1998a). Success in combat depends mainly on antagonistic mechanisms (Boddy, 2000). Included in combat are two types of interaction: defence and secondary resource capture. In the former the access to the primary resources gained is denied, and in the second access to a resource already gained is made available.

3.1.1.1.1 Mechanisms of combative interactions

Antagonism: This interaction is at distance and is mediated by diffusible or volatile compounds. Sometimes one mycelium is degenerated and replaced by the other, and it is common in agar cultures to have mutual inhibition (Boddy, 2000) Mutual inhibition could possibly involve the reciprocal exchange of chemical signals (Rayner and Webber, 1984).

Hyphal contact: This interaction occurs when one hypha makes contact with another hypha or spore. The result of this contact can be death of the contacted area (Boddy, 2000), which is followed by changes in membrane permeability, increased vacuolation and eventually lysis (Rayner and Webber, 1984).

Mycoparasitism: This includes interactions in which one hypha makes contact

with another, there is recognition and a direct attack takes place which can be by penetration or appresorium production and continued growth over the hyphal surface of the host (Chet, *et al.*, 1997; Boddy, 2000).

In ecological and evolutionary terms, competition is a determinant of the niche and not the contrary. When two different fungal species share a niche it is likely that competition will take place (Cooke and Rayner, 1984). When competition takes place, one competing species may exclude the other from particular habitats, this is called competitive exclusion. Alternatively, they may co-exist sharing the resources (Begon *et al.*, 1996). Under this situation, it is important to define two different concepts: the niche of a species in the absence of competitors from other species is called the fundamental niche which is defined by the combination of optimal conditions and resources to maintain a viable population. In the presence of competitors, one species may be restricted to a realized niche, which is determined by which competing species are present. When there is coexistence of competitors, a differentiation of realized niches can be the result. Under these conditions competition is almost undetectable (Begon *et al.*, 1996).

3.1.1.2 Neutralism and Mutualisms

There are basically two types of fungal neutralisms. In the first one there is no benefit or harm for the species involved. In the second type one of them benefits from the activities of the other without conferring any benefit or harm in return (Rayner and Webber, 1984). The first type of neutralism is passive, although it is rare among fungi (Cooke and Rayner, 1984).

Mutualistic associations are those in which each species involved benefits from the activities of the other. Due to the great quantity of fungal species in the environment, it is possible that this interaction, as well as the second type of neutralism, may occur by chance. However, there is also the possibility that this interaction can be used as a means of survival by one or both species involved (Cooke and Rayner, 1984).

3.1.2 Effect of environmental factors on the outcome of interactions

Environmental factors play an important role in the outcome of some interactions, e.g. for some fungi that colonize maize grains, water activity and temperature are key factors (Lee and Magan, 1999a, b; Boddy, 2000; Kok and Papert, 2002).

The interaction between the competitiveness of some fungi and environmental factors are complex. For example, *Aspergillus ochraceus* (Wilhelm) produces ochratoxin as a defensive response. However, on some occasions when growing in the presence of other fungi its growth rate was reduced but it did not form ochratoxin as a defensive response. Ochratoxin was produced only under particular temperature and water activity conditions and only in the presence of specific fungal species (Lee and Magan, 1999a). This demonstrates that the defence mechanisms for this fungus are present but are only triggered under specific abiotic conditions or species presence.

Different approaches have been used to assess competitive interactions between fungi; including the development of an Index of dominance values, comparison of growth rates (e.g. Marin *et al.*, 1998b; Lee and Magan, 1999a; Jacobs *et al.*, 2003), hyphal interactions (e.g. Berry *et al.*, 1993; Griffith *et al.*, 1994 a, b and c; Barlocher, 1991; Kuter, 1984) and development of a Niche overlap index using *in vitro* carbon source utilization experiments (Marin *et al.*, 1998b).

3.1.3 Interactions between entomopathogenic fungi

The majority of the research on interactions between fungi has focussed on phytopathogenic fungi. There are few studies on the interactions between species of entomopathogenic fungi (e.g. Thomas *et al.*, 2003; Inglis *et al.*, 1997, Fargues and Bon, 2004, Rostás and Hilker, 2003). *Zoophthora radicans* and *P. blunckii* can both infect *P. xylostella* larvae and both pathogens have been recorded infecting the same population of *P. xylostella* (Riethmacher and Kranz, 1994; Velasco *et al.*, 2000). When these pathogens share a resource, in

this case the host *P. xylostella*, it is very likely that both species will compete on some occasions, or have some mechanisms to co-exist in the same environment (crucifers). To understand how these two species are able to co-exist in the crucifer-*P. xylostella* ecosystem an understanding of the interactions between these fungi is required

The possibility of intra-specific interactions among isolates of each species is also very likely to occur. If competition is the outcome of intra-specific interactions, this competition may be stronger than inter-specific interactions because genetically different isolates of the same species are competing for exactly the same resource (Wicklow *et al*, 2003)

In the experiment described in this chapter, the possible interactions that may occur between *Z. radicans* and *P. blunckii* isolates *in vitro* were determined at two temperatures. These isolates were selected according to their growth profiles averaged over the four temperatures evaluated (2.3.5.). Isolates with greater, intermediate and smaller growth were selected. Geographic origin was also considered when selecting isolates, making sure that Mexican isolates from the same region were selected, because coming from the same region it was likely that these were interacting in the field.

3.2 MATERIAL AND METHODS

3.2.1 Fungal isolates

The experiment was done using six *P. blunckii* isolates, nine *Z. radicans* isolates and one *Pandora* sp. isolate, selected according to their temperature attributes (Chapter 2) (Table 3.1). All the isolates were retrieved from storage (2.4.1.1) and grown on SEMA (2.4.1.2). They were never sub-cultured more than three times before use.

Table 3.1. Isolates used for the *in vitro* interaction experiment.

Species	Isolate	Country
Zoophthora radicans	NW168	Japan
	NW250	Malaysia
	NW325	Kenya
	NW328	New Zealand
	NW353	Mexico
	NW364	Australia
	NW382	Mexico
	NW386	Mexico
	NW410	Australia
Pandora blunckii	NW381	Mexico
	ARSEF217	Germany
	ARSEF6306	Mexico
	ARSEF6310	Mexico
	ARSEF6311	Mexico
	ARSEF6293	Mexico
Pandora sp.	ARSEF3201	Philippines

3.2.2 Experimental design

For this experiment, 136 combinations of isolates were compared at each of two temperatures, 20 and 25 °C, and each treatment was set up in the same way. A 5 mm colony plug was taken from the growing edge of each of two different isolates (14 day old colonies) and placed 2.5 cm apart from each other in the centre of a Petri dish containing 24 ml of SEMA (Fig. 3.1). Control treatments contained two plugs from the same isolate. The radial growth of all plugs was measured every two days for 14 days. The treatments were allocated in four shelves in each incubator in a completely randomised design. Three replicates of each combination were made on each of three occasions and the temperature for each incubator was changed at least once.

The radial growth for each colony was recorded on two axes with four measurement lines (Fig. 3.1). Seven measurements were carried out during the experiment. However, only two measurement dates were used for the analysis. 96 and 192 hours after inoculation. These times were selected as at 96 hours there were still four measurement lines for each original plug and the colonies had not yet contacted each other, and 192 hours because after this time all the colonies had contacted each other and had only three measurement lines. At the remaining times, there was a lot of variability in the number of measurement lines. When making comparisons between the sizes of colonies at these time points, colony size represented the average of the four or three radii at 96 and 192 hours after inoculation respectively.

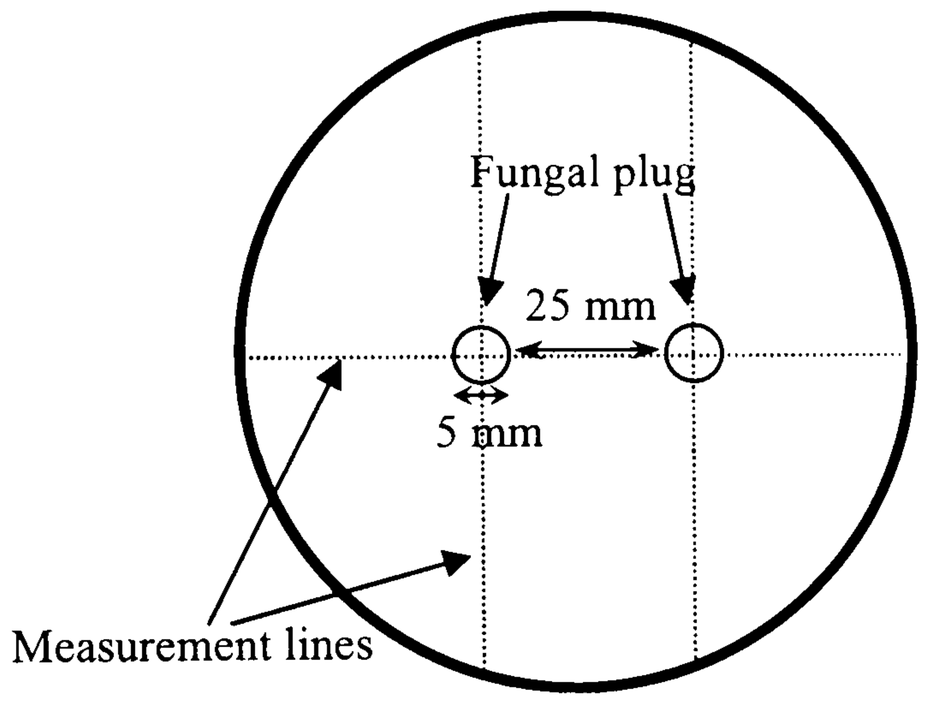


Figure 3.1. Position of the fungal plugs in the Petri dish (9 cm diameter) for the *in vitro* interaction experiment.

3.2.3 Statistical analysis

The data from this experiment were analysed using ANOVA in GenStat v. 6.0. A separate analysis was done for each isolate. Each dataset, therefore, consisted of measurements from one colony per Petri dish representing colony growth of that isolate in the presence of the other isolates. To analyse the variation due to isolate differences, this was partitioned into three sets of orthogonal contrasts (A, B and C) (Fig.3.2).

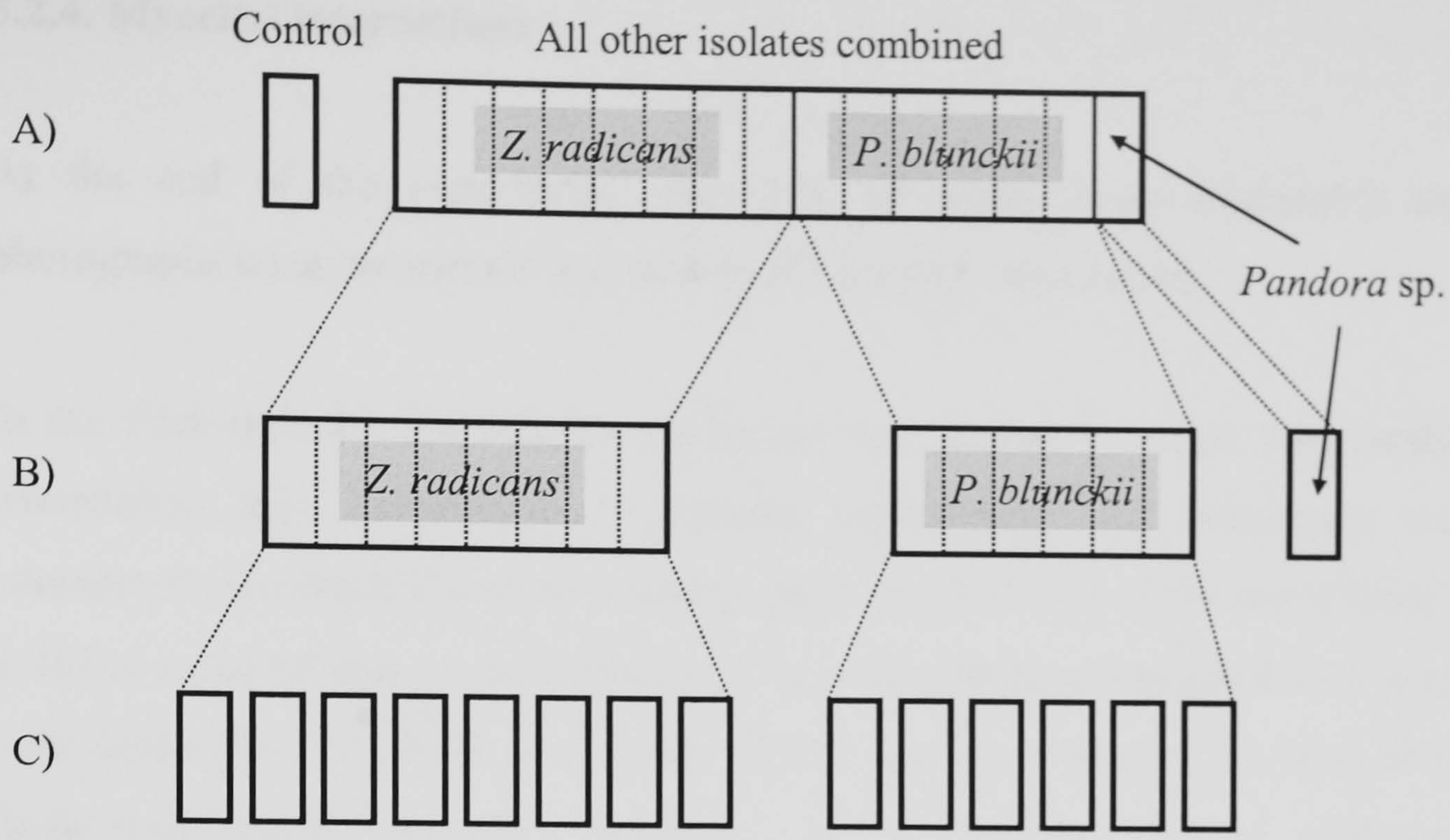


Figure 3.2. Schematic representation of the contrasts used in the statistic analysis. In each case the means being compared are illustrated by the bold line square, showing how data of individual treatments were combined in the analysis. A similar structure was used for all other isolates. A) Comparison between the mean colony size of a given isolate (control) and the mean colony size of that isolate grown in the presence of all other isolates combined (1 degree of freedom (d.f.)), B) Comparison amongst mean colony size of a given isolate grown in the presence of isolates from each species combined (2 d.f.) and C) Comparison of mean colony sizes of a given isolate grown in the presence of each isolate within each species (7 d.f. for *Z. radicans* and 5 d.f. for *P. blunckii*).

In total, the analysis of the data arising from this experiment will therefore involve 288 statistical tests, each at the 5% level. We would therefore expect 14.4 (0.05*288) of these tests to be significant even if the null hypothesis of no differences is actually true (false positive results or type I errors). In order to compare the observed number of significant tests, denoted K, with this expected number, a False Discovery Rate (FDR) was computed as:

FDR = (100*14.4)/K.

3.2.4. Mycelial interactions

At the end of the experiment (day 14), all plates were examined and photographs taken to assess the outcome of mycelial interactions.

In the intra-specific interactions, outcomes were scored as either somatically compatible, non compatible or partially compatible. An interaction was considered as compatible when paired isolates touched each other and formed a uniform mycelial mat or incompatible when they became separated by a zone with distinctive mycelium (Barlocher, 1991). Partial compatibility referred to those combinations that were inconsistent in the outcomes amongst replicates of the same combination. Based on the results, tables were created to summarise all the possible outcomes of interactions at both temperatures. All the inter-specific interactions were described as deadlock, replacement or partial replacement (Boddy, 2000). The presence of some morphological changes such as stationary barrages and the production of intracellular pigment were also recorded (Boddy, 2000). The outcomes were also compared at 20 and 25 °C.

3.3 RESULTS

The main treatment effect and the interaction between treatment and temperature are described here because these are biologically more relevant to the interactions between isolates. The main effect of temperature alone was not considered in detail here.

3.3.1 Interactions 96 hours post inoculation (p.i.)

3.3.1.1 Comparison between mean control colony size and mean colony size of a given isolate grown in the presence of all other isolates combined

The *Z. radicans* isolates NW325, NW382 and NW168 showed a significant difference in the mean colony size between the control colony and its growth against all other isolates combined (Table 3.2).

Table 3.2. Comparison of the mean control colony size of a given isolate and mean colony size of that isolate grown in the presence of all other isolates combined 96 hours p.i., and its interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Zoophthora radicans					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
NW325	All other isolates (AOI)	1	58	4.03	0.049
	Temperature	1	2	4.80	0.160
	AOI.Temperature	1	58	2.35	0.131
NW386	All other isolates (AOI)	1	57	0.93	0.338
	Temperature	1	2	5.15	0.151
	AOI.Temperature	1	57	0.15	0.701
NW353	All other isolates (AOI)	1	58	0.22	0.639
	Temperature	1	2	2.46	0.258
	AOI.Temperature	1	58	0.17	0.682
NW328	All other isolates (AOI)	1	58	0.21	0.647
	Temperature	1	2	9.17	0.094
	AOI.Temperature	1	58	1.82	0.182
NW364	All other isolates (AOI)	1	57	0.17	0.681
	Temperature	1	2	28.15	0.034
	AOI.Temperature	1	57	1.94	0.169
NW410	All other isolates (AOI)	1	56	1.41	0.240
	Temperature	1	2	7.33	0.114
	AOI.Temperature	1	56	0.02	0.901
NW382	All other isolates (AOI)	1	57	5.38	0.024
	Temperature	1	2	114.22	0.009
	AOI.Temperature	1	57	4.87	0.031
NW250	All other isolates (AOI)	1	58	3.29	0.079
	Temperature	1	2	1.11	0.403
	AOI.Temperature	1	58	0.16	0.694
NW168	All other isolates (AOI)	1	58	0.47	0.497
	Temperature	1	2	3.62	0.197
	AOI.Temperature	1	58	5.41	0.023

Table 3.2. Continued.

Pandora blunckii					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
ARSEF6293	All other isolates (AOI)	1	57	0.45	0.507
	Temperature	1	2	40.35	0.024
	AOI.Temperature	1	57	1.66	0.203
ARSEF6310	All other isolates (AOI)	1	58	0.58	0.451
	Temperature	1	2	4.87	0.158
	AOI.Temperature	1	58	0.15	0.703
NW381	All other isolates (AOI)	1	58	0.04	0.843
	Temperature	1	2	35.70	0.027
	AOI.Temperature	1	58	0.02	0.887
ARSEF6311	All other isolates (AOI)	1	30	1.97	0.171
	Temperature	1	2	26.32	0.123
	AOI.Temperature	1	30	3.73	0.063
ARSEF217	All other isolates (AOI)	1	57	0.06	0.812
	Temperature	1	2	1.92	0.300
	AOI.Temperature	1	57	0.17	0.679
ARSEF6306	All other isolates (AOI)	1	57	0.50	0.484
	Temperature	1	2	0.06	0.828
	AOI.Temperature	1	57	0.05	0.825
***Pandora* sp.**					
	Treatment	**DF**	**RDF**	**VR**	**P**
ARSEF3201	All other isolates (AOI)	1	58	2.12	0.151
	Temperature	1	2	0.69	0.495
	AOI.Temperature	1	58	0.00	0.971

The growth of isolate NW325 was stimulated when grown in presence of all other isolates and this was consistent at both temperatures (Fig. 3.3a). The growth of isolate NW382 was inhibited by all other isolates at 20 °C but not at 25 °C (Fig. 3.3b). The growth of isolate NW168 was inhibited at 20 °C but stimulated at 25 °C (Fig. 3.3c). No effect on any *P. blunckii* or *Pandora* sp. isolates was found.

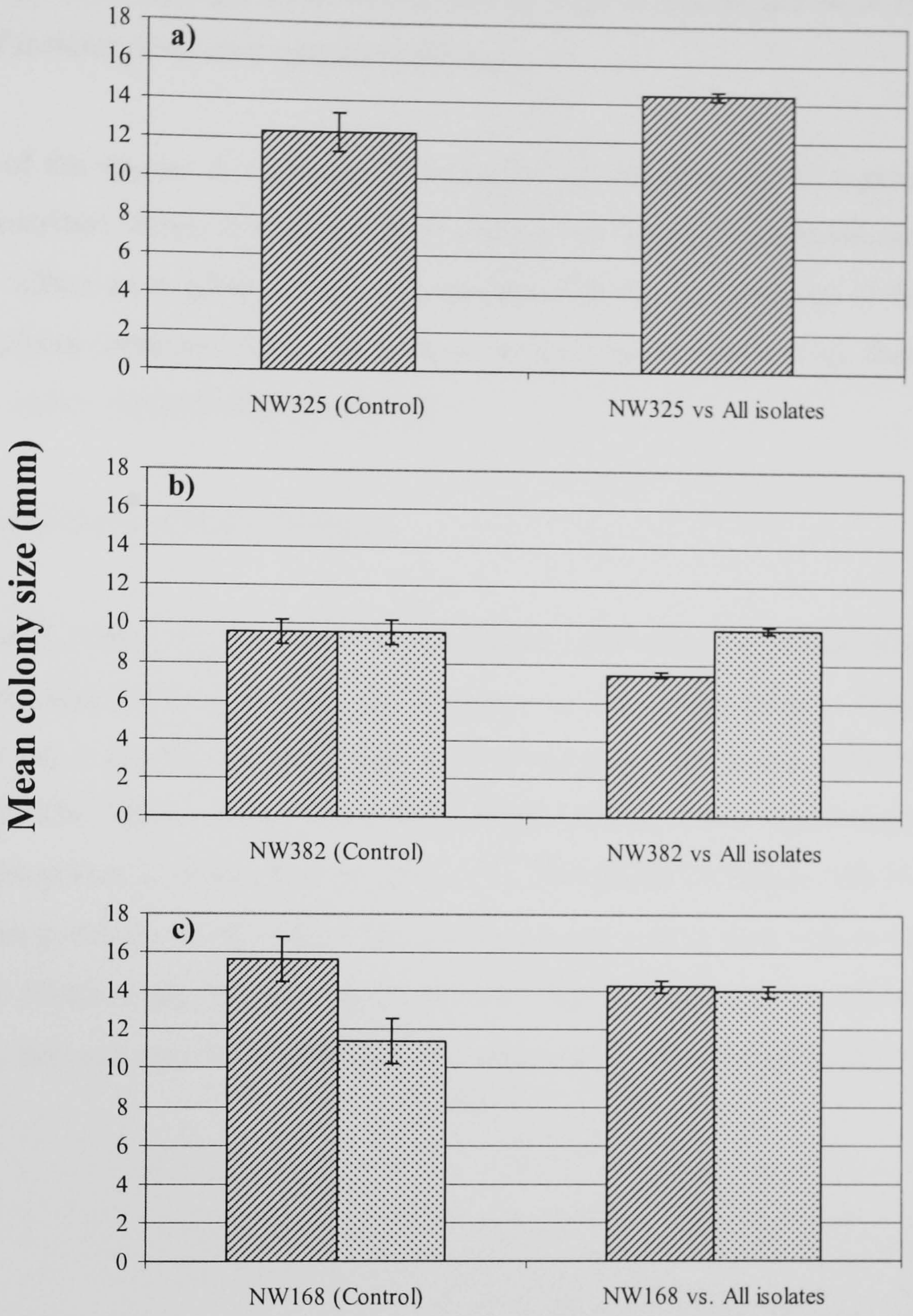


Figure 3.3. Mean colony sizes (mm) 96 hours p.i. for three *Zoophthora radicans* isolates growing in presence of itself (control colony) and in the presence of all other isolates combined. a) Mean colony sizes of isolate NW325 averaged over the two temperature conditions. Mean colony sizes of isolates b) NW382 and c) NW168, at 20 °C ▨ and 25 °C ▩. Error bars represent one standard error of the mean (SEM).

3.3.1.2 Comparison amongst mean colony size of a given isolate grown in the presence of isolates from each species combined

The effect of the species *Z. radicans, P. blunckii* and *Pandora* sp. on a given isolate is described. When *Z. radicans* or *P. blunckii* as a species are mentioned causing an effect on a given isolate, the species effect is the average of the different isolates combined from that species. In the case of *Pandora* sp. there is only one isolate representing this species.

Effect on Zoophthora radicans *isolates*

Isolates NW410 and NW382 showed significant differences between their mean colony sizes when grown in the presence of the three species. These differences had a significant interaction with temperature for isolate NW382 (Table 3.3). The mean colony sizes from both isolates were significantly smaller in the presence of *Pandora* sp. (Fig. 3.4). This result for isolate NW382 was consistent only at 20 °C, where the smallest mean colony size was in the presence of *Pandora* sp. followed by *P. blunckii* and the largest mean colony size was in the presence of *Z. radicans*. No differences were found at 25 °C (Fig. 3.4b).

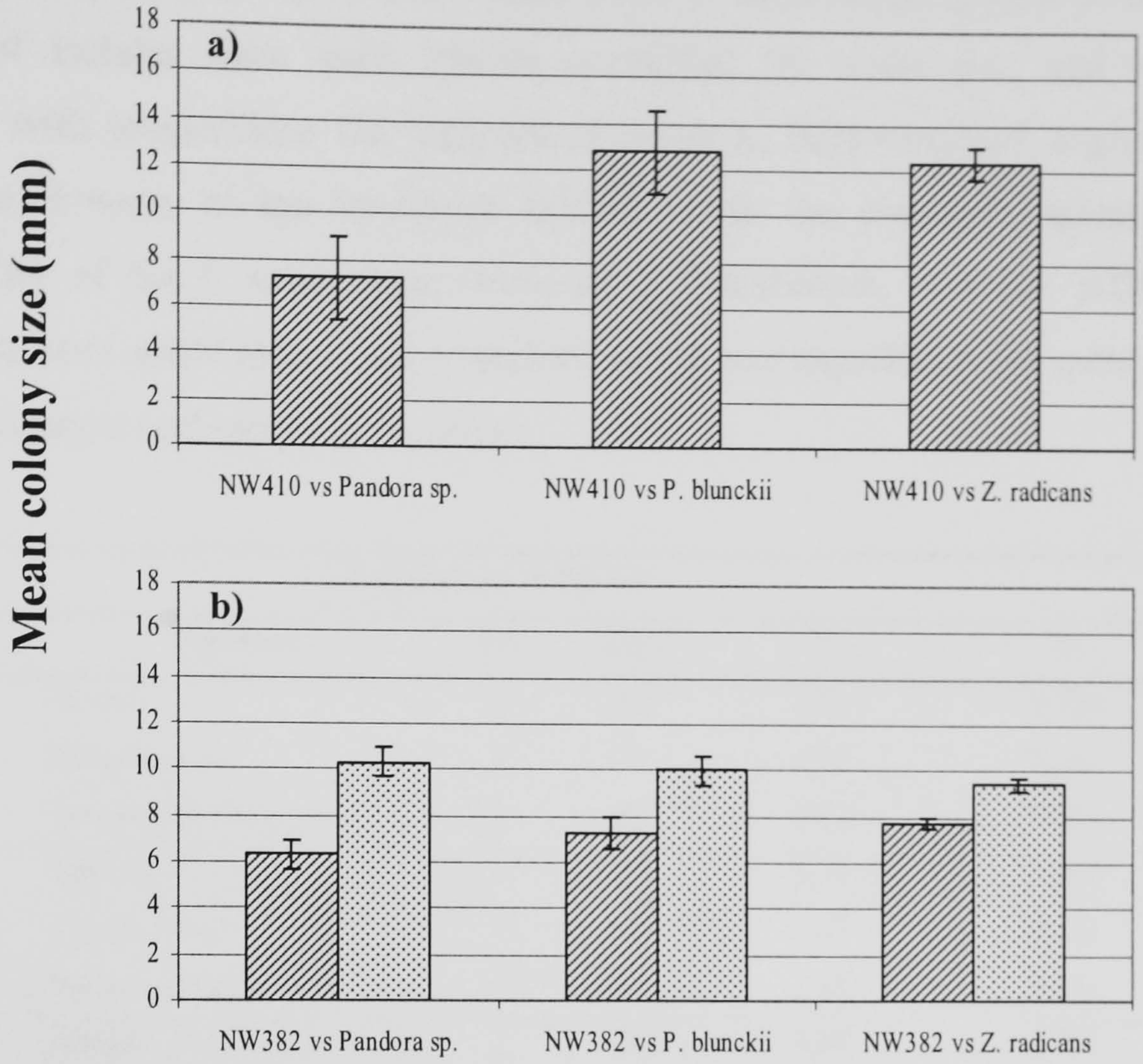


Figure 3.4. Mean colony size (mm) 96 hours p.i. for two *Zoophthora radicans* isolates growing in presence of the three species. a) Mean colony sizes of isolate NW410 averaged over the two temperature conditions. b) Mean colony sizes of isolate NW382 at 20 °C ▨ and 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Table 3.3. Comparison of the mean colony sizes of each isolate grown in the presence of isolates from each species combined 96 hours p.i., and its interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Zoophthora radicans					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
NW325	Species	2	58	1.15	0.324
	Temperature	1	2	4.80	0.160
	Species.Temperature	2	58	0.51	0.606
NW386	Species	2	57	0.22	0.807
	Temperature	1	2	5.15	0.151
	Species.Temperature	2	57	3.13	0.051
NW353	Species	2	58	0.26	0.773
	Temperature	1	2	2.46	0.258
	Species.Temperature	2	58	1.32	0.276
NW328	Species	2	58	0.75	0.475
	Temperature	1	2	9.17	0.094
	Species.Temperature	2	58	1.26	0.292
NW364	Species	2	57	1.01	0.370
	Temperature	1	2	28.15	0.034
	Species.Temperature	2	57	0.77	0.470
NW410	Species	2	56	4.21	0.020
	Temperature	1	2	7.33	0.114
	Species.Temperature	2	56	1.25	0.294
NW382	Species	2	57	0.30	0.741
	Temperature	1	2	114.22	0.009
	Species.Temperature	2	57	4.04	0.023
NW250	Species	2	58	2.64	0.080
	Temperature	1	2	1.11	0.403
	Species.Temperature	2	58	0.02	0.983
NW168	Species	2	58	1.32	0.274
	Temperature	1	2	3.62	0.197
	Species.Temperature	2	58	1.80	0.175

Table 3.3. Continued.

Pandora blunckii					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
ARSEF6293	Species	2	57	0.64	0.530
	Temperature	1	2	40.35	0.024
	Species.Temperature	2	57	0.23	0.796
ARSEF6310	Species	2	58	1.24	0.296
	Temperature	1	2	4.87	0.158
	Species.Temperature	2	58	0.63	0.537
NW381	Species	2	58	1.47	0.239
	Temperature	1	2	35.70	0.027
	Species.Temperature	2	58	0.31	0.736
ARSEF6311	Species	2	30	12.73	<0.001
	Temperature	1	2	26.32	0.123
	Species.Temperature	2	30	13.62	<0.001
ARSEF217	Species	2	57	1.18	0.314
	Temperature	1	2	1.92	0.300
	Species.Temperature	2	57	1.31	0.277
ARSEF6306	Species	2	57	4.53	0.015
	Temperature	1	2	0.06	0.828
	Species.Temperature	2	57	4.15	0.021
***Pandora* sp.**					
	Treatment	**DF**	**RDF**	**F**	**P**
ARSEF3201	Species	1	58	1.85	0.180
	Temperature	1	2	0.69	0.495
	Species.Temperature	1	58	0.50	0.483

Effect on Pandora blunckii *isolates*

Significant differences were found in the mean colony size of *P. blunckii* isolates ARSEF6311 and ARSEF6306 when grown in the presence of the three species (Table 3.3). The results for both isolates also showed a significant interaction with temperature (Table 3.3). Isolate ARSEF6311 achieved the smallest colony size in the presence of *Pandora* sp. followed by *P. blunckii* and the largest colony size in the presence of *Z. radicans*. However, these results were different at each temperature. At 20 °C this isolate had smaller mean colony size in the presence of *P. blunckii* and *Pandora* sp. compared to *Z.*

radicans, but at 25 °C the smallest colony size was in presence of *Pandora* sp. compared to the other species (Fig. 3.5a). Isolate ARSEF6306 achieved the smallest colony size in the presence of *Pandora* sp. which was consistent at both temperatures. However, the largest colony size was achieved in the presence of *P. blunckii* at 20 °C and in the presence of *Z. radicans* at 25 °C (Fig. 3.5b).

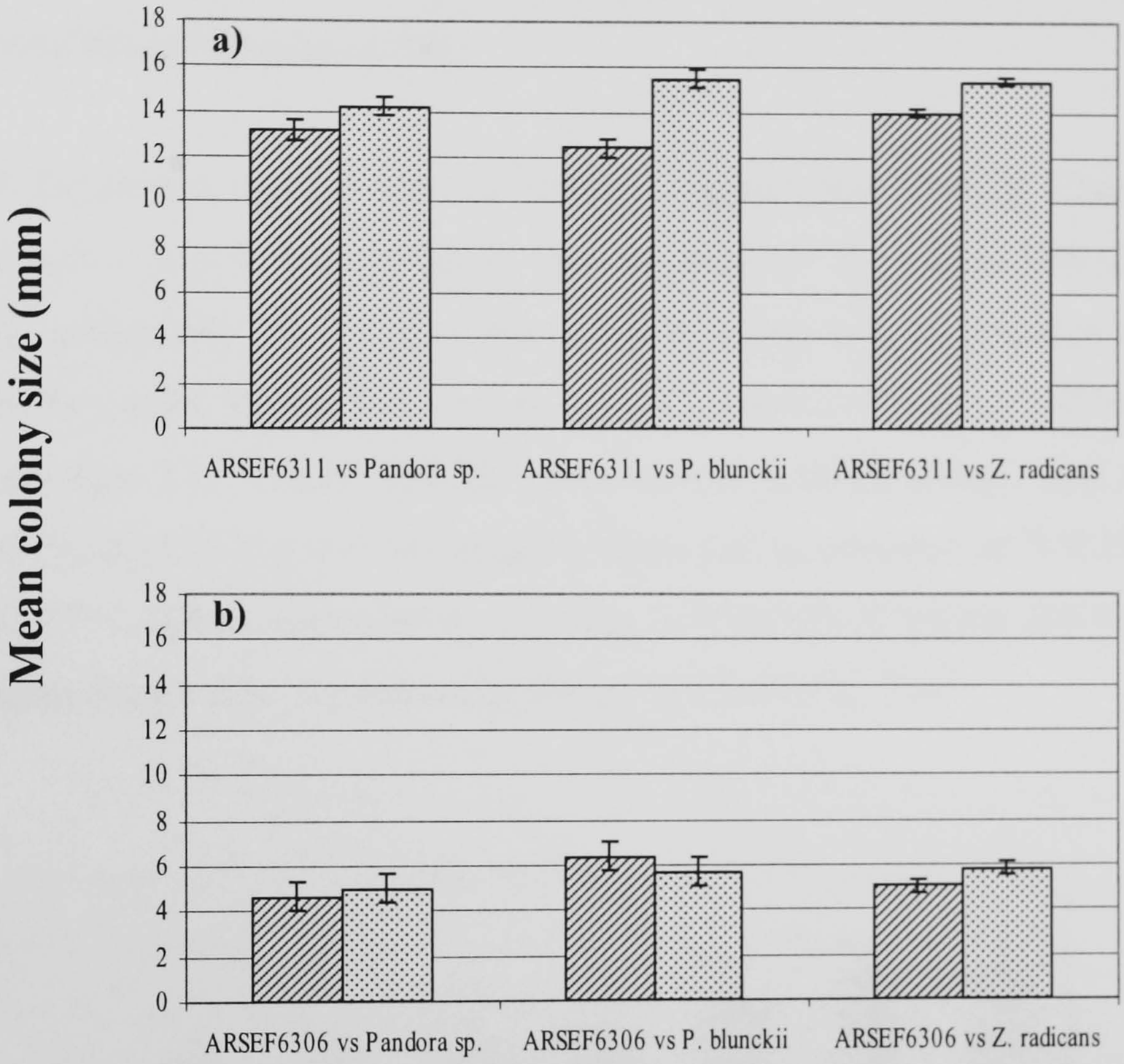


Figure 3.5. Mean colony sizes (mm) 96 hours p.i. for two *Pandora blunckii* isolates growing in the presence of the three species at the two temperature conditions. a) ARSEF6311, b) ARSEF6306. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Effect on Pandora *sp. isolate*

No differences were found in the mean colony size of the the *Pandora* sp. isolate when growth in the presence of *Z. radicans* or *P. blunckii* (Table 3.3)

3.3.1.3 Comparison of mean colony sizes of a given isolate grown in the presence of each isolate within each species

Only the interactions between *P. blunckii* and *Z. radicans* isolates were compared because there was only one isolate of the *Pandora* sp. and its effect on the other isolates was already evaluated in the previous comparisons.

Effect on Zoophthora radicans *isolates*

Significant differences were found for isolates NW386 and NW328. These differences had a significant interaction with temperature for isolate NW328 (Table 3.4). Isolate NW386 obtained the smallest colony size in presence of isolate NW382 and the largest mean colony size in presence of isolates NW364 and NW168 (Fig. 3.6). Isolate NW320 obtained the smallest colony size in presence of isolate NW386 and the largest colony size in presence of NW325 at 20 C. At 25 C, the smallest colony size was in presence of isolate NW325 and the largest colony size in presence of isolate NW168 (Fig. 3.7).

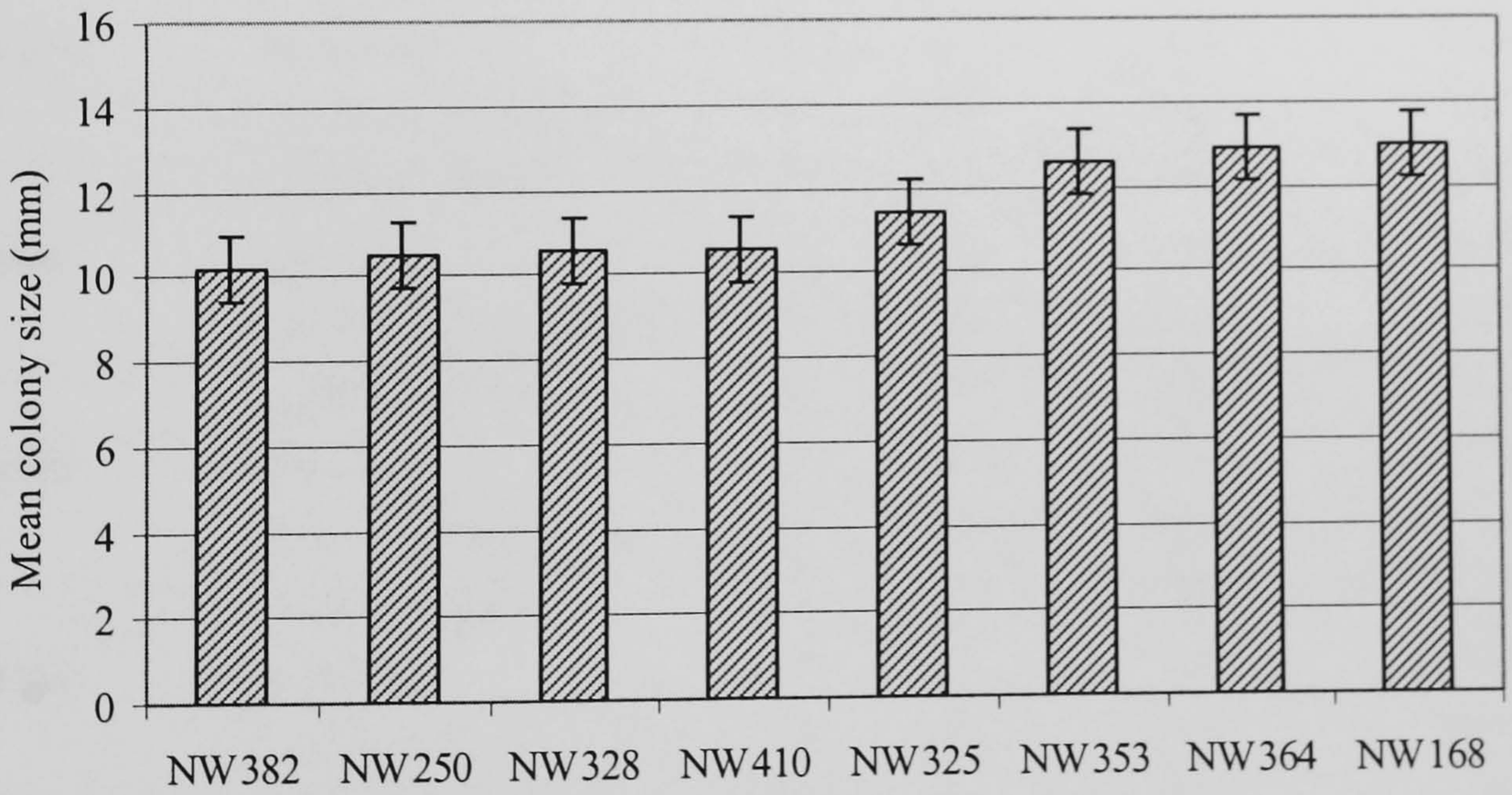


Figure 3.6. Mean colony sizes (mm) 96 hours p.i. for *Zoophthora radicans* isolate NW386 growing in the presence of con-specific isolates averaged over the two temperatures. Error bars represent one standard error of the mean (SEM).

Table 3.4. Comparison of the mean colony sizes of the *Zoophthora radicans* isolates grown in the presence of con-specific isolates 96 hours p.i., and its interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
NW325	*Z. radicans* isolates	7	58	1.65	0.140
	Temperature	1	2	4.80	0.160
	Z. radicans. Temperature	7	58	0.73	0.645
NW386	*Z. radicans* isolates	7	57	2.19	0.048
	Temperature	1	2	5.15	0.151
	Z. radicans. Temperature	7	57	0.92	0.498
NW353	*Z. radicans* isolates	7	58	0.91	0.504
	Temperature	1	2	2.46	0.258
	Z. radicans. Temperature	7	58	0.92	0.495
NW328	*Z. radicans* isolates	7	58	0.93	0.488
	Temperature	1	2	9.17	0.094
	Z. radicans. Temperature	7	58	2.52	0.025
NW364	*Z. radicans* isolates	7	57	0.45	0.868
	Temperature	1	2	28.15	0.034
	Z. radicans. Temperature	7	57	0.52	0.813
NW410	*Z. radicans* isolates	7	56	0.36	0.920
	Temperature	1	2	7.33	0.114
	Z. radicans. Temperature	7	56	1.15	0.349
NW382	*Z. radicans* isolates	7	57	0.42	0.888
	Temperature	1	2	114.22	0.009
	Z. radicans. Temperature	7	57	1.59	0.157
NW250	*Z. radicans* isolates	7	58	0.56	0.788
	Temperature	1	2	1.11	0.403
	Z. radicans. Temperature	7	58	0.55	0.797
NW168	*Z. radicans* isolates	7	58	1.22	0.309
	Temperature	1	2	3.62	0.197
	Z. radicans. Temperature	7	58	1.27	0.280

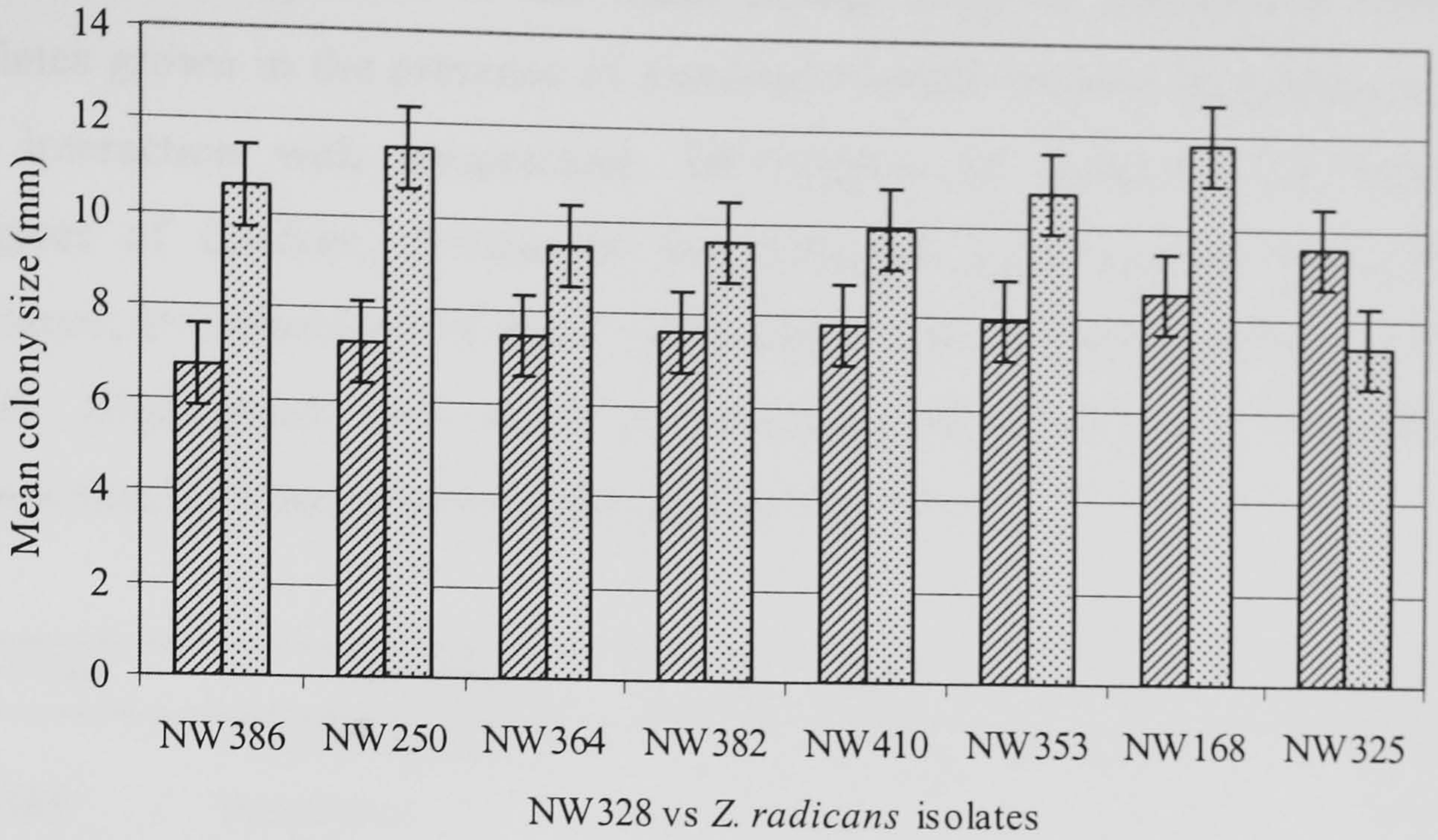


Figure 3.7. Mean colony sizes (mm) 96 hours p.i. for *Zoophthora radicans* isolate NW328 growing in the presence of each con-specific isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

A significant difference in mean colony size was found only for isolate NW410, when grown in the presence of different *P. blunckii* isolates (Table 3.5). The smallest colony size was achieved when grown in the presence of ARSEF6293, which was consistent at both temperatures, and the largest mean colony size was achieved in the presence of isolate ARSEF6311. At 25 °C, the largest mean colony size was achieved when grown in the presence of isolate ARSEF6311, and the smallest mean colony size was in the presence of isolate ARSEF217. At 20 °C, the smallest mean colony size was achieved in the presence of isolate ARSEF6293 and the largest colony size in the presence of isolate NW381 (Fig. 3.8).

Table 3.5. Comparison of the mean colony sizes of *Zoophthora radicans* isolates grown in the presence of *Pandora blunckii* isolates 96 hours p.i., and its interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
NW325	*P. blunckii* isolates	5	58	0.38	0.861
	Temperature	1	2	4.80	0.160
	P. blunckii. Temperature	5	58	1.79	0.129
NW386	*P. blunckii* isolates	5	57	1.33	0.263
	Temperature	1	2	5.15	0.151
	P. blunckii. Temperature	5	57	0.80	0.551
NW353	*P. blunckii* isolates	5	58	2.02	0.089
	Temperature	1	2	2.46	0.258
	P. blunckii. Temperature	5	58	0.92	0.472
NW328	*P. blunckii* isolates	5	58	0.55	0.741
	Temperature	1	2	9.17	0.094
	P. blunckii. Temperature	5	58	1.45	0.219
NW364	*P. blunckii* isolates	5	57	0.72	0.614
	Temperature	1	2	28.15	0.034
	P. blunckii. Temperature	5	57	0.86	0.516
NW410	*P. blunckii* isolates	5	56	2.86	0.023
	Temperature	1	2	7.33	0.114
	P. blunckii. Temperature	5	56	2.65	0.032
NW382	*P. blunckii* isolates	5	57	0.82	0.543
	Temperature	1	2	114.22	0.009
	P. blunckii. Temperature	5	57	1.41	0.235
NW250	*P. blunckii* isolates	5	58	1.37	0.250
	Temperature	1	2	1.11	0.403
	P. blunckii. Temperature	5	58	1.23	0.305
NW168	*P. blunckii* isolates	5	58	1.62	0.169
	Temperature	1	2	3.62	0.197
	P. blunckii. Temperature	5	58	1.27	0.287

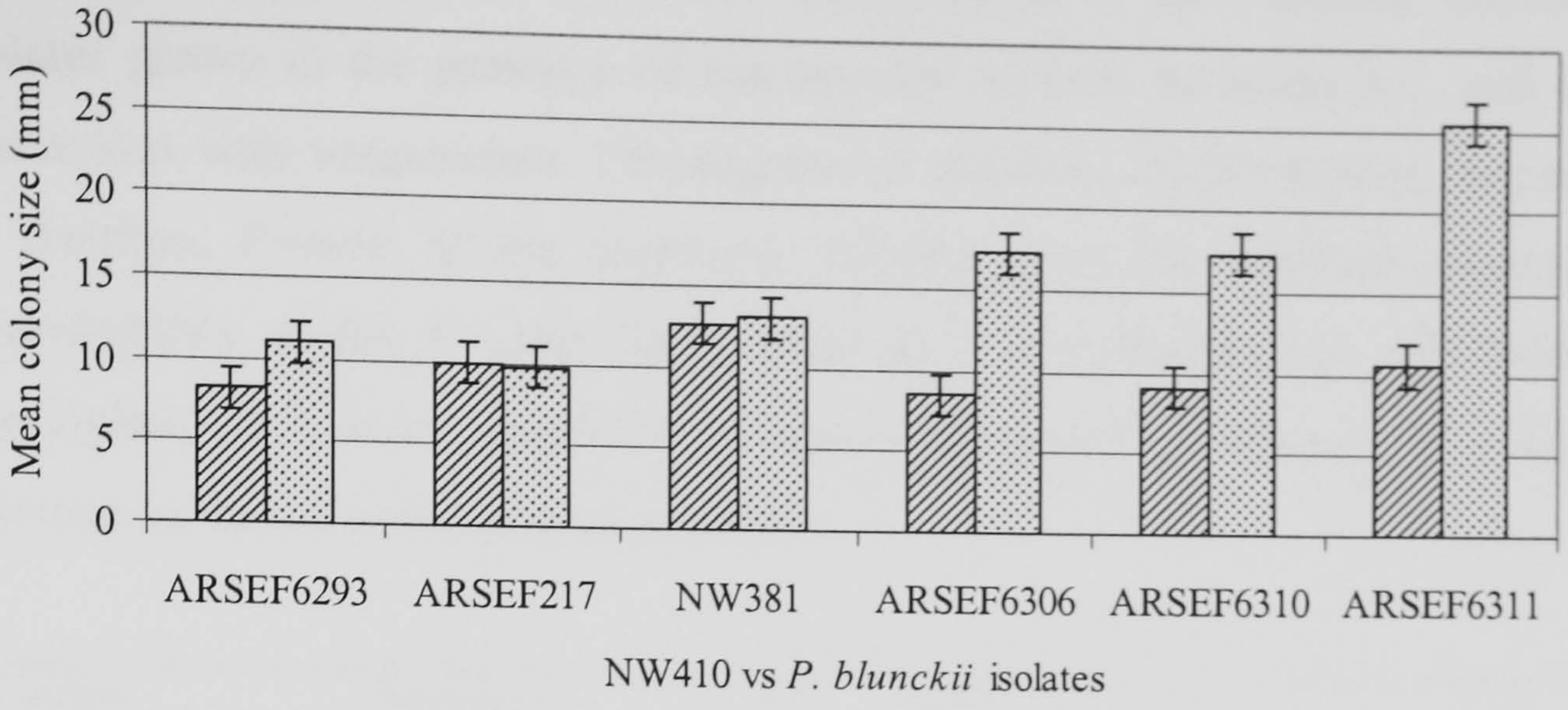


Figure 3.8. Mean colony sizes (mm) 96 hours p.i. for *Zoophthora radicans* isolate NW410 growing in the presence of each of the *Pandora blunckii* isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Effect on Pandora blunckii *isolates*

Isolates NW381 and ARSEF6311 showed significant differences in their mean colony sizes when grown in the presence of con-specific isolates (Table 3.6). At 20 °C isolate NW381, achieved the smallest mean colony size in the presence of isolate ARSEF6311 and the largest mean colony size in the presence of isolate ARSEF6293. At 25 °C, the smallest mean colony size was achieved in the presence of isolate ARSEF6293 and the largest mean colony size in the presence of isolate ARSEF6306 (Fig. 3.9). Isolate ARSEF6311 achieved the smallest mean colony size in the presence of isolate ARSEF6293, and the largest mean colony size in the presence of isolate ARSEF6306 regardless the temperature (Fig. 3.10).

Table 3.6. Comparison of the mean colony sizes of the *Pandora blunckii* isolates grown in the presence of con-specific isolates 96 hours p.i., and its interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
ARSEF6293	*P. blunckii* isolates	4	57	1.00	0.416
	Temperature	1	2	40.35	0.024
	P. blunckii. Temperature	4	57	0.45	0.773
ARSEF6310	*P. blunckii* isolates	4	58	0.65	0.629
	Temperature	1	2	4.87	0.158
	P. blunckii. Temperature	4	58	0.99	0.421
NW381	*P. blunckii* isolates	4	58	1.65	0.175
	Temperature	1	2	35.70	0.027
	P. blunckii. Temperature	4	58	3.29	0.017
ARSEF6311	*P. blunckii* isolates	4	30	2.87	0.040
	Temperature	1	2	26.32	0.123
	P. blunckii. Temperature	4	30	2.32	0.080
ARSEF217	*P. blunckii* isolates	4	57	0.72	0.582
	Temperature	1	2	1.92	0.300
	P. blunckii. Temperature	4	57	0.35	0.842
ARSEF6306	*P. blunckii* isolates	4	57	1.59	0.190
	Temperature	1	2	0.06	0.828
	P. blunckii. Temperature	4	57	1.29	0.286

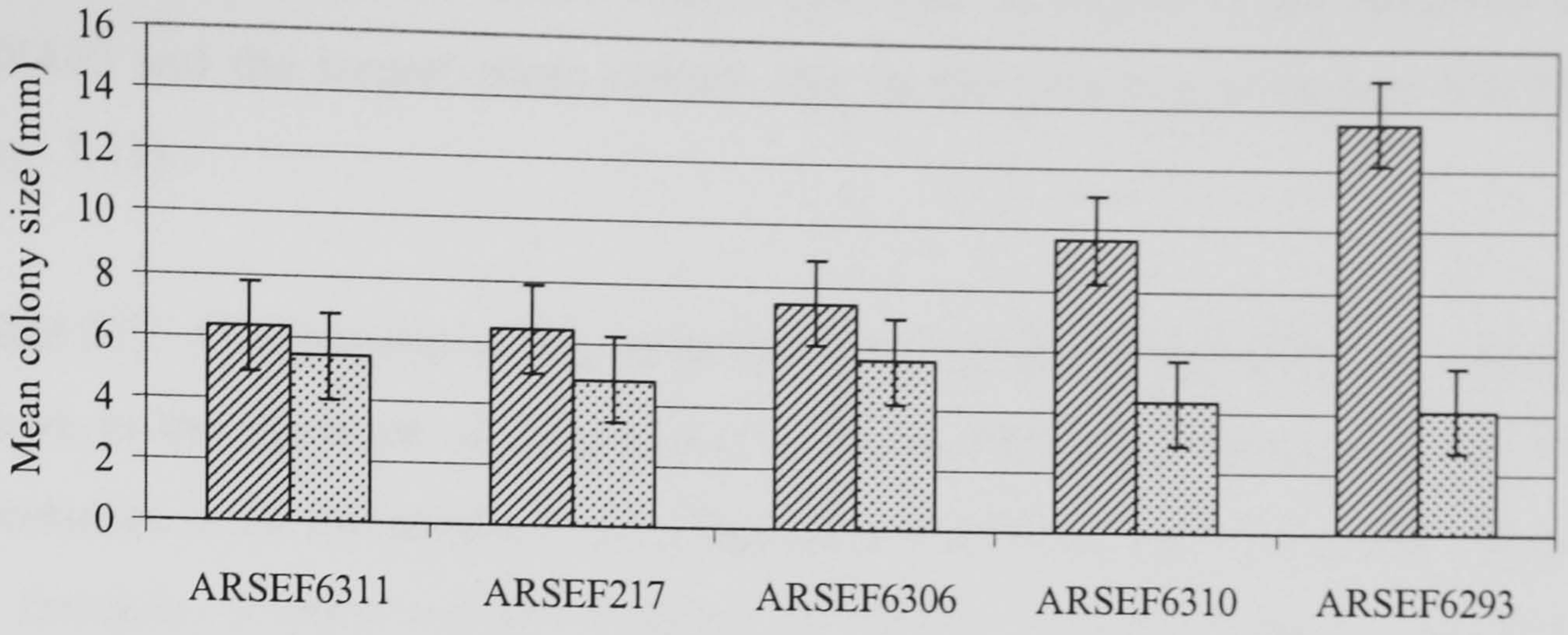


Figure 3.9. Mean colony sizes (mm) 96 hours p.i. for *Pandora blunckii* isolate NW381 growing in the presence of con-specific isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

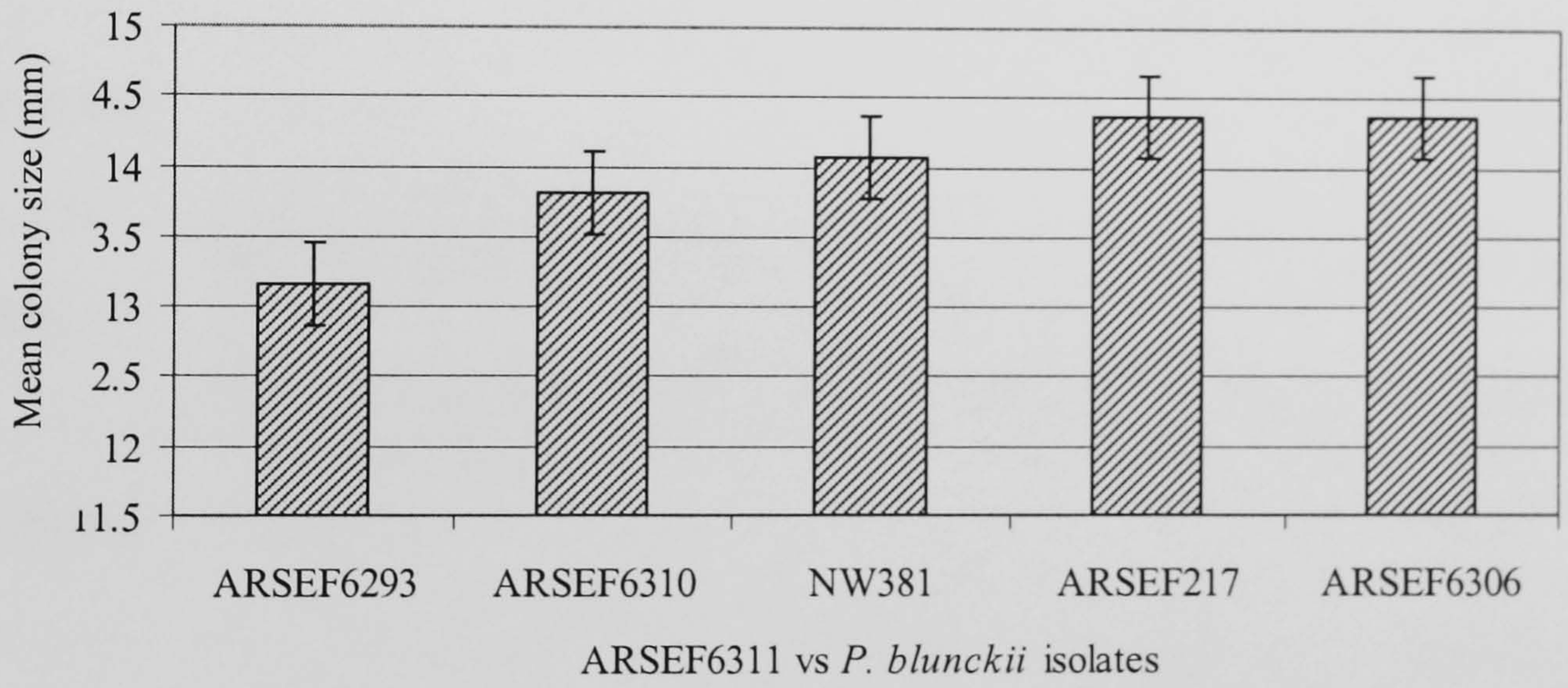


Figure 3.10. Mean colony sizes (mm) 96 hours post inoculation for *Pandora blunckii* isolate ARSEF6311 growing in the presence of con-specific isolates averaged over the two temperature conditions. Error bars represent one standard error of the mean (SEM).

Significant differences were found in the mean colony size of isolate NW381 when grown in the presence of different *Z. radicans* isolates. This effect had a significant interaction with temperature (Table 3.7). At 20 °C the smallest mean colony size was achieved in the presence of isolate NW168 and the largest mean colony size in the presence of two isolates, NW410 and NW382.

At 25 °C, the smallest mean colony size was achieved in the presence of NW410 and the largest mean colony size in the presence of isolate NW328 (Fig. 3.11).

Table 3.7. Comparison of the mean colony sizes of *Pandora blunckii* isolates grown in the presence of *Zoophthora radicans* isolates 96 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
	Z. radicans isolates	8	57	1.23	0.298
ARSEF6293	Temperature	1	2	40.35	0.024
	Z. radicans. Temperature	8	57	0.61	0.762
	Z. radicans isolates	8	58	0.50	0.852
ARSEF6310	Temperature	1	2	4.87	0.158
	Z. radicans. Temperature	8	58	1.15	0.344
	Z. radicans isolates	8	58	2.54	0.019
NW381	Temperature	1	2	35.70	0.027
	Z. radicans. Temperature	8	58	3.26	0.004
	Z. radicans isolates	8	30	1.83	0.110
ARSEF6311	Temperature	1	2	26.32	0.123
	Z. radicans. Temperature	8	30	0.65	0.733
	Z. radicans isolates	8	57	1.02	0.433
ARSEF217	Temperature	1	2	1.92	0.300
	Z. radicans. Temperature	8	57	0.87	0.549
	Z. radicans isolates	8	57	0.96	0.480
ARSEF6306	Temperature	1	2	0.06	0.828
	Z. radicans. Temperature	8	57	1.67	0.127

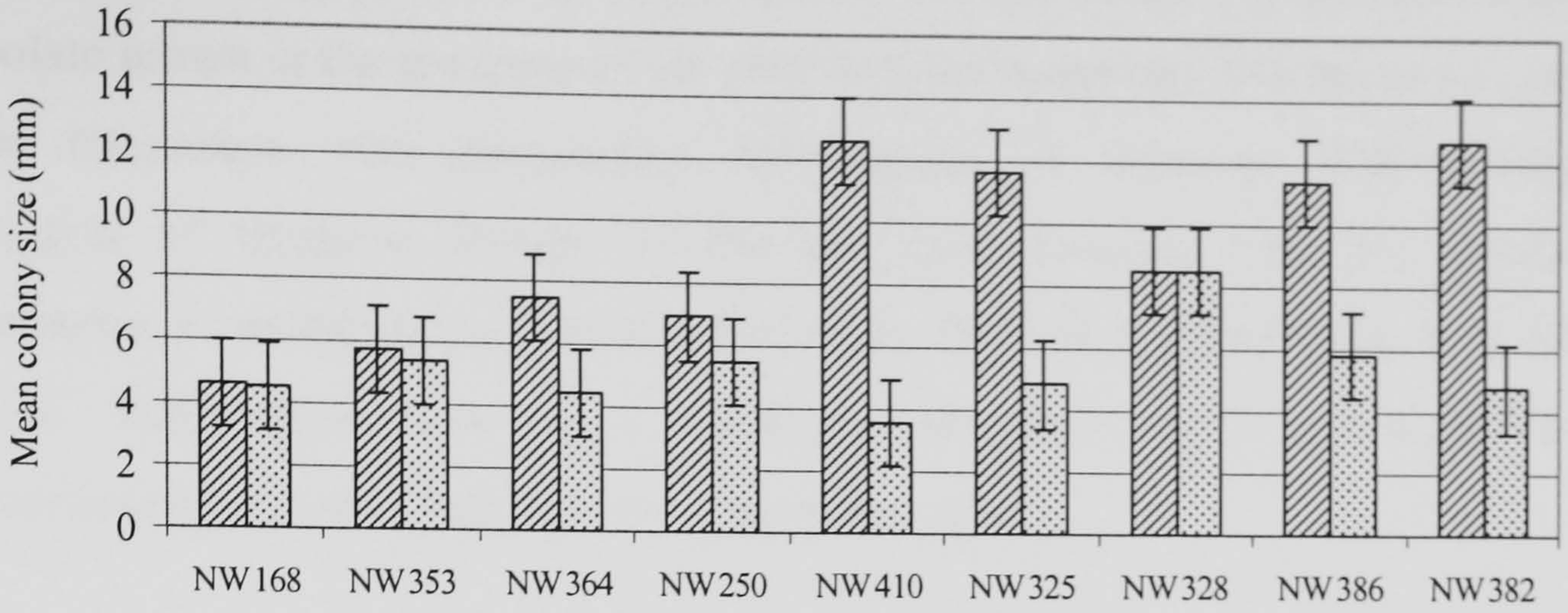


Figure 3.11. Mean colony sizes (mm) 96 hours p.i. for *Pandora blunckii* isolate NW381 growing in the presence of each *Zoophthora radicans* isolate at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

3.3.2 Interactions 192 hours post inoculation (p.i.)

3.3.2.1 Comparison between mean control colony size and mean colony size of a given isolate grown in the presence of all other isolates combined

For isolate NW382, a significant interaction with temperature was found in the difference between the mean colony size of the control colony and when grown in the presence of all other isolates combined (Table 3.8). This isolate was inhibited by all other isolates at 20°C, but not affected at 25 °C (Fig. 3.12).

Table 3.8. Comparison of the mean colony size and mean colony size of that isolate grown in the presence of all other isolates combined 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Zoophthora radicans					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
NW325	All other isolates (AOI)	1	55	0.15	0.699
	Temperature	1	2	15.24	0.060
	AOI.Temperature	1	55	0.00	0.965
NW386	All other isolates (AOI)	1	57	0.30	0.586
	Temperature	1	2	20.31	0.046
	AOI.Temperature	1	57	0.07	0.795
NW353	All other isolates (AOI)	1	58	0.53	0.471
	Temperature	1	2	0.62	0.513
	AOI.Temperature	1	58	0.17	0.684
NW328	All other isolates (AOI)	1	58	0.35	0.558
	Temperature	1	2	34.01	0.028
	AOI.Temperature	1	58	2.62	0.111
NW364	All other isolates (AOI)	1	57	0.01	0.941
	Temperature	1	2	17.71	0.052
	AOI.Temperature	1	57	1.52	0.223
NW410	All other isolates (AOI)	1	56	3.36	0.072
	Temperature	1	2	0.24	0.672
	AOI.Temperature	1	56	0.48	0.493
NW382	All other isolates (AOI)	1	57	3.59	0.063
	Temperature	1	2	36.19	0.027
	AOI.Temperature	1	57	5.08	0.028
NW250	All other isolates (AOI)	1	58	2.87	0.095
	Temperature	1	2	2.21	0.276
	AOI.Temperature	1	58	0.16	0.686
NW168	All other isolates (AOI)	1	58	0.01	0.905
	Temperature	1	2	7.65	0.110
	AOI.Temperature	1	58	2.85	0.097

Table 3.8. Continued.

Pandora blunckii					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
ARSEF6293	All other isolates (AOI)	1	57	0.26	0.612
	Temperature	1	2	30.74	0.031
	AOI.Temperature	1	57	0.00	0.957
ARSEF6310	All other isolates (AOI)	1	57	1.77	0.189
	Temperature	1	2	7.06	0.117
	AOI.Temperature	1	57	0.60	0.440
NW381	All other isolates (AOI)	1	56	0.07	0.786
	Temperature	1	2	51.25	0.019
	AOI.Temperature	1	56	1.34	0.251
ARSEF6311	All other isolates (AOI)	1	30	3.77	0.062
	Temperature	1	2	2.57	0.355
	AOI.Temperature	1	30	0.25	0.618
ARSEF217	All other isolates (AOI)	1	56	0.14	0.709
	Temperature	1	2	2.69	0.242
	AOI.Temperature	1	56	0.00	0.949
ARSEF6306	All other isolates (AOI)	1	57	0.66	0.421
	Temperature	1	2	0.01	0.941
	AOI.Temperature	1	57	0.01	0.933
***Pandora* sp.**					
	Treatment	**DF**	**RDF**	**F**	**P**
ARSEF3201	All other isolates (AOI)	1	57	0.51	0.477
	Temperature	1	2	0.63	0.510
	AOI.Temperature	1	57	0.04	0.838

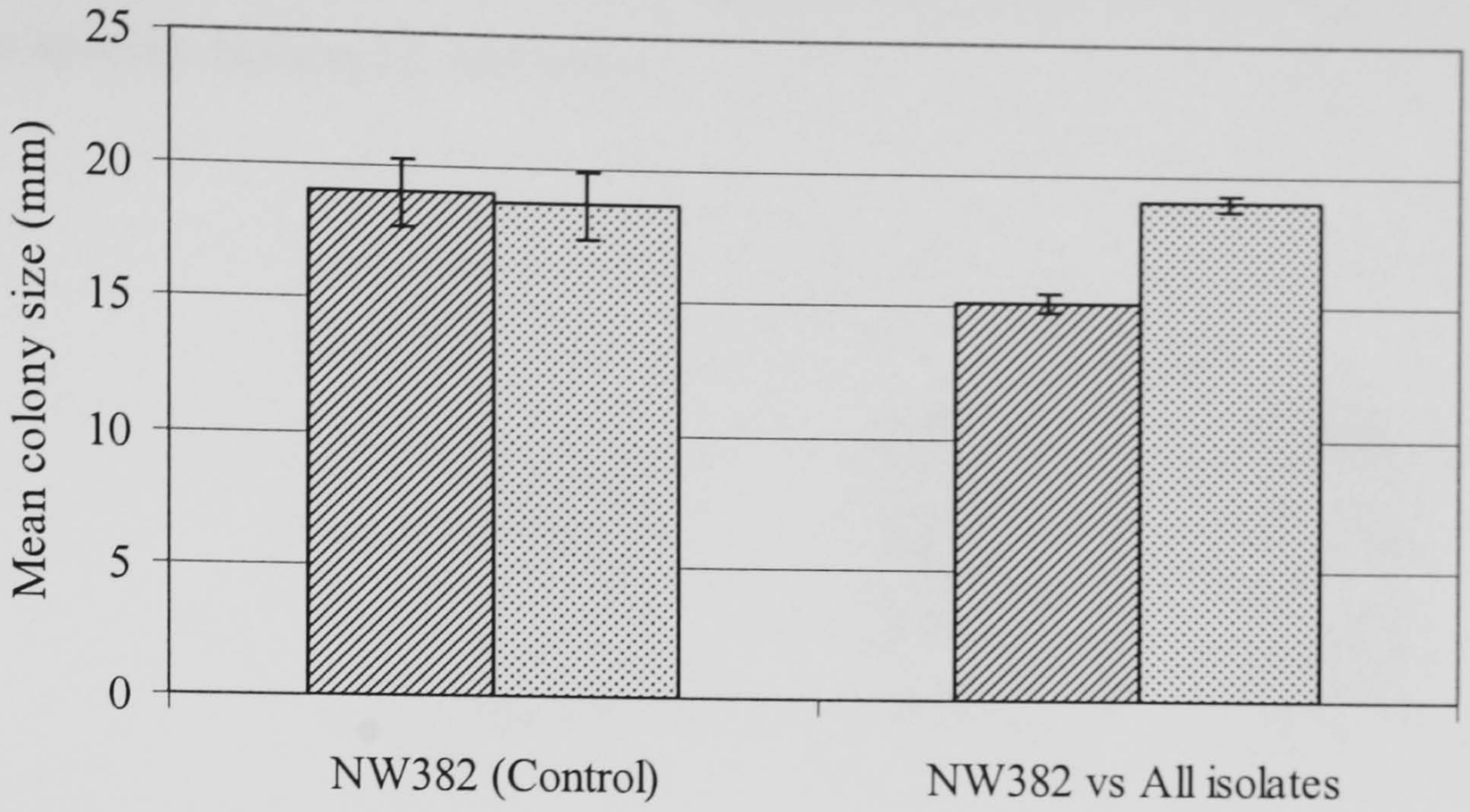


Figure 3.12. Mean colony sizes (mm) 192 hours p.i. for *Zoophthora radicans* isolate NW382 growing in the presence of itself (control colony) and in the presence of all other isolates combined at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

3.3.2.2 Comparison amongst the mean growth of a given isolate grown in the presence of isolates from each species combined

The effect of the species *Z. radicans, P. blunckii* and *Pandora* sp. on a given isolate is described. When *Z. radicans* or *P. blunckii* as a species are mentioned causing an effect on a given isolate, the species effect is the average of the different isolates combined from that species. In the case of *Pandora* sp. there is only one isolate representing this species.

Effect on Zoophthora radicans *isolates*

Significant differences were found in the mean colony sizes of *Z. radicans* isolates NW325, NW328, NW410, NW382, NW250 and NW168 when grown in the presence of the three different species (Table 3.9). Isolates NW328, NW250 and NW168 showed consistent results over the two temperatures. The two former isolates achieved the smallest mean colony size in the presence of *Pandora* sp. (Figs. 3.13a and b) and isolate NW168 achieved the smallest mean colony size in the presence of *Pandora* sp. and *P. blunckii* (Fig. 3.13c).

These three isolates achieved the largest mean colony size in the presence of con-specific isolates (*Z. radicans*).

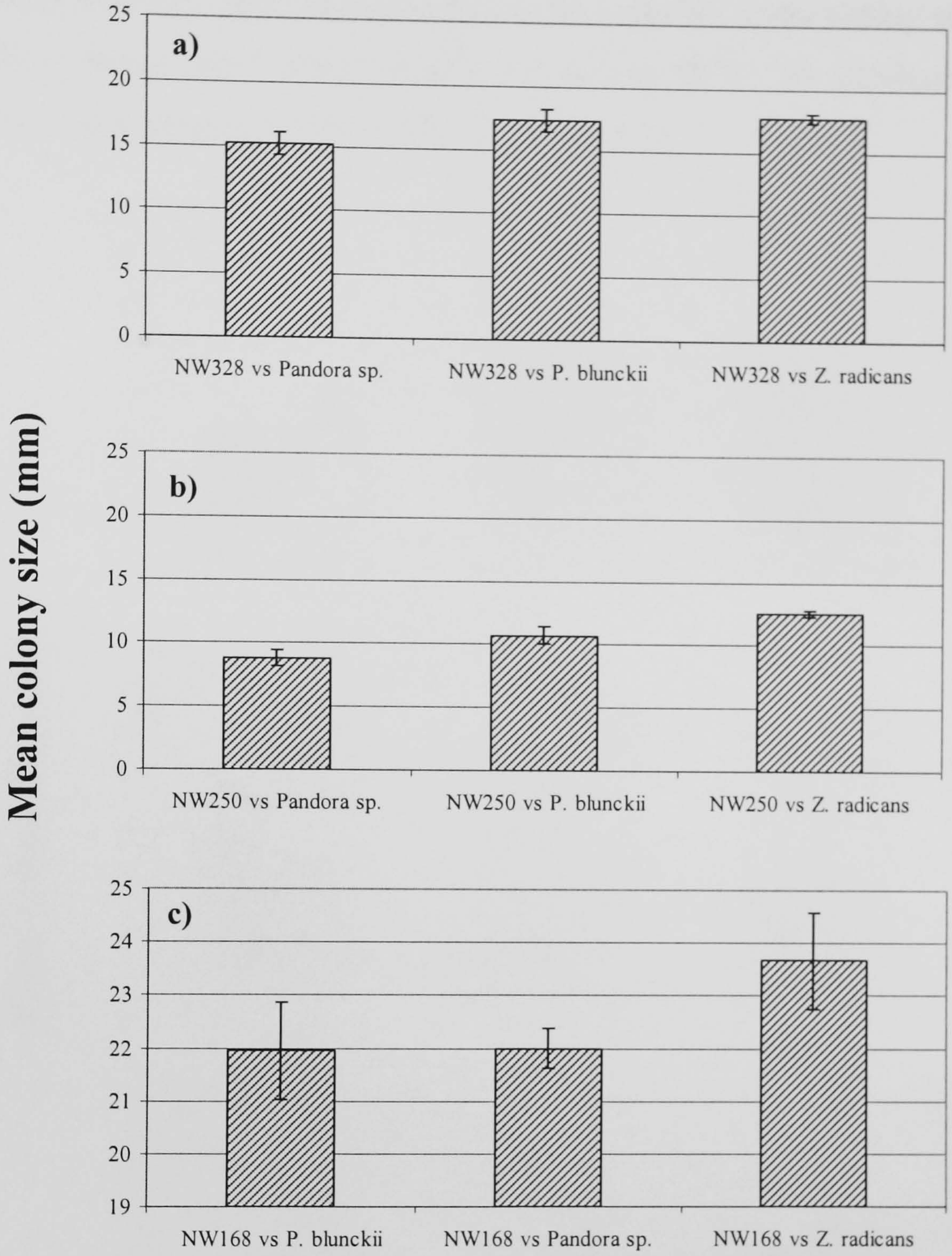


Figure 3.13. Mean colony sizes (mm) 192 hours p.i. for three *Zoophthora radicans* isolates growing in the presence of three species averaged over the two temperature conditions. a) NW328, b) NW250 and c) NW168. Error bars represent one standard error of the mean (SEM).

Overall for isolate NW410, the smallest mean colony size was achieved in the presence of the *Pandora* sp. isolate. This result was not consistent over the two temperatures; at 20 °C, the largest mean colony size was achieved in the

presence of *Z. radicans*, but in the presence of *P. blunckii* at 25 °C (Fig. 3.14a). The differences in the mean colony sizes of isolates NW325 and NW382 when grown in the presence of the three species had a significant interaction with temperature (Table 3.9). Both isolates achieved the smallest mean colony size when grown in the presence of *Pandora* sp. but only at 20 °C. No differences in colony size were observed at 25 °C (Fig. 3.14 b and c).

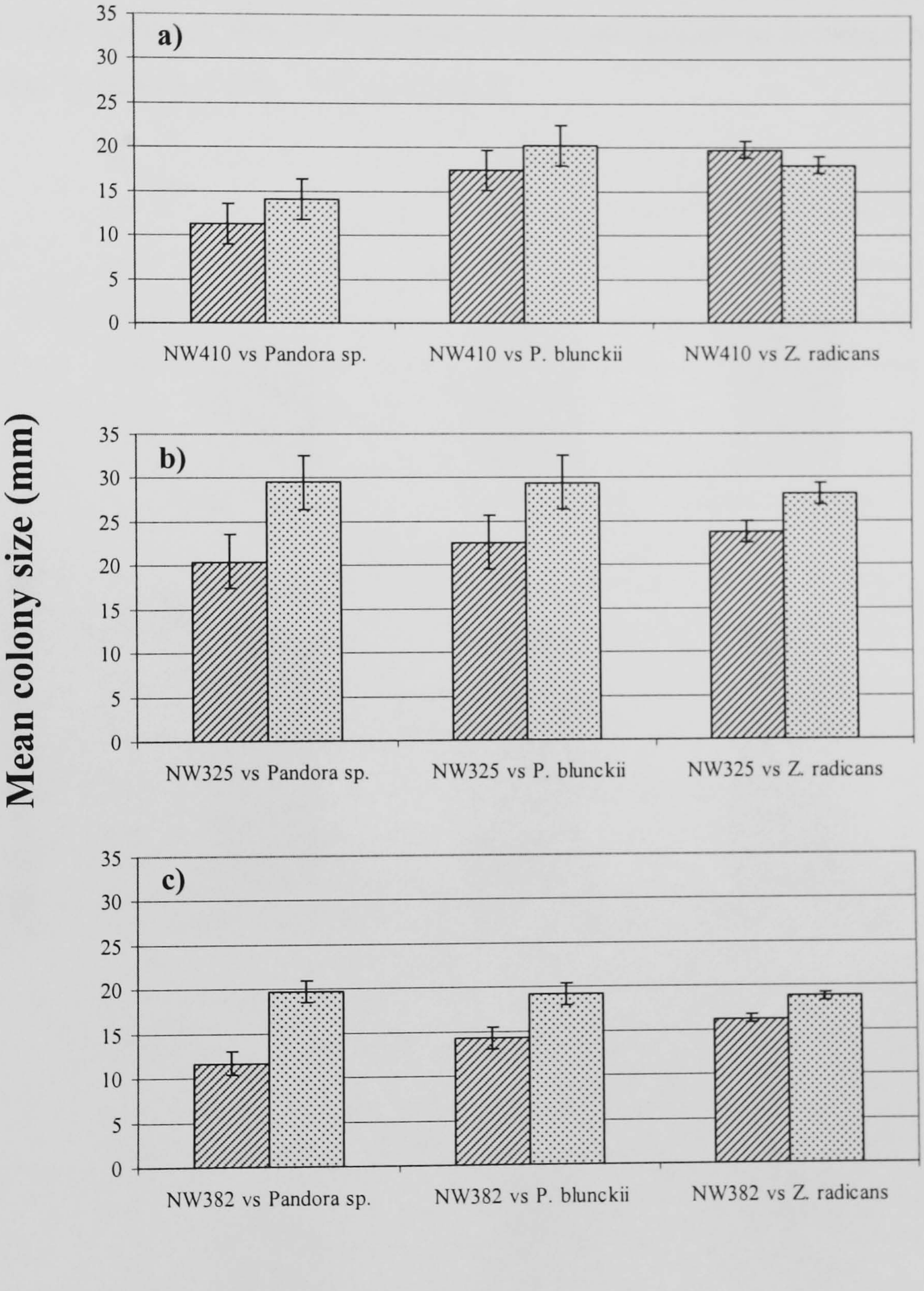


Figure 3.14. Mean colony sizes (mm) 192 hours p.i. for three *Zoophthora radicans* isolates growing in the presence of the three species at the two temperature conditions. a) NW410, b) NW325 and c) NW382. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Effect on Pandora blunckii *isolates*

Pandora blunckii isolates NW381, ARSEF6311 and ARSEF6306 showed significant differences in their mean colony sizes when grown in the presence of their con-specific isolates, *Pandora* sp. and *Z. radicans* (Table 3.9). The three isolates achieved the smallest mean colony size when grown in the presence of *Pandora* sp., followed by *P. blunckii* and the greatest growth with *Z. radicans*. Only isolate ARSEF6306 achieved the same growth in presence of *P. blunckii* and *Z. radicans* (Fig. 3.15 a, b and c).

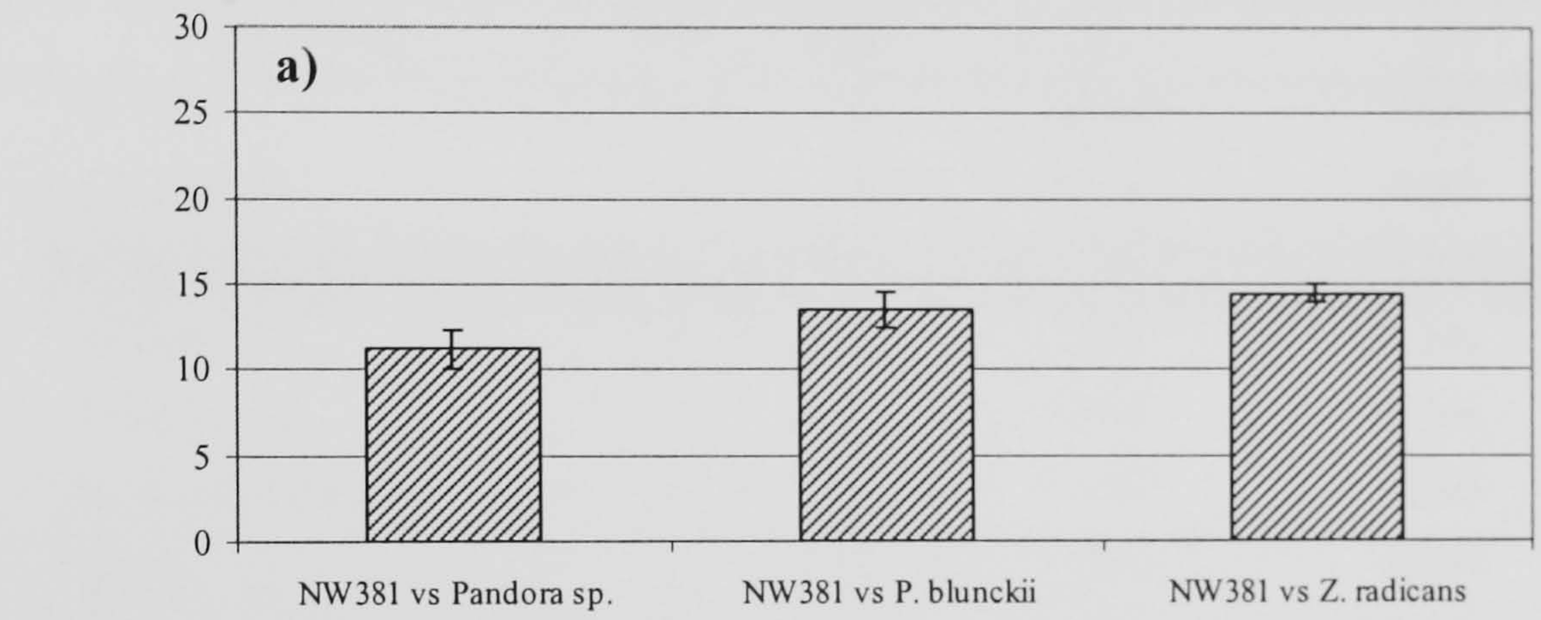


Figure 3.15. Mean colony sizes (mm) 192 hours p.i. for *Pandora blunckii* isolates, growing in the presence of three species averaged over the two temperature conditions. a) NW381, b) ARSEF6311 and c) ARSEF6306. Error bars represent one standard error of the mean (SEM).

Table 3.9. Comparison of the mean colony sizes of a given isolate grown in the presence of isolates from each species combined 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Zoophthora radicans					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
NW325	Species	2	55	0.38	0.686
	Temperature	1	2	15.24	0.060
	Species.Temperature	2	55	3.64	0.033
NW386	Species	2	57	2.01	0.143
	Temperature	1	2	20.31	0.046
	Species.Temperature	2	57	1.36	0.266
NW353	Species	2	58	1.43	0.247
	Temperature	1	2	0.62	0.513
	Species.Temperature	2	58	2.28	0.111
NW328	Species	2	58	3.47	0.038
	Temperature	1	2	34.01	0.028
	Species.Temperature	2	58	0.98	0.380
NW364	Species	2	57	2.64	0.080
	Temperature	1	2	17.71	0.052
	Species.Temperature	2	57	0.44	0.649
NW410	Species	2	56	6.73	0.002
	Temperature	1	2	0.24	0.672
	Species.Temperature	2	56	3.56	0.035
NW382	Species	2	57	2.50	0.091
	Temperature	1	2	36.19	0.027
	Species.Temperature	2	57	6.20	0.004
NW250	Species	2	58	22.24	<0.001
	Temperature	1	2	2.21	0.276
	Species.Temperature	2	58	0.69	0.504
NW168	Species	2	58	6.60	0.003
	Temperature	1	2	7.65	0.110
	Species.Temperature	2	58	0.41	0.665

Table 3.9. Continued.

Pandora blunckii					
Isolate	**Treatment**	**DF**	**RDF**	**F**	**P**
ARSEF6293	Species	2	57	1.75	0.184
	Temperature	1	2	30.74	0.031
	Species.Temperature	2	57	2.47	0.093
ARSEF6310	Species	2	57	2.11	0.131
	Temperature	1	2	7.06	0.117
	Species.Temperature	2	57	0.44	0.646
NW381	Species	2	56	4.62	0.014
	Temperature	1	2	51.25	0.019
	Species.Temperature	2	56	0.24	0.790
ARSEF6311	Species	2	30	4.69	0.017
	Temperature	1	2	2.57	0.355
	Species.Temperature	2	30	0.73	0.489
ARSEF217	Species	2	56	2.10	0.132
	Temperature	1	2	2.69	0.242
	Species.Temperature	2	56	0.99	0.380
ARSEF6306	Species	2	57	5.65	0.006
	Temperature	1	2	0.01	0.941
	Species.Temperature	2	57	1.79	0.176
***Pandora* sp.**					
	Treatment	**DF**	**RDF**	**F**	**P**
ARSEF3201	All other isolates (AOI)	1	57	5.58	0.022
	Temperature	1	2	0.63	0.510
	AOI.Temperature	1	57	0.59	0.446

Effects on Pandora *sp isolate*

Significant differences were found between the mean colony size of the *Pandora* sp isolate when grown in the presence of *Z. radicans* and *P. blunckii* (Table 3.9). The smallest mean colony size was achieved when grown in the presence of *P. blunckii* compared to the presence of *Z. radicans* (Fig. 3.16)

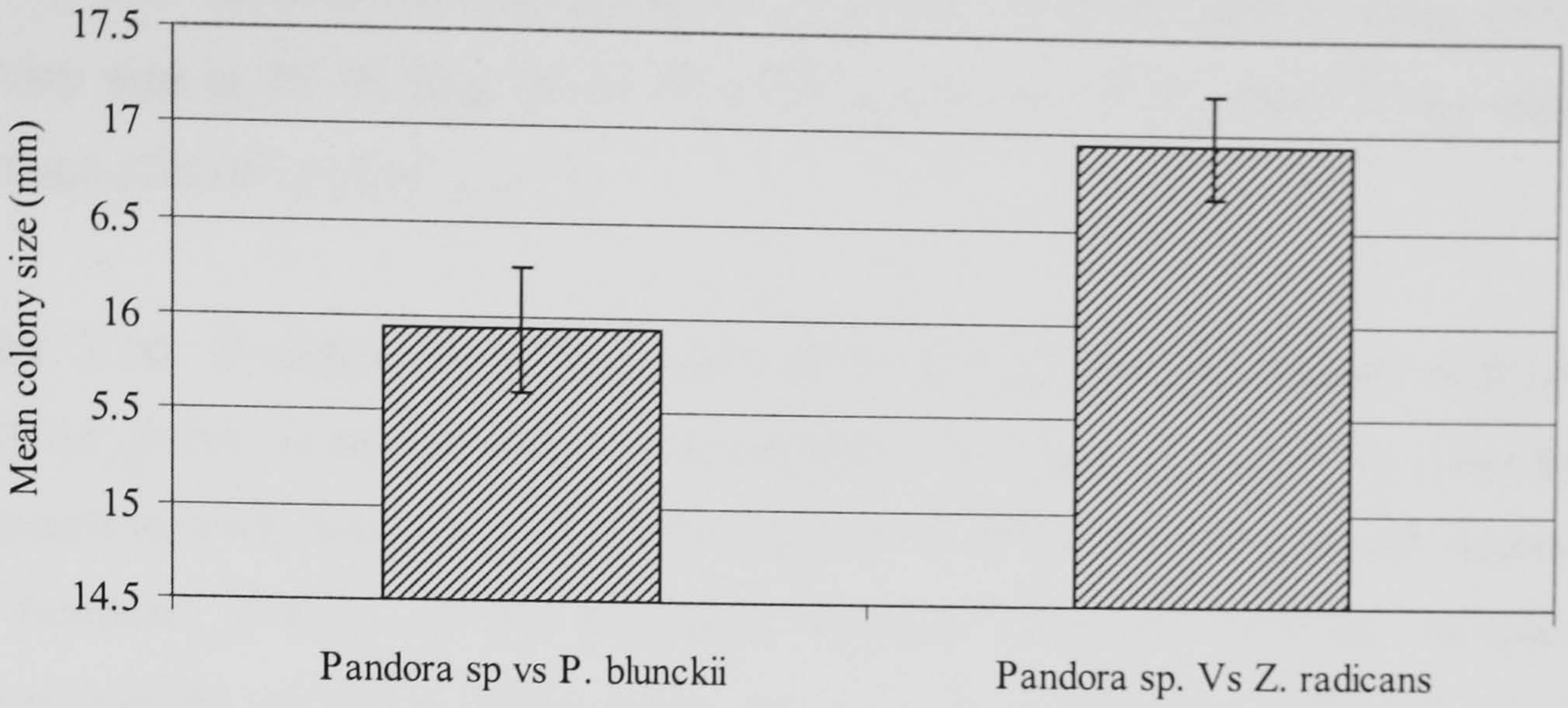


Figure 3.16. Mean colony sizes (mm) 192 hours p.i. for the *Pandora sp.* isolate growing in the presence of *Zoophthora radicans* and *Pandora blunckii* averaged over the two temperature conditions. Error bars represent one standard error of the mean (SEM).

3.3.2.3 Comparison of mean colony sizes of a given isolate grown in the presence of each isolate within each species

Effect on Zoophthora radicans *isolates*

Zoophthora radicans isolates NW328, NW382, NW250 and NW168 showed significant differences in their mean colony sizes when grown in the presence of con-specific isolates. These results were consistent at both temperatures except for isolate NW168, where its effect had a significant interaction with temperature (Table 3.10). Isolate NW328 achieved the smallest mean colony size when grown in the presence of isolate NW364 and the largest mean colony size in the presence of isolate NW168 (Fig. 3.17a). Isolate NW382 achieved the smallest mean colony size when grown in the presence of isolate NW325 and the largest mean colony size in the presence of isolate NW250 (Fig. 3.17b). Isolate NW250 achieved the smallest mean colony size when grown in the presence of isolate NW410, and the largest mean colony size when grown in the presence of isolate NW382 (Fig. 3.17c). The effect of the different con-specific isolates on the mean colony size of NW168 was highly variable. The mean colony size of NW168 in the presence of isolates NW353 and NW364 was the same at both temperatures. A smaller mean colony size at 20 °C than at

25 °C was achieved in the presence of isolate NW382 and a larger mean colony size at 20 °C than at 25 °C when grown in the presence of the other con-specific isolates (Fig. 3.18).

Table 3.10. Comparison of the mean colony sizes of the *Zoophthora radicans* isolates grown in the presence of con-specific isolates 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
NW325	*Z. radicans* isolates	7	55	1.82	0.102
	Temperature	1	2	15.24	0.060
	Z. radicans.Temperature	7	55	0.66	0.708
NW386	*Z. radicans* isolates	7	57	1.05	0.408
	Temperature	1	2	20.31	0.046
	Z. radicans.Temperature	7	57	0.65	0.711
NW353	*Z. radicans* isolates	7	58	1.99	0.072
	Temperature	1	2	0.62	0.513
	Z. radicans.Temperature	7	58	1.73	0.119
NW328	*Z. radicans* isolates	7	58	2.79	0.014
	Temperature	1	2	34.01	0.028
	Z. radicans.Temperature	7	58	2.02	0.068
NW364	*Z. radicans* isolates	7	57	1.11	0.369
	Temperature	1	2	17.71	0.052
	Z. radicans.Temperature	7	57	1.23	0.303
NW410	*Z. radicans* isolates	7	56	1.04	0.412
	Temperature	1	2	0.24	0.672
	Z. radicans.Temperature	7	56	0.88	0.528
NW382	*Z. radicans* isolates	7	57	2.25	0.043
	Temperature	1	2	36.19	0.027
	Z. radicans.Temperature	7	57	1.71	0.125
NW250	*Z. radicans* isolates	7	58	2.26	0.042
	Temperature	1	2	2.21	0.276
	Z. radicans.Temperature	7	58	0.38	0.913
NW168	*Z. radicans* isolates	7	58	0.27	0.961
	Temperature	1	2	7.65	0.110
	Z. radicans.Temperature	7	58	2.49	0.026

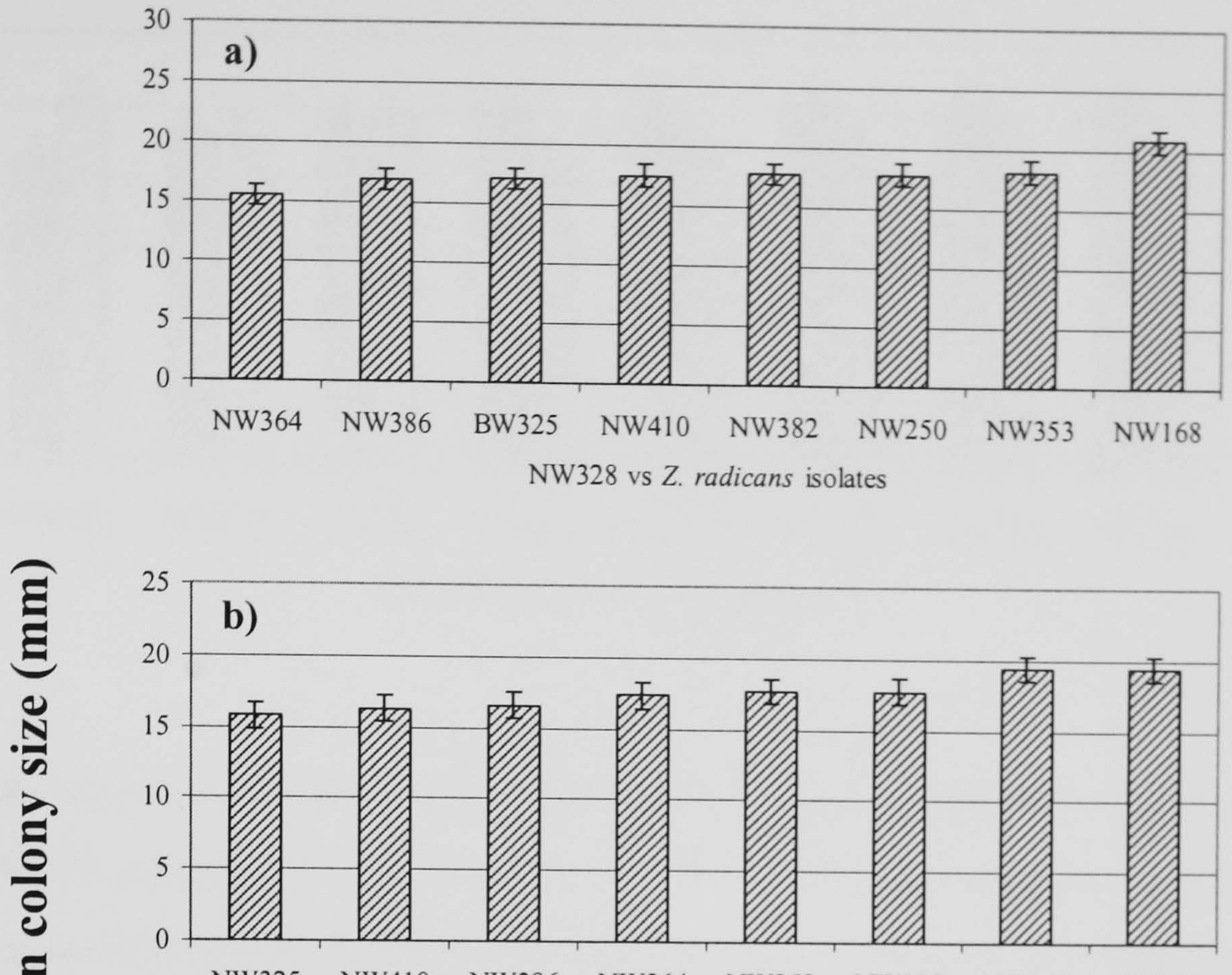


Figure 3.17. Mean colony sizes (mm) 192 hours p.i. for three *Zoophthora radicans* isolates growing in the presence of con-specific isolates averaged over the two temperature conditions. a) NW328, b) NW382 and c) NW250. Error bars represent one standard error of the mean (SEM).

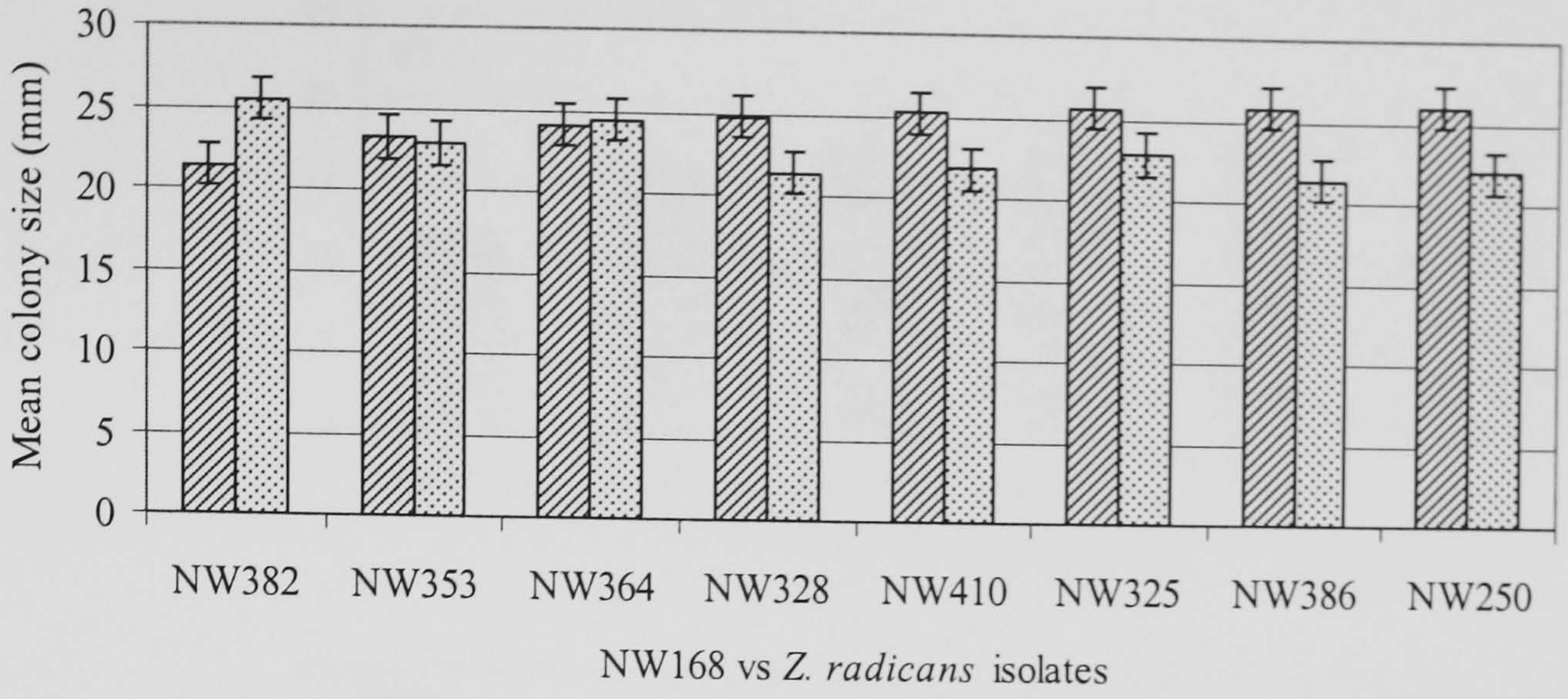


Figure 3.18. Mean colony sizes (mm) 192 hours p.i. for *Zoophthora radicans* isolate NW168 growing in the presence of con-specific isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

The *Z. radicans* isolates NW353, NW328, NW410, NW382, NW250 and NW168 showed significant differences in their mean colony sizes when grown in the presence of different *P. blunckii* isolates (Table 3.11).

The results obtained for isolates NW353, NW410, NW382 and NW250 were consistent over the two temperatures. All these isolates achieved the smallest mean colony size when grown in the presence of *P. blunckii* isolate ARSEF6293. Isolate NW250 also achieved the smallest mean colony size when grown in the presence of isolate ARSEF217 (Fig. 3.19 a, b, c and d).

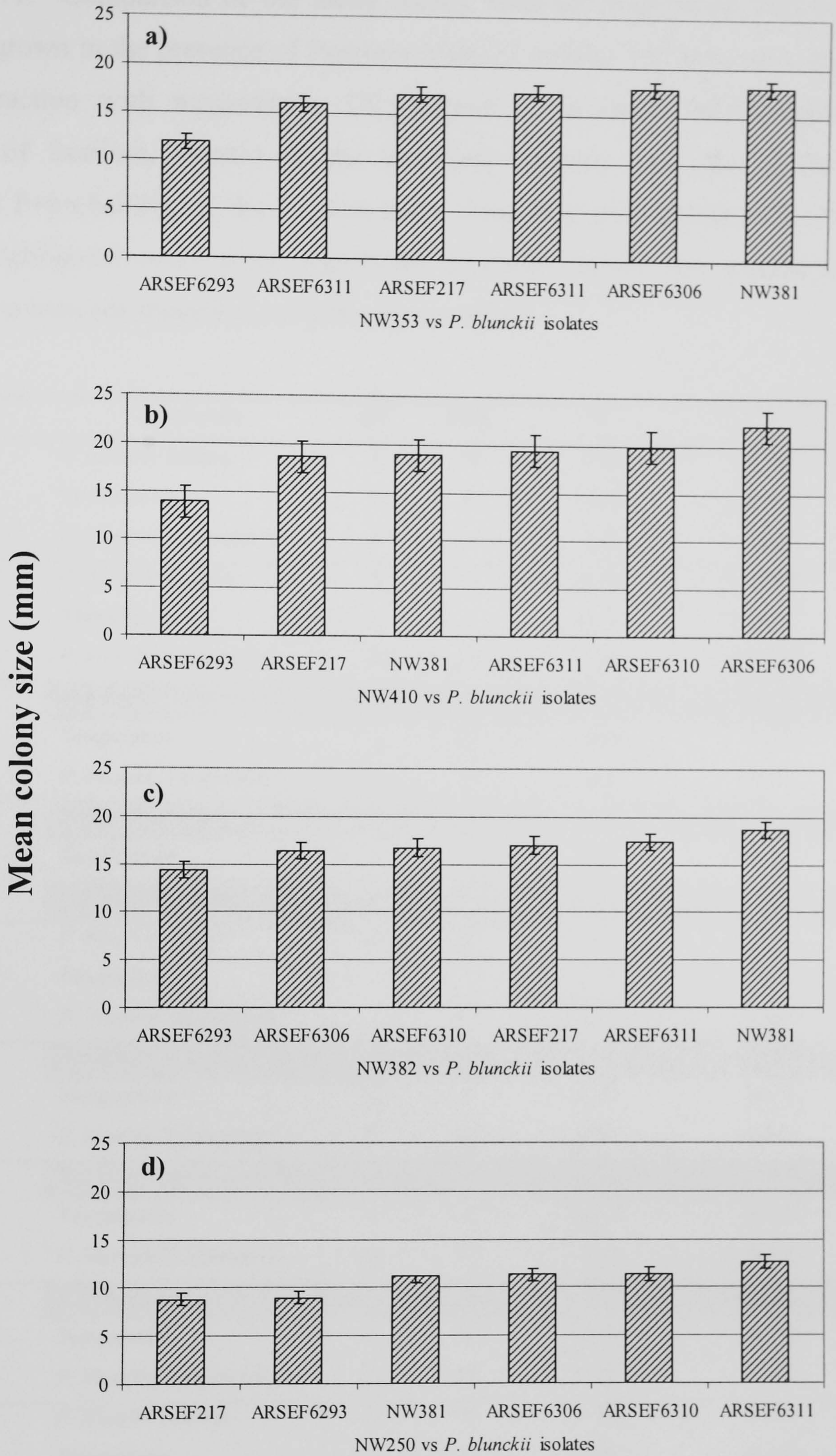


Figure 3.19. Mean colony sizes (mm) 192 hours p.i. for four *Zoophthora radicans* isolates growing in the presence of each *Pandora blunckii* isolate averaged over the two temperature conditions. a) NW353, b) NW410, c) NW382 and d) NW250. Error bars represent one standard error of the mean (SEM).

Table 3.11. Comparison of the mean colony sizes of *Zoophthora radicans* isolates grown in the presence of *Pandora blunckii* isolates 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
NW325	*P. blunckii* isolates	5	55	1.20	0.322
	Temperature	1	2	15.24	0.060
	P. blunckii.Temperature	5	55	1.27	0.289
NW386	*P. blunckii* isolates	5	57	2.03	0.089
	Temperature	1	2	20.31	0.046
	P. blunckii.Temperature	5	57	0.41	0.842
NW353	*P. blunckii* isolates	5	58	8.22	<0.001
	Temperature	1	2	0.62	0.513
	P. blunckii.Temperature	5	58	1.10	0.371
NW328	*P. blunckii* isolates	5	58	3.55	0.007
	Temperature	1	2	34.01	0.028
	P. blunckii.Temperature	5	58	2.52	0.039
NW364	*P. blunckii* isolates	5	57	1.15	0.345
	Temperature	1	2	17.71	0.052
	P. blunckii.Temperature	5	57	0.72	0.608
NW410	*P. blunckii* isolates	5	56	2.75	0.027
	Temperature	1	2	0.24	0.672
	P. blunckii.Temperature	5	56	0.67	0.648
NW382	*P. blunckii* isolates	5	57	2.52	0.040
	Temperature	1	2	36.19	0.027
	P. blunckii.Temperature	5	57	0.67	0.646
NW250	*P. blunckii* isolates	5	58	4.13	0.003
	Temperature	1	2	2.21	0.276
	P. blunckii.Temperature	5	58	0.52	0.760
NW168	*P. blunckii* isolates	5	58	1.85	0.118
	Temperature	1	2	7.65	0.110
	P. blunckii.Temperature	5	58	3.23	0.012

Isolate NW328 also achieved the smallest mean colony size when grown in the presence of isolate ARSEF6293, which was consistent at both temperatures. The mean colony size when grown in the presence of the other *P. blunckii* isolates were also consistent over the two temperatures except with isolate ARSEF271, where the mean colony size of isolate NW328 was smaller at 20 °C than at 25 °C (Fig. 3.20).

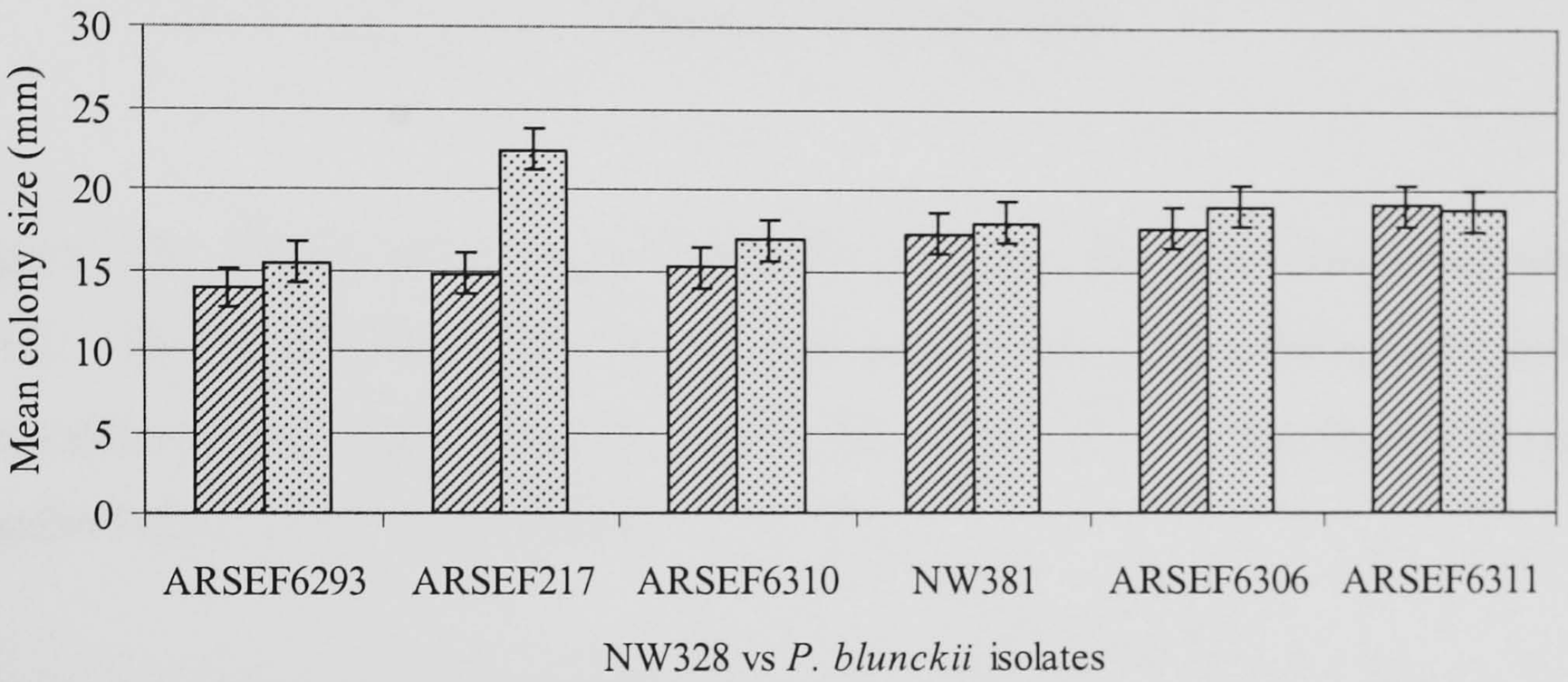


Figure 3.20. Mean colony sizes (mm) 192 hours p.i. for *Zoophthora radicans* isolate NW328 growing in the presence of all *Pandora blunckii* isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

The mean colony sizes of isolate NW168 when grown in the presence of each *P. blunckii* isolate were inconsistent over the two temperatures. For example, when grown in the presence of isolates ARSEF6293, ARSEF217 and ARSEF6311, the smallest mean colony size was at 25 °C rather than at 20 °C. The effect of the remaining isolates ARSEF6306, ARSESF6310 and NW381 were similar at both temperatures (Fig. 3.21).

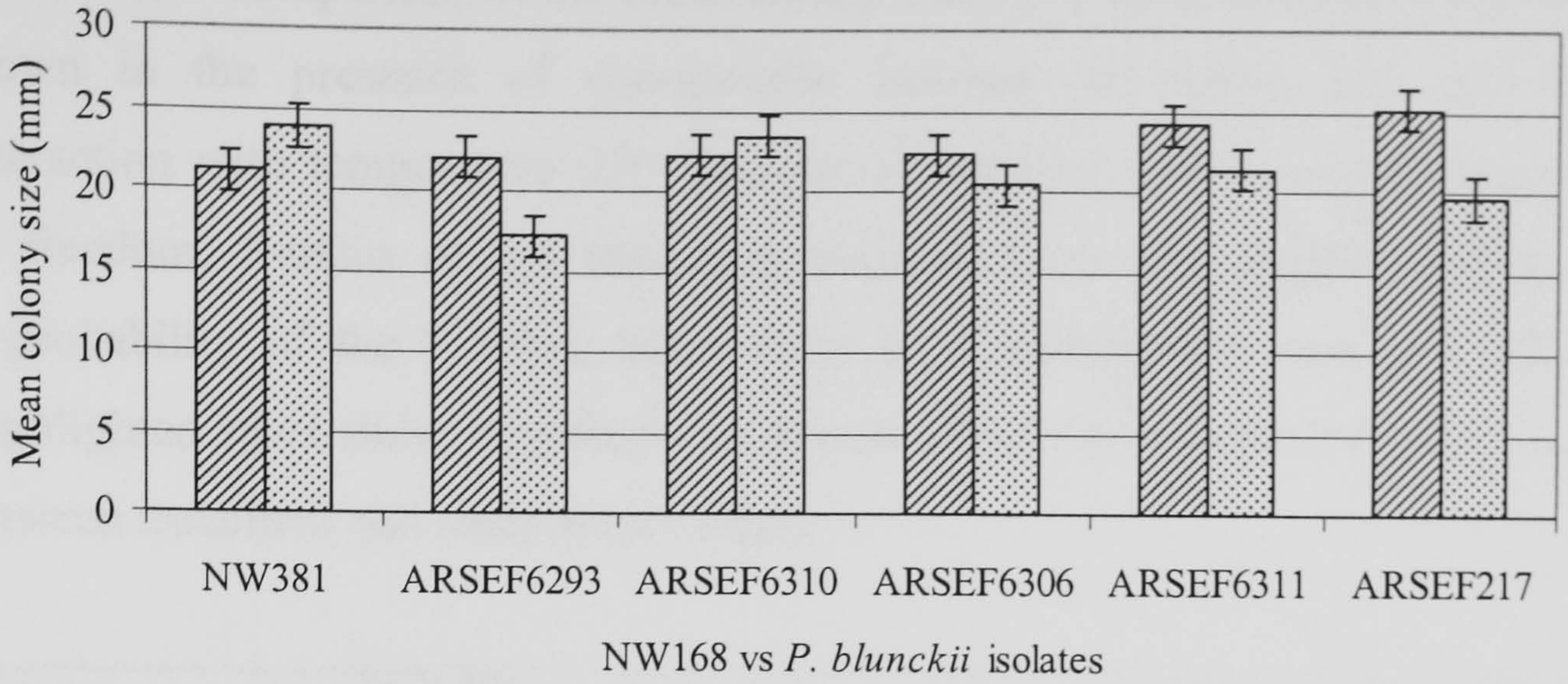


Figure 3.21. Mean colony sizes (mm) 192 hours p.i. for *Zoophthora radicans* isolate NW168 growing in the presence of each *Pandora blunckii* isolate at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Effect on Pandora blunckii *isolates*

Pandora blunckii isolates ARSEF6311, ARSEF6306 and NW381 showed significant differences in their mean colony sizes when growth in the presence of con-specific isolates (Table 3.12). Isolates ARSEF6311 and ARSEF6306 achieved the smallest mean colony size when grown in the presence of isolate ARSEF6293. This result was consistent at both temperatures (Fig. 3.22). Results for isolate NW381 were variable between the two temperatures, but overall, mean colony sizes were smaller at 25 °C than at 20 °C (Fig. 3.23).

Table 3.12. Comparison of the mean colony sizes of *Pandora blunckii* isolates grown in the presence of con-specific isolates 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
ARSEF6293	*P. blunckii* isolates	4	57	0.99	0.423
	Temperature	1	2	30.74	0.031
	P. blunckii.Temperature	4	57	0.48	0.753
ARSEF6310	*P. blunckii* isolates	4	57	0.21	0.930
	Temperature	1	2	7.06	0.117
	P. blunckii.Temperature	4	57	0.30	0.878
NW381	*P. blunckii* isolates	4	56	0.84	0.507
	Temperature	1	2	51.25	0.019
	P. blunckii.Temperature	4	56	2.85	0.032
ARSEF6311	*P. blunckii* isolates	4	30	8.41	<0.001
	Temperature	1	2	2.57	0.355
	P. blunckii.Temperature	4	30	1.91	0.135
ARSEF217	*P. blunckii* isolates	4	56	0.38	0.823
	Temperature	1	2	2.69	0.242
	P. blunckii.Temperature	4	56	0.04	0.997
ARSEF6306	*P. blunckii* isolates	4	57	4.72	0.002
	Temperature	1	2	0.01	0.941
	P. blunckii.Temperature	4	57	1.47	0.222

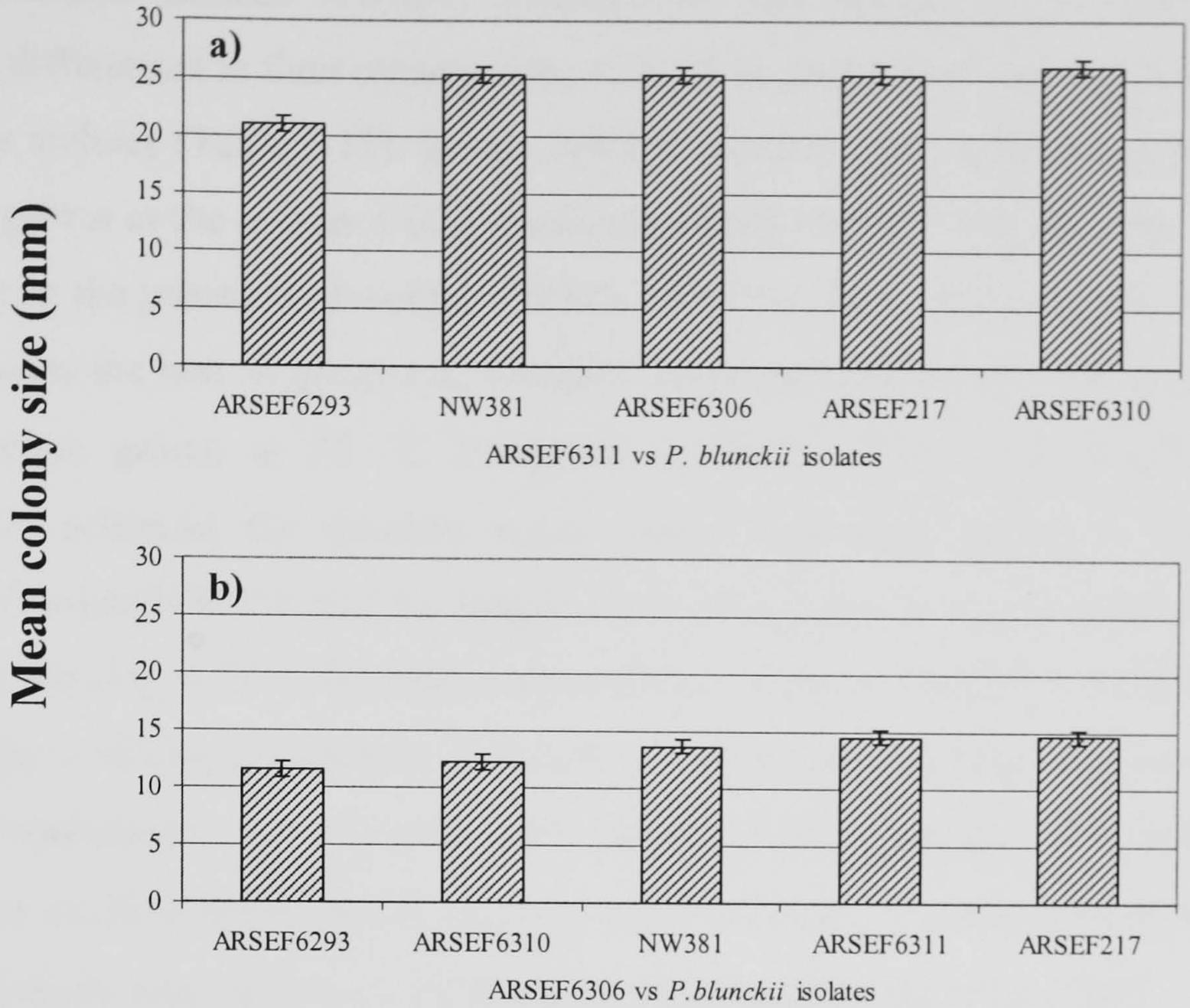


Figure 3.22. Mean colony sizes (mm) 192 hours p.i. for two *Pandora blunckii* isolates growing in the presence of con-specific isolates averaged over the two temperature conditions. a) ARSEF6311, b) ARSEF6306. Error bars represent one standard error of the mean (SEM).

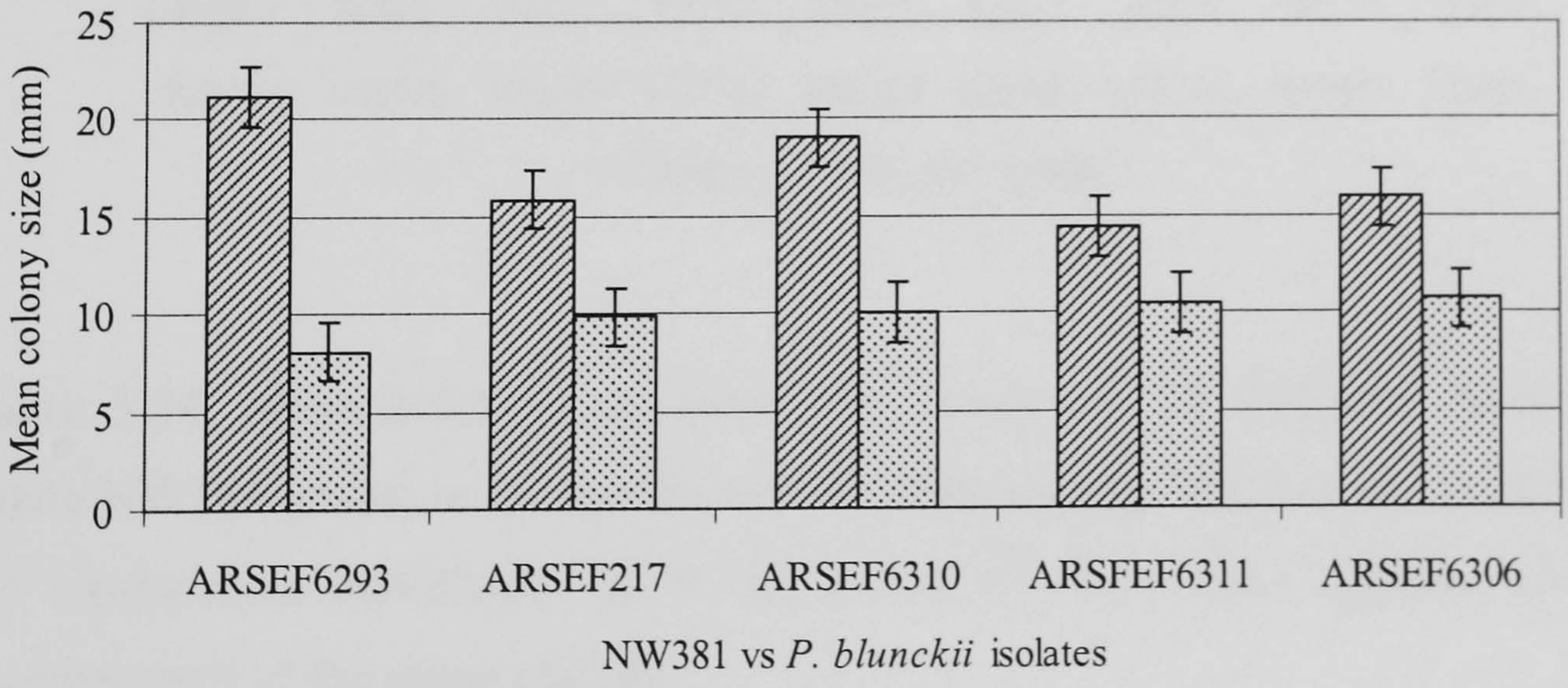


Figure 3.23. Mean colony sizes (mm) 192 hours p.i. for *Pandora blunckii* isolate NW381 growing in the presence of con-specific isolates at the two temperature conditions. 20 °C ▨, 25 °C ⊠. Error bars represent one standard error of the mean (SEM).

Pandora blunckii isolates NW381, ARSEF6306 and ARSEF6311 showed significant differences in their mean colony size when grown in the presence of *Z. radicans* isolates (Table 3.13). Isolate NW381 achieved the smallest colony size when grown in the presence of *Z. radicans* isolate NW168, and the largest colony size in the presence of isolate NW325. However, these results were not consistent over the two temperatures. Overall, the smallest mean colonies were obtained when grown at 25 °C compared to 20 °C (Fig. 3.24). Isolate ARSEF6306 achieved the smallest mean colony size when grown in the presence of isolate NW325, and the largest mean colony size in the presence of isolate NW168 (Fig. 3.25). The mean colony sizes of isolate ARSEF6311 when grown in the presence of different *Z. radicans* isolates were inconsistent over the two temperatures. For example, when grown in the presence of isolate NW386, the smallest mean colony size was achieved at 25 °C rather than at 20 °C but *vice versa* when grown in the presence of isolate NW328 (Fig. 3.26).

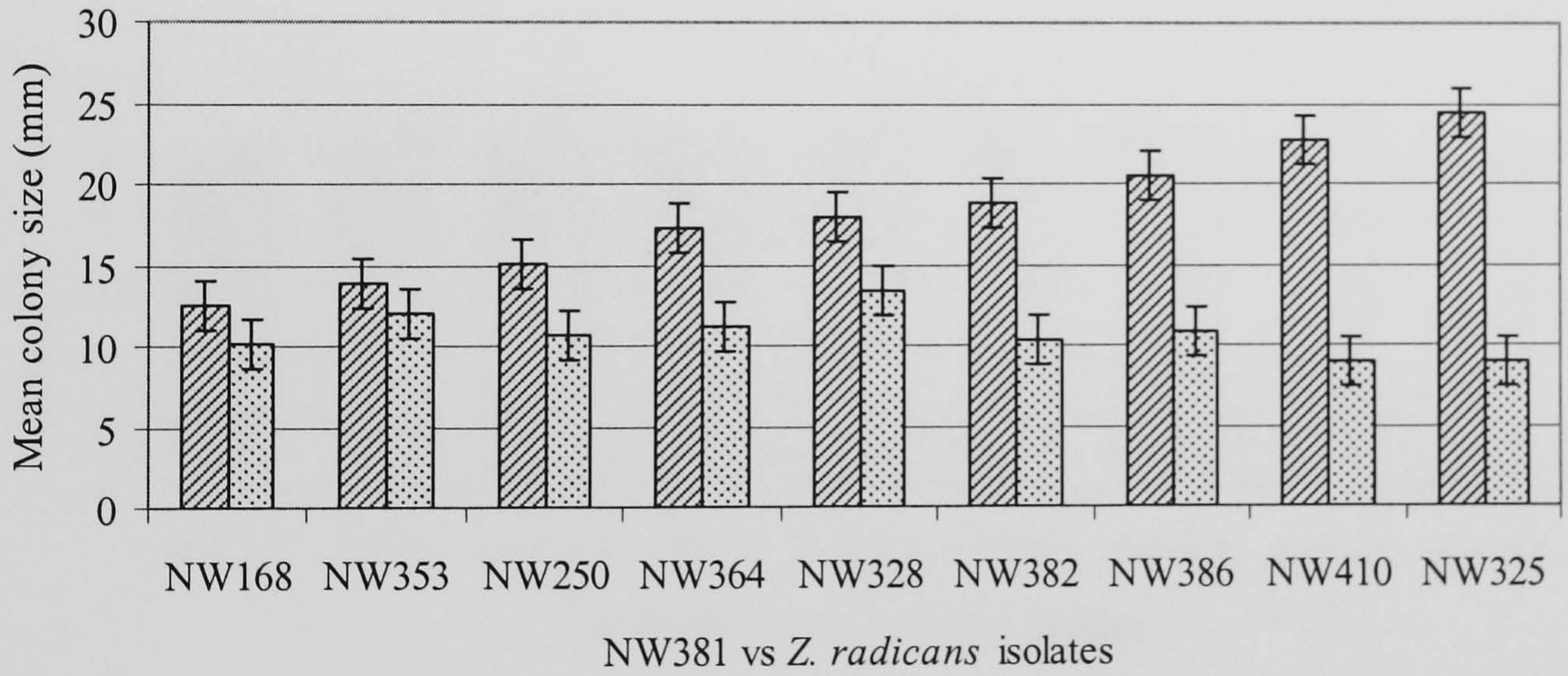


Figure 3.24. Mean colony sizes (mm) 192 hours p.i. for *Pandora blunckii* isolate NW381 grown in the presence of all *Zoophthora radicans* isolate at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

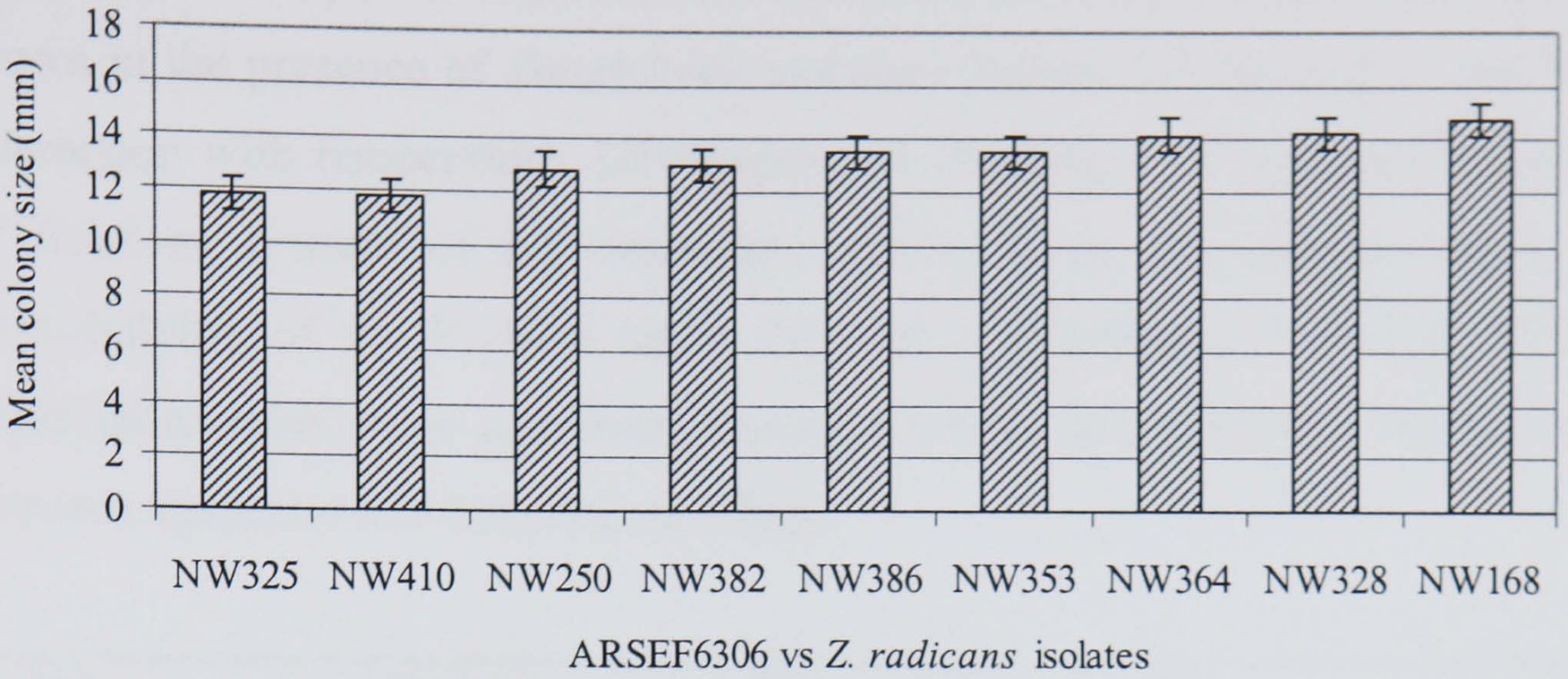


Figure 3.25. Mean colony sizes (mm) 192 hours p.i. for *Pandora blunckii* isolate ARSEF6306 grown in the presence of each *Zoophthora radicans* isolate averaged over the two temperatures conditions. Error bars represent one standard error of the mean (SEM).

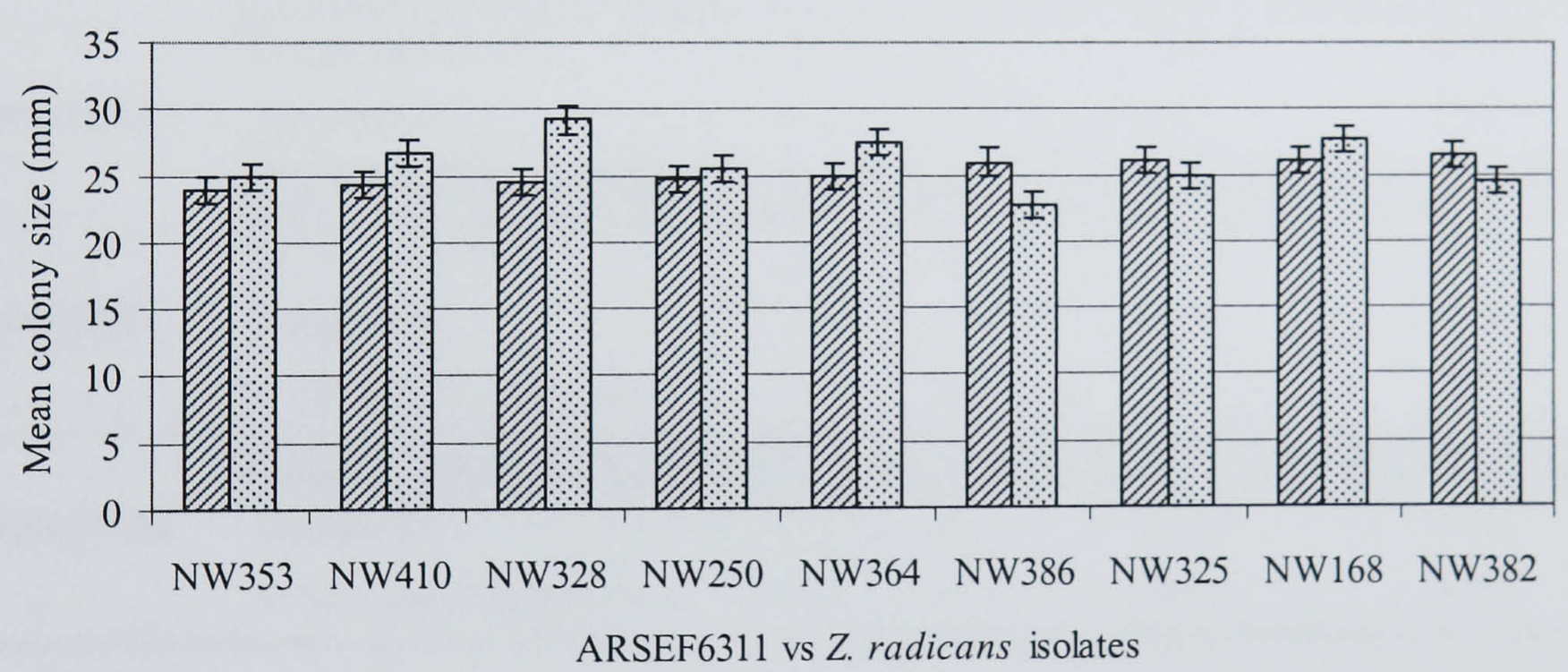


Figure 3.26. Mean colony sizes (mm) 192 hours p.i. for *Pandora blunckii* isolate ARSEF6311 grown in the presence of each *Zoophthora radicans* isolate at the two temperature conditions. 20 °C ▨, 25 °C ⊡. Error bars represent one standard error of the mean (SEM).

Table 3.13. Comparison of the mean colony sizes of *Pandora blunckii* isolates grown in the presence of *Zoophthora radicans* isolates 192 hours p.i., and the interaction with temperature. DF=degrees of freedom, RDF=residual degrees of freedom, F=ratio of the treatment variance over the residual variance, P=probability of the F value taken from an F distribution with DF, RDF. Highlighted rows show significant treatment effect and significant interaction between treatment and temperature effect.

Isolate	Treatment	DF	RDF	F	P
ARSEF6293	*Z. radicans* isolates	8	57	0.95	0.482
	Temperature	1	2	30.74	0.031
	Z. radicans.Temperature	8	57	0.84	0.568
ARSEF6310	*Z. radicans* isolates	8	57	0.77	0.630
	Temperature	1	2	7.06	0.117
	Z. radicans.Temperature	8	57	0.97	0.472
NW381	*Z. radicans* isolates	8	56	2.59	0.017
	Temperature	1	2	51.25	0.019
	Z. radicans.Temperature	8	56	4.95	<0.001
ARSEF6311	*Z. radicans* isolates	8	30	1.63	0.157
	Temperature	1	2	2.57	0.355
	Z. radicans.Temperature	8	30	3.07	0.012
ARSEF217	*Z. radicans* isolates	8	56	1.52	0.171
	Temperature	1	2	2.69	0.242
	Z. radicans.Temperature	8	56	0.99	0.451
ARSEF6306	*Z. radicans* isolates	8	57	2.75	0.012
	Temperature	1	2	0.01	0.941
	Z. radicans.Temperature	8	57	0.67	0.716

Of the 288 tests made, a total of 58 were found to be significant at the 5% level (i.e. about four times as many as the 14.4 expected by chance) giving an FDR of 24.82%. This gives a reasonable level of confidence that the results that were obtained have not occurred purely by chance.

3.3.3. Mycelial interactions

In all the control combinations, the colonies merged forming a uniform mycelial mat (Fig. 3.27a).

3.3.3.1. Intra-specific mycelial compatibility in Zoophthora radicans

Somatic incompatibilities were observed between some isolates (Fig. 3.27b, c and d). From the summary of compatibilities between isolates, it can be seen that isolate NW168 from Japan was incompatible with all other isolates at both temperatures (Table 3.14). One group of isolates were compatible among themselves at both temperatures and they included NW325, NW328, NW364 and NW410. A second group of compatible isolates at both temperatures were NW353, NW382 and NW386, all from Mexico. Isolate NW250 was inconsistent in its response to isolates in the first group, being compatible with isolates NW328 and NW410, but only partialy compatible with isolates NW325 and NW364 at 20 °C. At 25 °C NW250 was incompatible with all isolates in the first group except NW410 for which it was partially compatible. Isolates NW353 and NW382 were inconsistent demonstrating partial compatibilities or compatibilities that changed with the temperature e.g. isolate NW250 and NW353 were incompatible at 20 °C but compatible at 25 °C. Isolate NW386 demonstrated incompatibility with isolates in the first group at 20 °C but at 25 °C was compatible with isolates NW325 and NW328, and partially compatible with isolates NW364 and NW410. Incompatibility between isolates NW386 and NW250 was consistent at both temperatures (Table 3.14).

3.3.3.2. Intra-specific mycelial compatibility in Pandora blunckii

All the isolates of this species came from Mexico except ARSEF217 which came from Germany. Although most of the isolates came from the same geographical region, some mycelial incompatibilities were still observed. Isolate ARSEF217 from Germany was the most incompatible isolate at both temperatures. It failed to form a uniform mat of mycelium in any of the

combinations tested. Isolates ARSEF6310, ARSEF6311 and NW381 formed a group that were compatible amongst themselves at both temperatures. Isolates ARSEF6306 and ARSEF6293 were partially compatible with each other at 20 °C but completely compatible at 25 °C. These isolates were also inconsistent in their response to two of the isolates in the first group, ARSEF6310 and ARSEF6311, with which they were not compatible at 20 °C but partially compatible at 25 °C (Table 3.15).

Partial compatibility demonstrated by some isolate combinations was exemplified by different behaviour amongst replicates of the same isolate combination. For example, in one replicate of the ARSEF6293 and NW381 combination, the former isolate seemed to be replacing the latter isolate (Fig. 3.27f), but in the other replicates no such activity was observed. In the combination of ARSEF6293 and ARSEF6311, the latter isolate appeared to be antagonistic to ARSEF6293, based on the appearance of the ARSEF6293 colony which looked completely flat and lysed (Fig. 3.27e). This result was not observed in the other replicates.

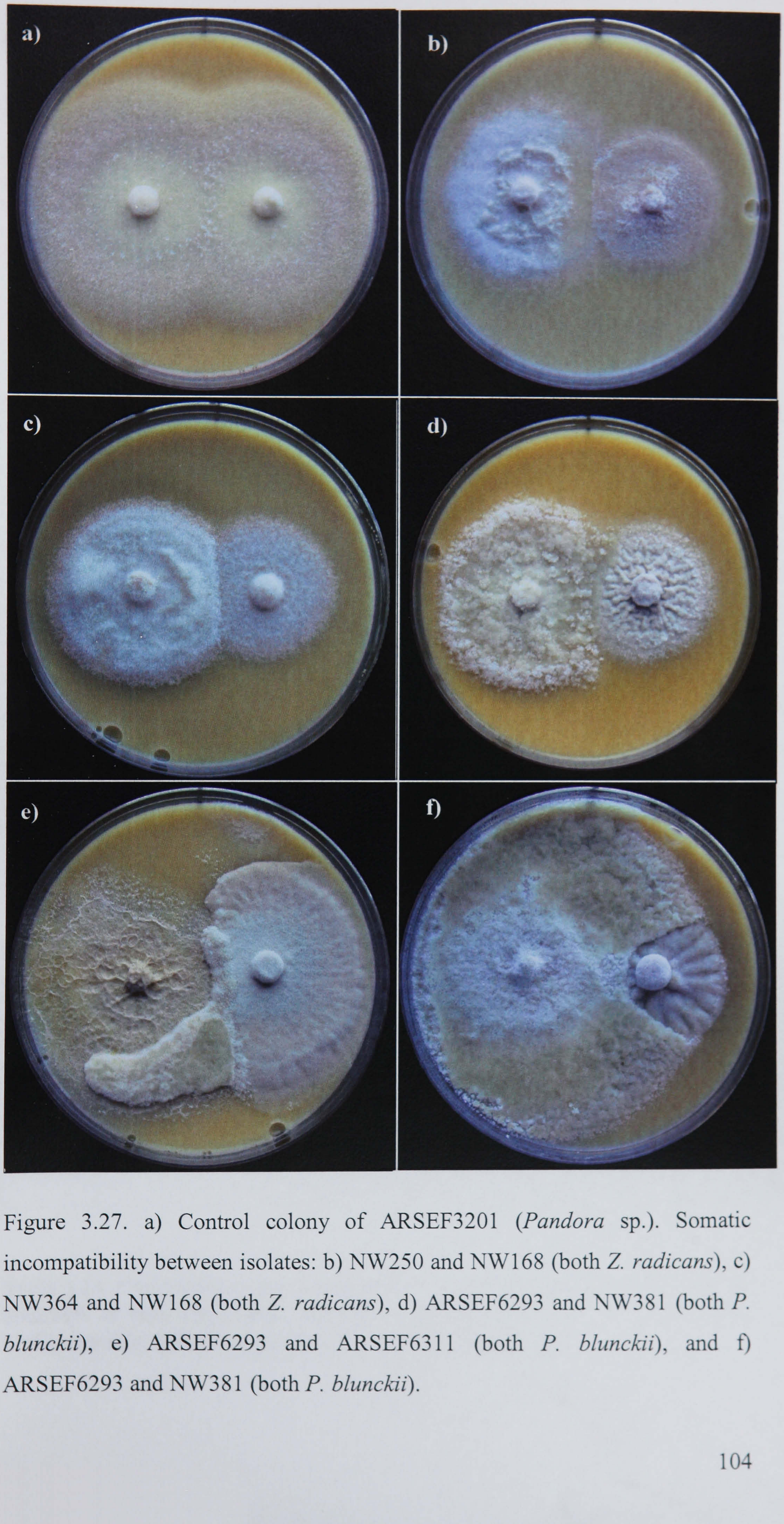


Figure 3.27. a) Control colony of ARSEF3201 (*Pandora* sp.). Somatic incompatibility between isolates: b) NW250 and NW168 (both *Z. radicans*), c) NW364 and NW168 (both *Z. radicans*), d) ARSEF6293 and NW381 (both *P. blunckii*), e) ARSEF6293 and ARSEF6311 (both *P. blunckii*), and f) ARSEF6293 and NW381 (both *P. blunckii*).

3.3.3.3. Inter-specific mycelial interactions

When the mycelial interactions were inter-specific, all the outcomes were incompatible. By 336 hours post inoculation at both temperatures all the combinations ended in deadlock (Fig. 3.28a, b, c, d and e). The presence of stationary barrages and pigmentation was observed at 25 °C. In the majority of these cases, these structures were formed between *P. blunckii* isolates such as ARSEF6310, ARSEF6311 and NW381 in the presence of *Z. radicans* isolates (e.g. Fig. 3.28d). However, *Z. radicans* isolates also produced pigmentation as a response to *P. blunckii* (Fig. 3.28e).

a)

	Kenya	New Zealand	Australia	Australia	Malaysia	Mexico	Mexico	Mexico	Japan
	NW325	NW328	NW364	NW410	NW250	NW353	NW382	NW386	NW168
NW325	CN	C	C	C	PC	NC	PC	NC	NC
NW328		CN	C	C	C	PC	NC	NC	NC
NW364			CN	C	PC	NC	PC	NC	NC
NW410				CN	C	PC	NC	NC	NC
NW250					CN	NC	NC	NC	NC
NW353						CN	C	C	NC
NW382							CN	PC	NC
NW386								CN	NC
NW168									CN

b)

	Kenya	New Zealand	Australia	Australia	Malaysia	Mexico	Mexico	Mexico	Japan
	NW325	NW328	NW364	NW410	NW250	NW353	NW382	NW386	NW168
NW325	CN	C	C	C	NC	PC	C	C	NC
NW328		CN	C	C	NC	PC	C	C	NC
NW364			CN	C	NC	PC	C	PC	NC
NW410				CN	PC	NC	PC	PC	NC
NW250					CN	C	NC	NC	NC
NW353						CN	C	C	NC
NW382							CN	C	NC
NW386								CN	NC
NW168									CN

Table 3.14. Compatibility amongst *Zoophthora radicans* isolates from different countries at two temperature conditions. a) 20 °C, b) 25 °C. CN=Control, C=Compatible, NC=Incompatible, PC=partially compatible.

a)

	Mexico	Mexico	Mexico	Mexico	Mexico	Germany
	ARSEF6310	ARSEF6311	NW381	ARSEF6306	ARSEF 6293	ARSEF217
ARSEF6310	CN	C	C	NC	NC	NC
ARSEF6311		CN	PC	NC	NC	NC
NW381			CN	NC	PC	NC
ARSEF6306				CN	PC	NC
ARSEF 6293					CN	NC
ARSEF217						CN

b)

	Mexico	Mexico	Mexico	Mexico	Mexico	Germany
	ARSEF6310	ARSEF6311	NW381	ARSEF6306	ARSEF 6293	ARSEF217
ARSEF6310	CN	C	C	PC	PC	NC
ARSEF6311		CN	C	PC	PC	NC
NW381			CN	PC	NC	NC
ARSEF6306				CN	C	NC
ARSEF 6293					CN	NC
ARSEF217						CN

Table 3.15. Compatibility amongst *Pandora blunckii* isolates from different countries at two temperature conditions. a) 20 °C, b) 25 °C. CN=Control, C=Compatible, NC=Incompatible, PC=partially compatible.

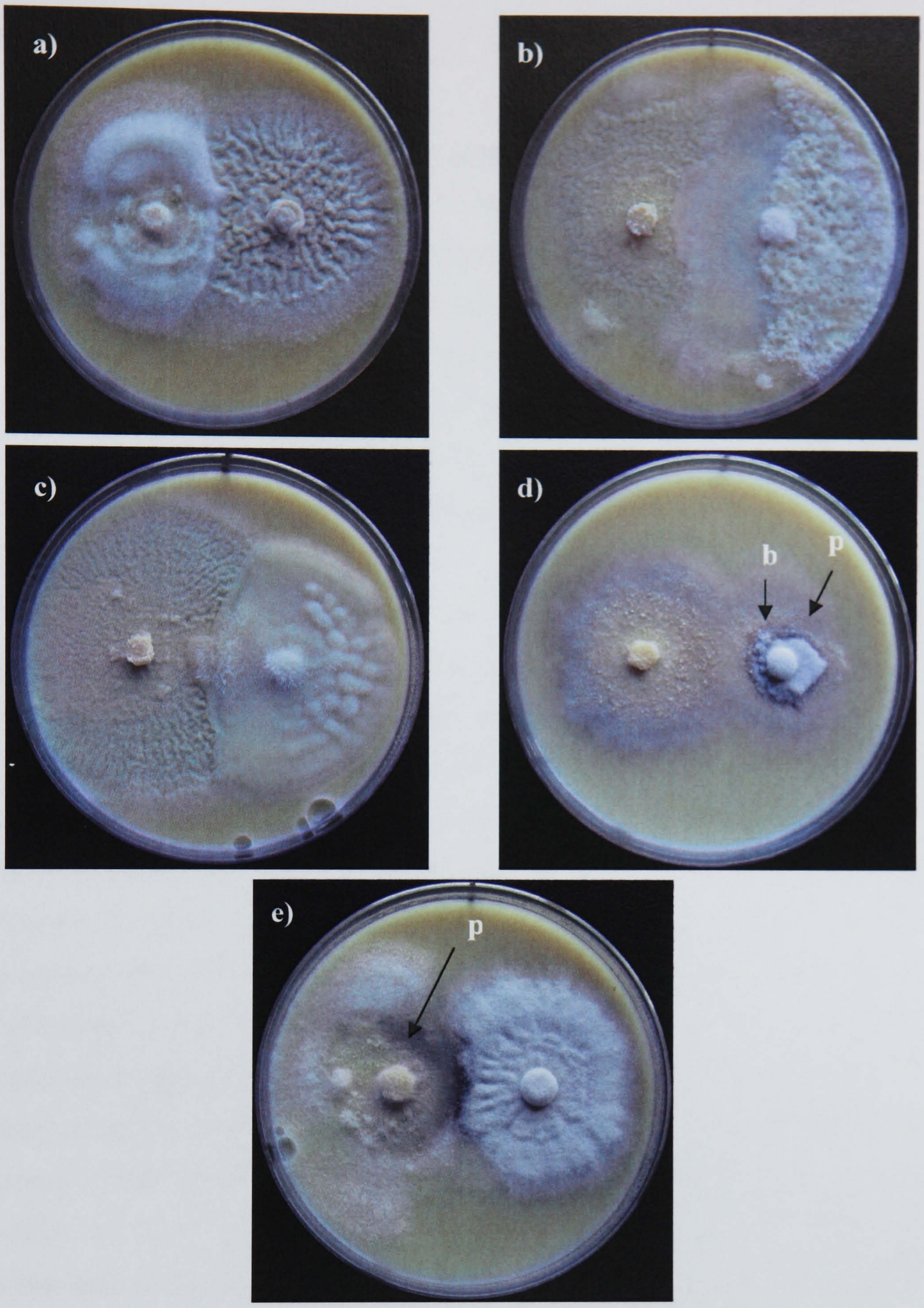


Figure 3.28. Inter-specific interactions showing deadlock: a) Isolates NW364 (*Z. radicans*) and NW381 (*P. blunckii*), b) Isolates NW386 (*Z. radicans*) and ARSEF6293 (*P. blunckii*), c) Isolates NW382 (*Z. radicans*) and ASREF217 (*P. blunckii*), d) Isolates NW382 (*Z. radicans*) and ARSEF6311 (*P. blunckii*), also it can be seen in this picture that isolate ARSEF6311 is producing a barrage and pigment as a result of the interaction with NW382 (b=barrage, p=pigment), e) Isolates NW328 (*Z. radicans*) and ARSEF6311 (*P. blunckii*), isolate NW328 producing a pigmentation as a response to the presence of ARSEF6311.

3.4. DISCUSSION

The experiments carried out in this chapter, although they were *in vitro* and may not fully represent what would be happening within the host, showed that the interactions between these two fungal species can be complex. There was a large variability amongst isolates of the same species in their response to the presence of a con-specific isolate or from other species. These interactions can be affected by external factors such as temperature and the absence or not of physical contact between the interacting isolates. The later was represented by the results obtained after 96 and 192 hours post-inoculation.

The fact that some significant effects were found at 96 hours, when both colonies had made no contact, could suggest the existence of some volatiles or diffusible compounds affecting the growth of the isolates. In previous studies, when volatiles were involved, the type of interaction was more likely to be antagonism, where one mycelium was degenerated and replaced by the other, or mutual inhibition where both colonies never contact each other, which is common in agar cultures (Boddy, 2000). In my study, the interactions began at a distance but all the combinations eventually contacted each other after 192 hours and no mycelium was degenerated or replaced. The absence of volatiles was also suggested by the fact that most of the interactions occurred at 192 hours after inoculation, when the interacting colonies had physical contact. The possible differences in the growth of isolates at 96 hours may be due to the ability of some isolates to grow faster in response to the presence of a competitor and use the majority of the resources leaving a limited amount for the other isolate and, therefore, limiting its growth rate compared to growth in the absence of competitors, this is called competition by exploitation.

All the isolates from *Z. radicans* and *P. blunckii* that showed significant differences in the mean colony size when grown in the presence of con-specific isolates or the other two species, had smallest colony sizes in the presence of *Pandora* sp. Growth of the *Pandora* sp. isolate was not affected by any isolate of the other two species. This suggests that this isolate is a strong competitor in the environment. Further *in vivo* experiments will need to be done to confirm

this and evaluate the effect of this isolate on the normal infection process of *P. blunckii* and *Z. radicans* isolates separately and in dual inoculation of *P. xylostella*. The *Z. radicans* isolates that showed significant differences in their mean colony size when grown in the presence of con-specific isolates or isolates of the other two species, were affected more by the presence of *P. blunckii* than by the presence of con-specific *Z. radicans* isolates. The *P. blunckii* isolates were only affected by the presence of *Pandora* sp. and not *Z. radicans* or other con-specific isolates of *P. blunckii*. These results suggest that *P. blunckii* may be more competitive than *Z. radicans*.

Initially, the *Pandora* sp. isolate was considered as *P. blunckii*. However after molecular analysis based on the ribosomal DNA (Chapter 4) it was confirmed that this isolate was from the genus *Pandora* (based on spore morphology) but not the species *P. blunckii* (based of the product size after a PCR amplification of the ITS region of the rDNA). In addition, in a bioassay (data not shown), this isolate was not pathogenic to *P. xylostella* larvae. This result is interesting because it demonstrates that *Z. radicans* and *P. blunckii* are not just interacting with each other when infecting *P. xylostella* but also with other species within the same ecosystem. Boddy (2000) has suggested in other systems that, although a co-occurring species may not be competing for the same host, its presence could modify the possible outcomes of any interactions in the same way as abiotic factors.

When the individual effect of the *P. blunckii* isolates on the *Z. radicans* isolates was compared, smallest mean colony sizes always occurred in the presence of *P. blunckii* isolate ARSEF6293. This suggests that the negative species effect of *P. blunckii* on *Z. radicans* was due to isolate ARSEF6293 alone. This also suggests that the isolate effect is more important that the species effect in interactions between *P. blunckii* and *Z. radicans*. Also, there were a few *Z. radicans* isolates that were not affected by any of *P. blunckii* isolates, for example isolate NW386.

Overall, intra-specific interactions were not common over all the combinations. There were only three significant intra-specific interactions among the *Z.*

radicans isolates. Mean colony sizes of isolates NW382, NW250 and NW168 were reduced in presence of isolates NW325, NW410 and NW382.

The outcomes of these interactions may be classified as a result of primary resource capture, which suggests initial access and influence over available resources are affected (Cooke and Rayner, 1984). However, this interaction is strongly influenced by the time of arrival. In this experiment, because both isolates were inoculated at the same time, the ability of some isolates to occupy more of the resources was likely to be directly related to a faster germination and growth rate.

Although the results suggest that *Z. radicans* was less competitive than P. *blunckii*, both pathogens can still be found in the same environment. This suggests that Z. *radicans* must have alternative survival strategies. Many of the *Z. radicans* isolates produced resting spores, particularly the Mexican isolates (Chapter 5), which allow this species to survive adverse conditions and germinate later or in the next season. Adverse conditions are usually considered to be abiotic conditions such as temperature and humidity. However, the presence of strong competitors could also contribute to an adverse environment. It is possible that *Z. radicans* produces resting spores as part of survival mechanisms to avoid direct competition with *P. blunckii* or other microorganisms present in the same environment. *Zoophthora radicans* isolate NW250 did not form resting spores. This isolate came from Malaysia and no *P. blunckii* was recorded at the time when the isolate was found or in subsequent collections (Pell J.K., personal communication), which may suggest that NW250 did not have to compete for a resource or that the environmental conditions were favourable for the majority of the time.

The outcomes of most of the interactions were not significantly affected by temperature. This is probably because only three species were used, and the range of temperature optima for these species was similar (20 and 25 °C). The effect of temperature may be more evident when interactions take place among more than three species (e.g. Marin *et al.*, 1998a, b; Lee and Magan, 1999a). The more species considered, the greater the chance that they will have a

diverse range of optimal temperatures and that temperature will have a greater effect. However, isolate level differences had an important role in some interactions. Isolates like ARSEF6293 always had the same effect on the other isolates regardless of the temperature. It is possible that isolates where the response to the presence of another isolate was affected by temperature may not be so successful in the field, because if the temperature conditions are not optimal these isolates would easily be out-competed by other species. The variability in the response of different isolates of the same species to temperature may be an important strategy for survival of that species because it allows the species to survive under different environmental conditions.

Mycelial interactions

Heterokaryon formation between different fungal individuals is an important part of the fungal life cycle. This also allows haploid fungi to enjoy the benefits of functional diploidy. Somatic or vegetative incompatibility represents a means to self and non-self recognition (Leslie, 1993). Compatible, individuals form a uniform mat of mycelium when grown together (Barlocher, 1991). Based on these definitions, some somatic incompatibilities were found amongst *Z. radicans* isolates and amongst *P. blunckii* isolates.

Zoopthora radicans isolates were clearly divided into four groups, the Mexican isolates, isolates from Kenya, Australia and New Zealand in another group, the Malaysian isolates and the isolate from Japan in a fourth group. This suggests that isolates within the same group originated from the same parental lines, even though, in the second group, they came from different countries. The isolate from Japan (NW168) was not compatible with any other *Z. radicans* isolates, which suggests that this isolate came from a completely different parental line. Isolate NW250 (Malaysia) showed partial compatibility with the second group (Kenya, Australia and New Zealand) (in at least one out of three replicates it was not compatible), which might be as a result of a lot of variability in the genetic background of this isolate.

Although the *P. blunckii* isolates came from the same region (except ARSEF217 from Germany), they also showed high somatic incompatibility amongst themselves. Isolate ARSEF217 definitely belonged to a different group because it did not form a uniform mat of mycelium when grown in the presence of any of the other isolates, and this was consistent at both temperatures.

At the mycelial level, somatic incompatibility was also associated with production of barrages and/or clear demarcation zones between isolates which may be formed when mycelia die (Leslie, 1993; Rayner, 1991; Cilliers *et al.*, 2000). In my observations no antagonistic activity was observed amongst isolates of the same species even when they belonged to different somatic compatibility groups. However, observations were not made microscopically and only those combinations with an obvious separation were considered to be incompatible. There is also a chance that a true somatic rejection might be confused with other phenomena such as mutual "staling" or nutrient depletion (Rayner, 1991). In these cases, a comparison with a control is important.

In the inter-specific interactions, although all the interactions ended in deadlock, some combinations seemed to demonstrate that partial replacement was underway which eventually ended in deadlock. This is difficult to confirm because it could also be as a result of differences in the speed of growth that allowed one isolate to occupy more space that the other. An example of this is isolate ARSEF6293 that always acquired more physical space than other isolates in the Petri dish, suggesting partial replacement. However, at the same time it had the greatest growth *in vitro* compared to the other *P. blunckii* isolates (Chapter 2) suggesting a speed of growth effect as well. In this experiment, however, only deadlock was considered because it was difficult to determine whether replacement or partial replacement had taken place.

The presence of stationary barrages and dark pigmentation was present in many inter-specific interactions but only at 25 °C. Although it is difficult to be certain about which species is forming the barrier in most of the combinations, some assumptions were considered. The first and easiest assumption is when

the pigment is within the colony (Fig 3.46e). When the barrier and the pigmentation is in the border line of both colonies (3.46d). normally one colony is bigger than the other, this suggest that the bigger colony was more successful in using the resources gaining physical space from the smaller colony, so it is assumed that the smaller colony is forming the barrier to try to stop the bigger colony replacing it. Based on this, some *P. blunckii* isolates made what appeared to be barriers around the colony as a response to the invasion of some *Z. radicans* isolates. Similar responses have been reported by other authors for other fungal genera and the barrier is termed a "stationary barrage" resistant to invasion (Boddy, 2000). A barrage can be the result of the encounter of two incompatible strains or species that grow into each other and interact in an antagonistic way; this consists of a central region that contains dead or dying cells and a dark pigment may also be deposited (Leslie, 1993). Pigments have been suggested to slow or prevent the advance of other fungi (Griffith *et al.*, 1994a). The fact that the stationary barrages and pigmentation were present mainly at 25 °C and caused by the presence of *Z. radicans* could be related to the optimal temperature of *Z. radicans* that is 25 °C (Chapter 2). At this temperature, *Z. radicans* isolates showed greater growth than *P. blunckii* isolates, which suggests that under optimal temperature conditions some isolates of this species may be more competitive than some isolates of *P. blunckii*. It is likely that in the field, *Z. radicans* under optimal temperatures can competitively exclude *P. blunckii* from dual inoculated larvae.

In conclusion, the results suggest that *P. blunckii* is more competitive than *Z. radicans*. It is clear, however, that much of this effect is due to isolate ARSEF6293. The existence of other microorganisms in the same environment of *P. xylestella*, even when they do not compete for the same host, can affect the possible interactions that may occur between *P. blunckii* and *Z. radicans*. Temperature did not have a large effect on the interactions, this may be because the *Z. radicans* and *P. blunckii* had similar optimal growth temperatures. Although the number of intra-specific interactions was small compared to the inter-specific interactions, mycelial incompatibility was found amongst isolates within each species, which suggest different parental lines.

therefore, the genetic variability of these isolates was further investigated (Chapter 4).

It is difficult to know *a priori* if the results obtained in this *in vitro* experiment will be reflected *in vivo*. As mentioned before, biotic and abiotic factors can modify the result of an interaction. Included in the biotic factors are good dispersal, rapid spore germination, the ability to use the resources by production of suitable enzymes, tolerance to adverse conditions (Boddy, 2000; Rayner and Webber, 1994), but also virulence. Virulence can be very variable among isolates of the same species (e.g. Pell *et al.*, 1993a), and could also have an important role in the outcome of an interaction *in vivo*. The more information that is obtained about the interactions among these two species *in vitro* and *in vivo*, the more possibilities there will be to understand the ecology of these two species and their interaction with *P. xylostella*.

To achieve this, the relative virulence of isolates selected for their contrasting *in vitro* competitive abilities (this chapter) were estimated as *in vivo* competing ability (Chapter 5).

As demonstrated in this chapter, the outcomes of interactions may change with the time that fungi are in contact. This is likely to happen inside the host during a dual infection. To evaluate this, isolates from each species must be recognized inside the host and the amount of mycelium quantified from each species. However, this cannot be investigated using conventional methods such as morphological differentiation of mycelia. Therefore, quantitative real time PCR was developed and used to detect changes in the amount of DNA of one species in the presence of the other during the course of a dual infection (Chapter 6).

CHAPTER 4. GENETIC VARIABILITY OF *Zoophthora radicans* AND *Pandora blunckii* ISOLATES

ABSTRACT

The genetic variation within and between *P. blunckii* isolates from *P. xylostella* and *Z. radicans* isolates from both *P. xylostella* and other insect hosts was investigated. The DNA sequences that encode for ribosomal RNA, more specifically, the internal transcribed spacer (rDNA-ITS) were analysed using PCR-RFLP for 22 isolates of *P. blunckii* and 39 isolates of *Z. radicans*. For *P. blunckii*, all the isolates were from the same host (*P. xylostella*) and from Mexico (21 isolates) and Germany (one isolate). The *Z. radicans* isolates were more diverse in geographical and host of origin. The size of the rDNA-ITS region from these two species was different, 1550 bp for *Z. radicans* and 2100 bp for *P. blunckii.* No rDNA-ITS region size variation was found amongst isolates of the same species. The PCR-RFLP analysis showed that *Z. radicans* was genetically more diverse than *P. blunckii,* although no direct relationship was found with host or geographical origin. The genetic variation within the *Z. radicans* isolates was further confirmed by the sequence information of the rDNA-ITS region obtained from selected isolates. The relationship between the genetic variability within both fungal species and host specificity or ecological adaptation is discussed.

4.1 INTRODUCTION

Characterisation of isolates based only on morphological attributes is often insufficient to detect differences between, or within species. In recent years, attention has increasingly focussed on the DNA molecule as a source of informative polymorphisms. There are many techniques that help to identify polymorphisms but one of the most important is the Polymerase Chain Reaction (PCR). This technique results in the *in vitro* amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotides (primers) and a thermostable DNA polymerase, the

electrophoretic separation of the amplified fragments and the detection of polymorphic banding by staining. However, it would be wrong to think that this technique is a "stand alone" characterisation method. It should be used in conjunction with morphological, biochemical and biological properties (Karp *et al.*, 1996).

4.1.1 Some molecular techniques used for screening fungal diversity

There is a range of techniques that can be used as well as genes that can be targeted to evaluate diversity or evolutionary relationships among species or taxa. Some techniques are described here, particularly those used to assess diversity at a population level.

4.1.1.1 RFLP (Restriction Fragment Length Polymorphisms)

This technique has been widely used at different taxonomic levels for fungal systematics and a large selection of mitochondrial and chromosomal genes have been used. These include those derived from the ribosomal RNA genes, and have produced important information on relationships within and between fungal species (Paterson and Bridge, 1994).

The technique uses enzymes which recognise and cut specific nucleotide sequences (4-6 base-pair sequences), reducing large DNA pieces to fragments with smaller sizes. A polymorphism occurs when the mutation of a single base-pair results in the loss or creation of a restriction site, or when by insertion or deletion, the size of a restriction fragment is altered. The fragments as a result of a digestion are separated by gel electrophoresis (Brettshneider, 1998). In this technique, the total genomic DNA is digested with restriction enzymes, and as a result large quantities of good quality DNA are required. Fortunately, this limitation has been removed with the development of PCR for amplification DNA (Karp *et al.*, 1996).

4.1.1.2 Random amplified microsatellites (RAMS)

This technique combines the use of microsatellites or simple sequence repeats (SSR) and random amplified polymorphic DNA analysis (RAPD). Random amplified microsatellites analysis (RAMS) amplifies microsatellites randomly and, instead of using arbitrary sequence primers as in RAPDs, uses primers that recognise interspaced repetitive sequences (microsatellites) that can be found in all eukaryotic genomes. In RAMS analysis, the DNA between distal ends of two closely located microsatellites is amplified and the results can be visualised by gel electrophoresis (Zietkiewicz *et al.*, 1994; Hantula and Muller, 1997). The primers used in RAMS analysis are unique sequences (2 to 4 bases) anchored to the 3' or 5' end of the $(CA)_n$ repeats. Although there are other repeats, the $(CA)_n$ repeat is the most common in the human genome and appears to be present in other eukaryotes also. This design avoids the risk of primers attaching within a $(CA)_n$ repeat which provides specificity and reduces the number of genomic regions amplified to only those matching the unique sequence anchored to the $(CA)_n$ (Zietkiewicz *et al.*, 1994).

RAMS have been primarily used to detect variability in animals and plants (Zietkiewicz *et al.*, 1994). However, their utility to detect variability in fungi has also been confirmed (Hantula *et al.*, 1996; Hantula and Muller, 1997; Tymon and Pell, 2005).

4.1.1.3 Enterobacterial repetitive intergenic consensus (ERIC)

The existence of highly conserved sequences called enterobacterial repetitive intergenic consensus (ERIC), was described by Hulton *et al.*, (1991). They identified 14 in *Escherichia coli*, *Salmonella typhimurium* and three other enterobacterial species. These were 126 bp long and found on transcribed regions of the genome. The use of this sequence was then proposed as a fingerprinting tool for bacterial genomes and primers were developed. The primers attach randomly and amplify repetitive sequences that occur throughout the genome producing a multiple band pattern (Versalovic *et al.*, 1991). ERIC primers have also been used on the nematophagous fungus,

Pochonia chlamydosporia (Goddard) Zare and Gams, to detect genetic variation among isolates of the same species (Arora *et al.*, 1996; Morton *et al.*, 2003).

4.1.2 Ribosomal RNA

Comparative studies of the nucleotide sequence of ribosomal RNA genes have been extensively used to analyse phylogenetic relationships as well as variability among species or even populations (White *et al.*, 1990).

The ribosomes are cellular organelles directly involved in protein synthesis. They are made of RNA and protein and are produced in the nucleus. There are three major classes of RNA, all of which participate in protein synthesis: ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). All of these are synthesised under the direction of the DNA in a process called transcription. The rRNA and proteins are the major components of the ribosome. These are cellular organelles directly involved in protein synthesis. The ribosome can be divided into unequally sized subunits; the small subunit that consists of an 18S rRNA molecule and 21 different polypeptides, and the large subunit that contains a 5S and a 28S rRNA together with 31 different polypeptides. These subunits attach together to the mRNA to produce proteins (Voet and Voet, 1995). The genes encoding for these subunits are separated by internal transcribed spacer regions (ITS), and the whole gene cluster is separated from another by intergenic spacer sequences (IGS) (Bridge, 2002).

The DNA sequences that encode for ribosomal RNAs have been extensively used to study taxonomic and genetic variation in fungi (Bruns *et al.*, 1991). The great cellular demand for rRNA can only be satisfied through the production of multiple copies of the genes that encode them (Voet and Voet, 1995).

The ribosomal RNA gene cluster is found in both nuclei and mitochondria, and consists of highly conserved and variable regions (Bridge and Arora, 1998). The small-subunit sequences evolve relatively slowly and are useful for studying distantly related organisms. The internal transcribed spacer (ITS)

region and intergenic spacers (IGS) of the nuclear rRNA repeat units evolve faster and may vary among species within a genus or even among populations of the same species (White *et al.*, 1990).

4.1.2.1 Internal transcribed spacers (ITS)

The ITS region consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterisation studies on fungi for four main reasons:

1. It is usually short (e.g. 500 – 800 bp) and can be easily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rRNA subunit genes (White *et al.*, 1990).
2. The multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or highly degraded DNA.
3. The ITS region may be highly variable among morphologically similar species, and ITS-RFLP restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis.
4. Many researchers have used this region to develop species-specific primers, due to its diversity between species and high copy number, making it an ideal target for PCR amplification (Bridge and Arora, 1998).

4.1.3 Molecular characterisation of entomophthoralean fungi

Although there has been some research done to describe phylogenetic relationships among the different entomophthoralean genera (e.g. Jensen *et al.*, 1998; Nielsen *et al.*, 2001), one of the main interests has been to find genetic variability among isolates of the same species. The two species most extensively studied are *Zoophthora radicans* and *Pandora neoaphidis*. Research carried out on *Z. radicans* using electrophoresis of isoenzymes and other taxonumerical aspects found intra-specific variation among *Z. radicans* isolates with a host-specificity grouping (Milner and Mahon, 1983; Glare *et al.*,

1987). More recently, this was also confirmed by Hodge *et al.* (1995) using RAPD-PCR techniques which demonstrated variability among *Z. radicans* isolates. They found a clear relationship among isolates from the same host and attempted to follow the establishment of exotic isolates in the field. Intra-specific variability was also found by Tymon *et al.* (2004) in different entomphthoralean species, including *Z. radicans* using PCR amplification of the ITS region and restriction analysis.

Although there has been no research on genetic variability in *Pandora blunckii*, there has been a lot of work done on *Pandora neoaphidis*, using different molecular techniques. Using ITS-PCR amplification, differences in the size of the ITS region were found (Rohel *et al.*, 1997; Sierotzky *et al.*, 2000). However, recently no variability was found in the sequence of the ITS region among different *P. neoaphidis* isolates suggesting it to be a very conserved region in this species (Tymon *et al.*, 2004). This suggests that the previous size variation found by Rohel *et al.* (1997) and Sierotzky *et al.* (2000) could be the result of working with misidentified isolates. A large variability among isolates was found using the RAPD-PCR technique (Rohel *et al.*, 1997; Sierotzky *et al.*, 2000; Nielsen *et al.*, 2001; Tymon *et al.*, 2004). Different approaches have been used such as inter-simple sequence repeats (ISSR) and ERIC analysis (Tymon and Pell, 2005) where variability was larger using ERIC than ISSR technique.

It is important to know the genetic characteristics of different isolates either for use in microbial control, to track released isolates, or to try to understand the host specificity of an isolate (Driver and Milner, 1998). It is also important to try to understand the possible interactions between species or isolates within the same host population as this will help provide a clearer understanding of the ecology and distribution of the species among the host population which will eventually help in the development of successful microbial control programmes.

The aim of this study was to detect genetic variation within and between *P. blunckii* isolates from *P. xylostella* larvae and *Z. radicans* isolates from *P.*

xylostella and other insect hosts. The isolates from both species were mainly collected from Mexico but isolates from other geographical areas were added to the study.

The sequence information obtained in this study was used for the development of species-specific primers as a tool for monitoring each species in dual inoculated larvae during the infection process (Chapter 6). It also helped to confirm genetic variability amongst isolates that showed mycelial incompatibility within each species previously investigated (Chapter 3).

4.2 MATERIALS AND METHODS

4.2.1 Fungal isolates

For this study, 22 isolates of *P. blunckii* (Table 4.1) and 39 isolates of *Z. radicans* were used (Table 4.2). After retrieval from liquid nitrogen (2.2.1.1.), all the isolates were subcultured onto SEMA (2.2.1.2) in 9 cm triple vented Petri dishes and grown for 15 days at 20 °C in darkness.

4.2.2 Production of mycelium for DNA extraction

All the isolates were grown in liquid media containing Yeast Extract, Milk and Glucose (YEMG) (Sierotzki *et al.*, 2000). Eight 5 mm plugs of fungus from the growing edge of 15 day old cultures were inoculated into 25 ml of sterile medium in a 250 ml flask and incubated on a shaker.

Pandora blunckii isolates were grown at 200 r.p.m. and *Zoophthora radicans* at 250 r.p.m., both at 22 °C for 4 to 6 days. The mycelium was harvested by suction filtration through sterile Whatman No. 1 filter paper in a Buchner funnel. The mycelium was washed twice with sterile distilled water and any residual agar plugs removed. All the mycelium for each isolate was transferred to sterile bijou bottles. The mycelium was frozen overnight, and then freeze dried for 24 hours before DNA extraction.

Table 4.1. Isolates of *Pandora blunckii* used to evaluate genetic variability. NW isolates came from the Rothamsted Research collection, ARSEF isolates came from the USDA-ARSEF culture collection, curated by Dr. Richard Humber.

ISOLATE	HOST	COUNTRY
NW 344	*Plutella xylostella* (Lepidoptera: Plutellidae)	Mexico
NW 345	" "	" "
NW 346	" "	" "
NW 347	" "	" "
NW 348	" "	" "
NW 349	" "	" "
NW 350	" "	" "
NW 352	" "	" "
NW 380	" "	" "
NW 381	" "	" "
NW 383	" "	" "
NW 384	" "	" "
NW 385	" "	" "
ARSEF6293	" "	" "
ARSEF6306	" "	" "
ARSEF6309	" "	" "
ARSEF 6305	" "	" "
ARSEF 6310	" "	" "
ARSEF 6311	" "	" "
NW468	" "	" "
ARSEF1004	" "	Philippines
ARSEF217	" "	Germany

Table 4.2. Isolates of *Zoophthora radicans* used to evaluate genetic variability. NW isolates came from the Rothamsted Research collection, ARSEF isolates came from the USDA-ARSEF culture collection, curated by Dr. Richard Humber.

ISOLATE	HOST	COUNTRY
NW 353	*Plutella xylostella* (Lepidoptera: Plutellidae)	Mexico
NW 378	" "	" "
NW 379	" "	" "
NW 382	" "	" "
NW 386	" "	" "
NW 325	" "	Kenya
NW 168	" "	Japan
NW 328	" "	N. Zealand
NW 329	" "	" "
NW 361	" "	" "
NW 364	" "	Australia
NW 410	" "	" "
NW 182	" "	Taiwan
ARSEF 2893	" "	Philippines
NW 175	" "	Malaysia
NW 323	" "	" "
NW 250	" "	" "
NW 324	" "	" "
ARSEF 1100	" "	" "
ARSEF 4782	*Epinotia aporema* (Lepidoptera: Tortricidae)	Uruguay
ARSEF 1674	*Ptycholoma aeriferana* (Lepidoptera: Tortricidae)	Japan
ARSEF 68	*Tortrix viridana (*Lepidoptera: Tortricidae)	France
NW 358	Unidentified Leafroller	N. Zealand
NW 359	*Eutorna phaulacosma* (Lepidoptera: Oecophoridae	" "
NW 475	*Heliothis sp.* (Lepidoptera:Noctuidae)	" "
NW 169	*Choristoneura fumiferana* (Lepidoptera:Tortricidae)	USA
ARSEF 44	*C. fumiferana* (Lepidoptera:Tortricidae)	" "
ARSEF 1341	*Pieris brassicae* (Lepidoptera:Pieridae)	Poland
ARSEF 2411	*Sesamia inferens* (Lepidoptera:Noctuidae)	Java
ARSEF 6003	*Trichoplusia ni* (Lepidoptera:Noctuidae)	Mexico
ARSEF 1699	*Cnaphalocrocis medinalis* (Lepidoptera:Pyralidae)	Philippines
NW 398	*Drepanosiphon aceris* (Homopetera:Drepanosiphidae)	Switzerland
NW 399	*Metapolophium dirhodum* (Homoptera:Aphididae)	U.S.A.
ARSEF 112	*Therioaphis maculata* (Homoptera:Aphididae)	Israel
ARSEF 685	*Nilaparvata lugens* (Homoptera:Delphacidae)	China
ARSEF 389	*Dicyphus pallidus* (Hemiptera:Miridae)	Switzerland
ARSEF 2666	*Delia radicum* (Diptera:Anthomyiidae)	Denmark
ARSEF 13	*Neodiprion tsugae* (Hymenoptera:Diprionidae)	Alaska
ARSEF 1336	Diptera:Nematocera	Poland

4.2.2.1 DNA extraction method

Between 0.03 and 0.04 g of freeze dried mycelium per isolate was weighed into a sterile 2 ml eppendorf tube. The balance was cleaned with 95 % alcohol between each isolate to avoid contamination. The DNA was extracted following the CTAB method (Zolan and Pukkila, 1986). The quantity and quality of the DNA was tested by electrophoresis on 1.5% Agarose gel in 1X TBE (0.089M Tris, 0.089M boric acid, 0.002MEDTA; pH 8) buffer stained with ethidium bromide (0.1 μg ml $^{-1}$) and photographed. The DNA for PCR reactions was diluted in the proportion of 1:50 using 1xTE (10mM Tris-HCL, 1 mM EDTA, pH 8) and stored at 4 °C until required.

4.2.3 PCR analysis

4.2.3.1 ITS-PCR amplification of Zoophthora radicans *and* Pandora blunckii *isolates*

The ITS region was amplified from all isolates of both species using the universal primer set ITS 5 and 4 (White *et al.*, 1990) (Table 4.3). PCR amplifications were done in 0.5 ml Eppendorf tubes in a total reaction volume of 20 μl. Each reaction contained 0.2 μM of each primer, 150 μM of each dNTP (MBI Fermentas, Helena Biosciences), 0.5 units of *Taq* polymerase (Roche Diagnostics Ltd, Lewes) in 1x PCR reaction buffer (Roche Diagnostics Ltd, Lewes) and 1 μl of diluted DNA. The controls contained sterile distilled water (SDW) instead of DNA.

Table 4.3. Primers used in the PCR reactions. *B= G, T or C, *D = G, A or T.

Primer name	Primer sequence	Reference
Nuclear ITS/ ITS 5	5'GGA AGT AAA AGT CGT AAC AAG G3'	White *et al.*, 1990
Nuclear ITS/ ITS 4	5'TCC TCC GCT TAT TGA TAT GC3'	White *et al.*, 1990
RAM / ACA	5'BDB$(ACA)_5$ *3'	Hantula *et al.*, 1996
RAM / CCA	5'DD$(CCA)_5$ *3'	Hantula *et al.*, 1996
ERIC / ERIC R2	5'AAG TAA GTG ACT GGG GTG AGC G3'	Versalovic *et al.*, 1991
ERIC / R1 CIRE	5'CAC TTA GGG GTC CTC GAA TGT3'	Versalovic *et al.*, 1991

Reactions were carried out in a Hybaid Express thermal cycler. The thermal cycling conditions were: one cycle of denaturation at 95 °C for 3 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 1 minute and extension at 72 °C for 1.5 minutes with a final extension at 72 °C for 5 minutes. The extension time for *P. blunckii* was modified and increased to two minutes because of its larger ITS region (2000 bp) compared to the *Z. radicans* ITS region (1500 bp).

The PCR products were visualized on 1.5 % agarose gels in 1X TBE. GeneRuler™ 1 Kb and 100 bp (MBI Fermentas, Helena Biosciences) size markers were used. The gels were stained with ethidium bromide (0.1 μg ml^{-1}) and photographed.

4.2.3.2 RFLP analysis for Zoophthora radicans *and* Pandora blunckii *isolates*

For restriction analysis (RFLP), 5 μl of ITS products were digested with 5 U of restriction enzyme (*Hind*III, *Dde*I, *Mbo*I, *Alu*I and *Cla*I for both species and *Taq*I additionally for *P. blunckii*) according to the manufacturers' instructions (Roche Diagnostics Ltd, Lewes). Restriction fragments were analysed in 1.5% agarose gel using 1x TBE buffer alongside DNA size markers. All the ITS-RFLP analysis for both species was carried out on at least three separate occasions to ensure consistency.

4.2.4 Cloning and sequencing of the ITS region of selected *Zoophthora radicans* and *Pandora blunckii* isolates

Cloning and sequencing of the ITS regions was done with isolates from both species selected according to the RFLP analysis. The following isolates were selected from *Z. radicans*: NW378, NW386, NW328, NW250, NW323, ARSEF2411, ARSEF6003, ARSEF1699 and NW399. For *P. blunckii* only ARSEF6293 and ARSEF6311 were selected.

4.2.4.1 PCR amplification of the ITS region

The PCR amplification of the ITS region for the selected isolates from both species was performed using the same components and thermal reaction conditions as described previously (4.2.3.1). The amplified ITS fragments from all the isolates were excised from agarose gels and purified using the QIAquick gel extraction kit following the manufacturers' instructions (Qiagen, Crawley).

4.2.4.2 Cloning and sequencing of the ITS fragments

The ITS products were cloned using a TOPO TA Cloning® Kit for sequencing (Invitrogen Ltd, Paisley, UK) and using the chemically competent *E. coli* cells method according to the manufacturers' instructions with some modifications. The complete process is described below.

Cloning reaction- This reaction contained 4 µl of the PCR product, 1µl of a salt solution (provided with the kit), 0.5 µl of TOPO Vector and sterile distilled water for a final volume of 10 µl. The components were mixed gently and incubated for 30 minutes at room temperature. All the reactions were then placed on ice while the next step was started.

Transforming competent cells- The PCR fragments were transformed into competent *E. coli* cells provided with the kit. An aliquote (4 µl) of the cloning reaction was added to 25 µl of One shot® Chemically Competent *E. coli* and mixed gently. The mixture was incubated on ice for 30 minutes. The *E. coli* cells were then "heat-shocked" by placing them for 30 seconds in a water bath set to 42 °C, then on ice for 30 minutes. Two hundred µl of room temperature SOC medium (provided with the kit) were added. The tubes were capped and shaken horizontally at 37 °C for 2 hours at 200 r.p.m.

Either 50 or 200 µl of the mix was spread onto sterile plates of Luria-Bertani Medium (LB medium) (1% Tryptone, 0.5% Yeast extract, 1% NaCl). The media also contained ampicillin (100mg/ml). Two different volumes (50 and 200 µl) were spread in order to have at least one plate of well spaced colonies.

The plates were incubated at 37 °C overnight, and then maintained at 4 °C to stop further growth.

After incubation, only the well separated colonies were selected to check for transformants. To do this, half of each selected colony was used for extraction of DNA using the method described by Klimyuk *et al.*, 1993.

1. The half colonies were introduced separately into 1.5 ml Eppendorf tubes each containing 40 µl 0.25 M NaOH. The tubes were placed into boiling water for 30 seconds.
2. The samples were neutralized by the addition of 40 µl of 0.25 M HCl and 20 µl of 0.5 M Tris HCl pH 8.0, 0.25 % (w/v) Nonidet P-40 and left in boiling water for a further 2 minutes.
3. The tubes were removed and placed on ice.

With the DNA extracted, PCR was performed with the M13 Forward (F) and M-13 Reverse (R) primers provided with the kit to check that the clone had the correct size insert. The PCR products were visualized on a 1% agarose gel in 0.5 x TBE buffer. According to the gel results, colonies containing the correct size product were selected. With the selected colonies the following steps were carried out:

Plasmid DNA Minipreps- The other half of each colony was inoculated into 10 ml of LB medium plus 10 µl of 100mg/ml ampicillin. The cultures were grown overnight at 37 °C and 250 r.p.m on a rotary shaker. The DNA plasmid was purified using the Qiaprep Spin Miniprep kit following the manufacturers' instructions (Qiagen Ltd, Crawley, UK). Three ml of each culture were used for plasmid purification.

Sequencing- The purified plasmids were sequenced using an ABI Prism BigDye cycle sequencing kit (PE Applied Biosystems Division, Warrington, UK). The DNA was sequenced in both directions using the M13F and M13R primers provided in the TOPO TA Cloning® Kit. The PCR conditions used were those recommended by the manufacturer of the ABI Prism BigDye kit.

Precipitation of the sequencing reaction- After the sequencing reaction, 10 µl of sterile distilled water were added to the sequencing reaction for a final volume of 20 µl. Then, 2 µl of 3M NaOAc pH5 and 2 µl of 125 mM EDTA were added plus 50 µl of filter sterilised 95% ethanol. The mix was vortexed and left at room temperature for 15 minutes. The mixture was then centrifuged for 30 minutes at 13K r.p.m. The supernatant was carefully discarded and 100 µl of filter sterilised 70% ethanol added. The solution was centrifuged again for 5 minutes and the supernatant discarded using a pipette. The pellet was left at room temperature for about 20 minutes to dry and then sent to the Department of Biochemistry and Sequencing at Oxford University for the sequence to be determined.

4.2.5 Analysis of genetic variation data

4.2.5.1 Analysis of RFLP patterns

For analysis of RFLP patterns, the results obtained using *Alu* I and *Dde* I were combined in one data set. The different banding patterns produced by the ITS–RFLP analysis were scored manually for presence (1) or absence (0) from gel photographs to produce binary data. Only bands that were consistent in at least three independent reactions were considered for analysis. The binary data were compared using Jaccard's coefficient, producing an unweighted pair group method with arithmatic mean (UPGMA) and neighbour-joining tree using the program Freetree (Hampl *et al.*, 2001). Using the same software (Freetree), the tree was resampled 100 times for bootstrap analysis. Tree files were read using Treeview (Page, 1996).

4.2.5.2 Analysis of ITS sequence data

ITS-PCR sequence traces were edited in the Standen program. Sequences were assembled using BioEdit (Hall, 1999). Multiple sequence alignments were carried out using the Clustal W program (Thompson *et al.*, 1994). Because it was not possible to obtain the entire sequence of the ITS region (the information for the central part was missing), for analysis purposes, the

sequences from both extremes were joined to form one sequence. Maximum likelihood, Maximum Parsimony and Neighbour joining analysis were carried out using the Phylogenetic Analysis Using Parsimony (PAUP) software ver. 4.0b10 for Macintosh (Swofford, 2001). The robustness of branches was estimated by bootstrap analysis with 1000 repeated samplings of the data (Felsenstein, 1985).

4.2.6 Additional molecular techniques used for *Pandora blunckii*

As no differences were detected with the majority of the restriction enzymes used (except for *Taq*I) in the RFLP analysis of the ITS region of *P. blunckii,* additional techniques were used in order to detect more variability amongst the isolates of this species.

4.2.6.1 Random amplified microsatellites (RAMS) technique

RAMS primers ACA and CCA (Hantula *et al.*, 1996) (Table 4.3) were tested on *P. blunckii* isolates NW344, NW345, NW346, ARSEF446 and ARSEF1004 and *on Z. radicans* isolates NW250 and NW378 for comparison.

Each RAMS reaction contained 200 µM of each dNTP (MBI Fermentas, Helena Biosciences) 1.5 mM $MgCl_2$, 0.5 µM primer, 0.5 units *Taq* polymerise in 1x PCR buffer (Roche diagnostics Ltd.) and 1 µl of diluted DNA and enough sterile distilled water for a total reaction volume of 20 µl. Reactions were carried out in a Hybaid Express thermal cycler. The thermal cycling conditions were: one cycle of denaturation at 94 °C for 2 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 51 °C for 1.5 minutes and extension at 72 °C for 2 minutes with a final extension at 72 °C for 8 minutes.

The PCR products were analysed on 1.5 % agarose gels in 1X TBE (0.089M Tris, 0.089M boric acid, 0.002 MEDTA; pH 8). GeneRuler™ 1 Kb and 100 bp (MBI Fermentas, Helena Biosciences) size markers were used. The gels were stained with ethidium bromide (0.1 µg ml^{-1}) and photographed. All the

reactions were done on at least two different occasions to ensure consistency of the results.

4.2.6.2 Enterobacterial repetitive intergenic consensus (ERIC) technique

ERIC primers (Versalic *et al.*, 1991) (Table 4.3) were tested on DNA from all *P. blunckii* isolates (Table 4.2).

Each ERIC PCR reaction contained 200 µM of each dNTP (MBI Fermentas, Helena Biosciences), 200 µM $MgCl_2$, 0.5 µM primer, 1 unit of *Taq* polymerise in 1x PCR buffer (Roche diagnostics Ltd.), 1 µl of diluted DNA and enough SDW for a total reaction volume of 20µl.

Reactions were made in a Hybaid Express thermal cycler. The thermal cycling conditions were: one cycle of denaturation at 94 °C for two minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 45.5 °C for 1.5 minutes and extension at 72 °C for 2 minutes with a final extension at 72 °C for eight minutes.

The PCR products were visualised on 1.5 % agarose gels in 1X TBE (0.089M Tris, 0.089M boric acid, 0.002MEDTA; pH 8). GeneRuler™ 1 Kb and 100 bp (MBI Fermentas, Helena Biosciences) size markers were used. The gels were stained with ethidium bromide (0.1 µg ml^{-1}) and photographed. All the reactions were done on at least two different occasions to ensure consistency of the results.

4.3 RESULTS

4.3.1 Production of mycelium for DNA extraction

The method used allowed extraction of sufficient quantities of PCR quality DNA.

4.3.2 PCR analysis

4.3.2.1 ITS-PCR amplification of Zoophthora radicans *and* Pandora blunckii *isolates*

The ITS5/4 primer set amplified the 3' end of the small sub-unit (SSU), the ITS1, 5.8S and ITS2 regions and the 5' end of the large sub-unit (LSU) of the ribosomal gene. The sizes of the products were different between the two species *ca* 1550 bp for *Z. radicans* (Fig. 4.1) and *ca* 2100 bp for *P. blunckii* (Fig. 4.2). No size variation in the ITS region was observed amongst isolates from each species. Isolates ARSEF915 and ARSEF3201, considered previously as *P. blunckii*, showed different sized ITS regions to the other *P. blunckii* isolates which were *ca* 800 bp and *ca* 750 bp respectively (Fig. 4.2). Isolate ARSEF3201 is the same isolate called *Pandora* sp. on Chapter 3.

4.3.2.2 RFLP analysis of Zoophthora radicans *and* Pandora blunckii *isolates*

The *Z. radicans* isolates showed genetic variability only with two out of the five restriction enzymes tested (*Alu* I and *Dde* I). With *Alu* I six groups with different patterns were found (RFLP groups) (Fig. 4.3, Table 4.4). No clear relationship was found with geographical origin or host. Isolates from *P. xylostella* were found in four of these groups according to their RFLP profile. The first group contained *P. xylostella* isolates from Mexico, Kenya, Australia, Taiwan and Philippines. Group two contained *P. xylostella* isolates from New Zealand and Australia. Group three contained *P. xylostella* isolates from Mexico and Japan and the fourth group contained *P. xylostella* isolates from Malaysia (Table 4.4.). Groups five and six contained one isolate each group and none of them were isolated from *P. xylostella.*

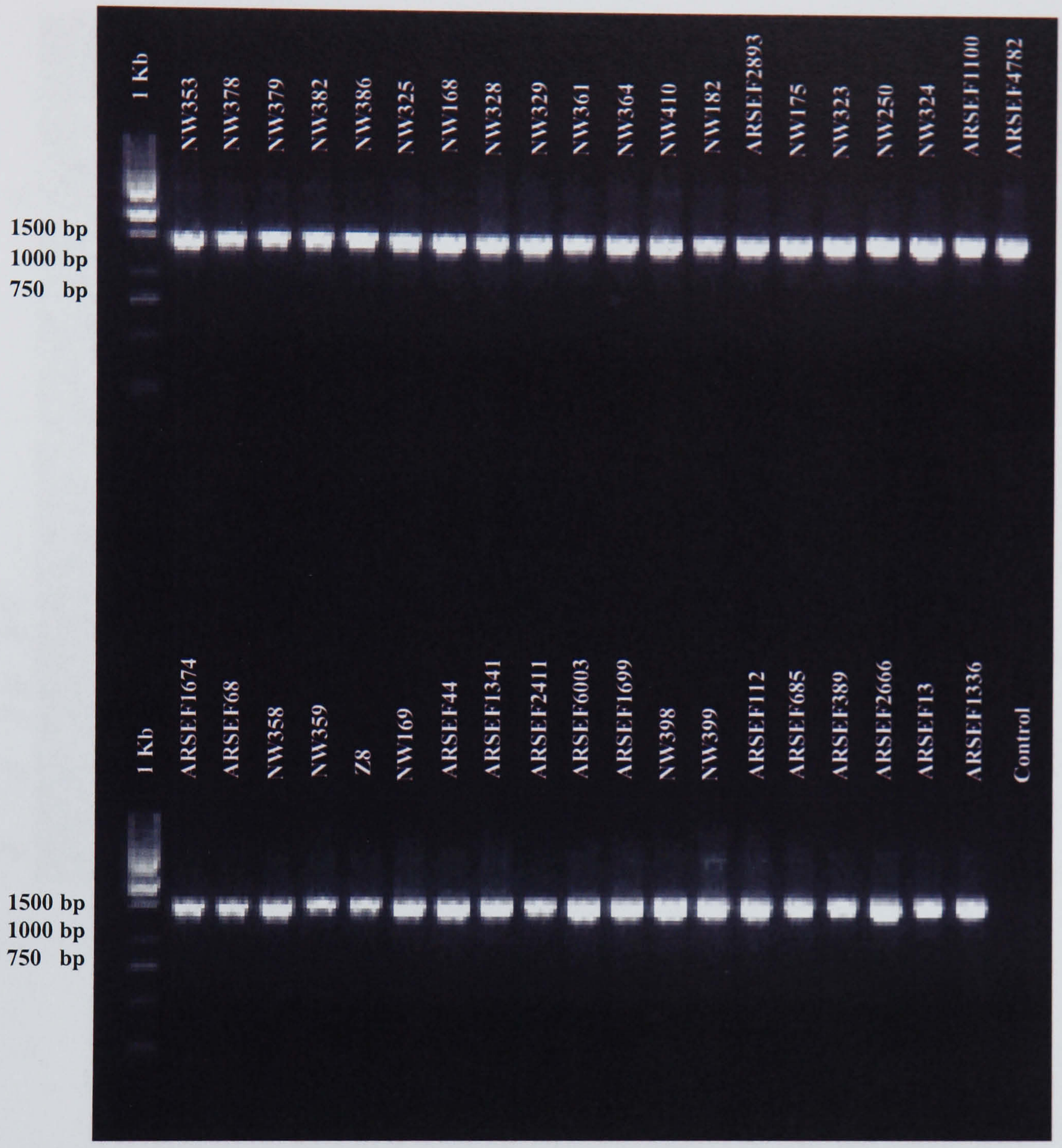


Figure 4.1. Amplification of the ITS regions of *Zoophthora radicans* isolates using primer set ITS 5 and 4. Isolate designations are given above each lane. Size marker = 1 Kb.

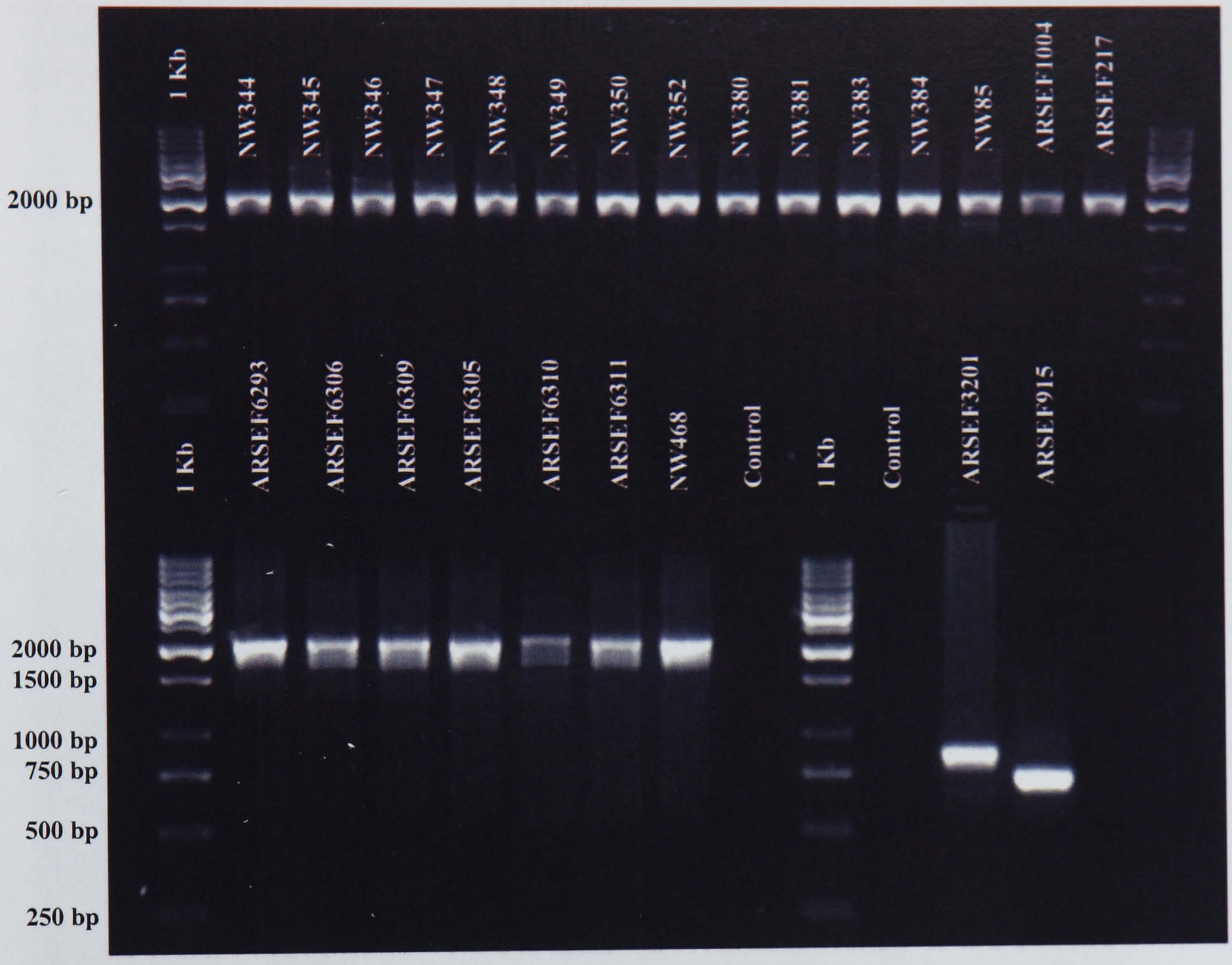


Figure 4.2. Amplification of the ITS regions of *Pandora blunckii* isolates using primer set ITS 5 and 4. Isolate designations are given above each lane. Size marker = 1 Kb.

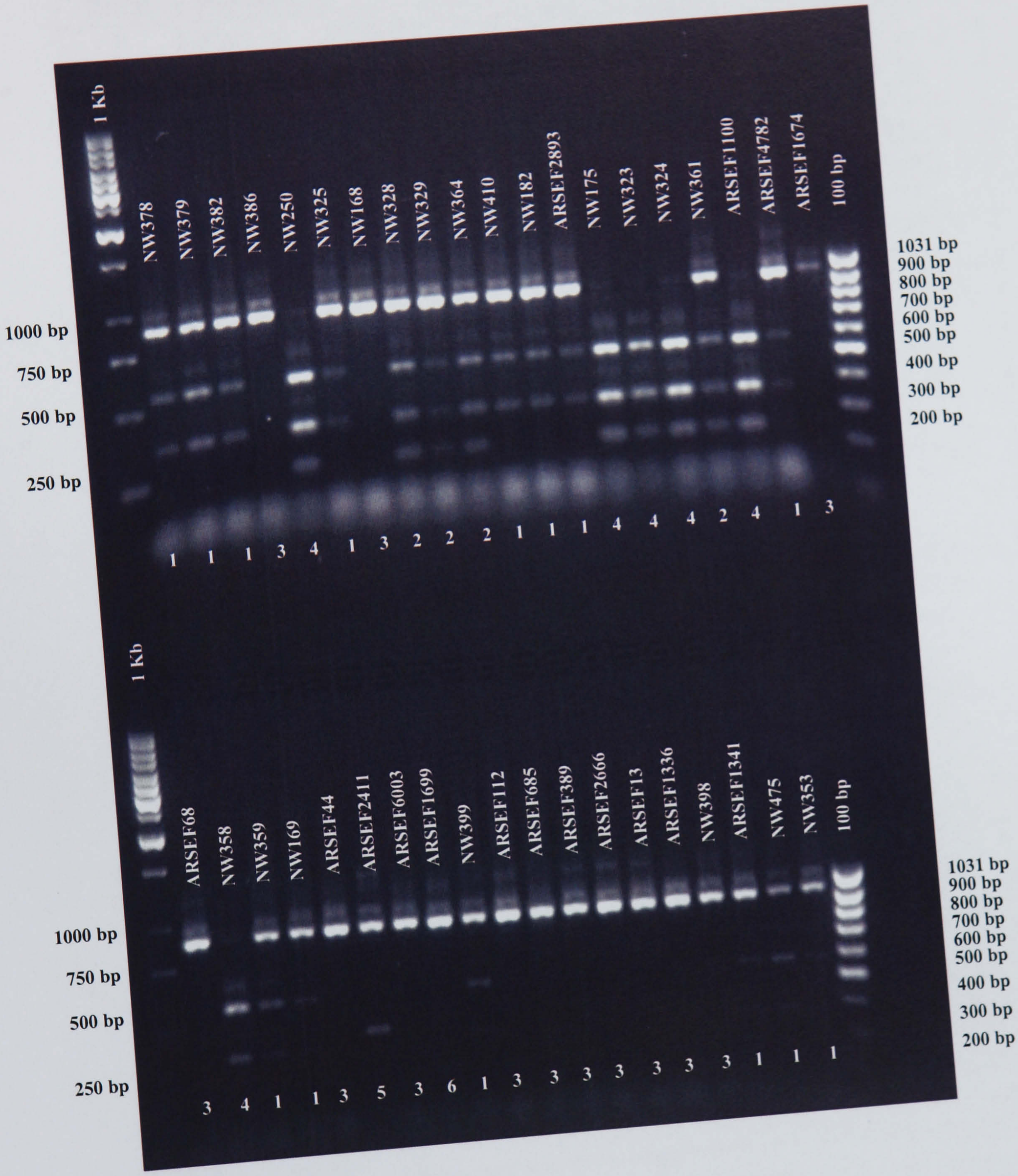


Figure 4.3. ITS-RFLP profiles of *Zoophthora radicans* isolates generated by the restriction enzyme *Alu* I. Isolate designations are given above each lane. RFLP group number is given below each lane. Size marker left = 1 Kb. Size marker right = 100 bp.

Table 4.4. RFLP groups obtained using the enzyme *Alu* I after RFLP analysis of the ITS region of all the *Zoophthora radicans* isolates (Table 4.2).

RFLP	ISOLATE	HOST	COUNTRY
1	NW 353	*Plutella xylostella* (Lepidoptera: Plutellidae)	Mexico
1	NW 378	" "	" "
1	NW 379	" "	" "
1	NW 382	" "	" "
1	NW 325	" "	Kenya
1	NW 410	" "	Australia
1	NW 182	" "	Taiwan
1	ARSEF 2893	" "	Philippines
1	ARSEF 4782	*Epinotia aporema* (Lepidoptera: Tortricidae)	Uruguay
1	NW 359	*Eutorna phaulacosma* (Lepidoptera: Oecophoridae	N. Zealand
1	NW 475	*Heliothis sp.* (Lepidoptera:Noctuidae)	" "
1	NW 169	*Choristoneura fumiferana* (Lepidoptera:Tortricidae)	U.S.A.
1	ARSEF 1341	*Pieris brassicae* (Lepidoptera:Pieridae)	Poland
1	NW 399	*Metapolophium dirhodum* (Homoptera:Aphididae)	U.S.A.
2	NW 328	*Plutella xylostella* (Lepidoptera: Plutellidae)	N. Zealand
2	NW 329	" "	" "
2	NW 361	" "	" "
2	NW 364	" "	Australia
3	NW 386	" "	Mexico
3	NW 168	" "	Japan
3	ARSEF 1674	*Ptycholoma aeriferana* (Lepidoptera: Tortricidae)	Japan
3	ARSEF 68	*Tortrix viridana (*Lepidoptera: Tortricidae)	France
3	ARSEF 44	*C. fumiferana* (Lepidoptera:Tortricidae)	USA
3	ARSEF 6003	*Trichoplusia ni* (Lepidoptera:Noctuidae)	Mexico
3	NW 398	*Drepanosiphon aceris* (Homopetera:Drepanosiphidae)	Switzerland
3	ARSEF 112	*Therioaphis maculata* (Homoptera:Aphididae)	Israel
3	ARSEF 685	*Nilaparvata lugens* (Homoptera:Delphacidae)	China
3	ARSEF 389	*Dicyphus pallidus* (Hemiptera:Miridae)	Switzerland
3	ARSEF 2666	*Delia radicum* (Diptera:Anthomyiidae)	Denmark
3	ARSEF 13	*Neodiprion tsugae* (Hymenoptera:Diprionidae)	Alaska
3	ARSEF 1336	Diptera:Nematocera	Poland
4	NW 175	*Plutella xylostella* (Lepidoptera: Plutellidae)	Malaysia
4	NW 323	" "	" "
4	NW 250	" "	" "
4	NW 324	" "	" "
4	ARSEF 1100	" "	" "
4	NW 358	Unidentified Leafroller	N. Zealand
5	ARSEF 2411	*Sesamia inferens* (Lepidoptera:Noctuidae)	Java
6	ARSEF 1699	*Cnaphalocrocis medinalis* (Lepidoptera:Pyralidae)	Philippines

Using the restriction enzyme *Dde* I nine RFLP groups were found and again, there was no clear relationship with geographical origin or host. Isolates from *P. xylostella* were found in the first three of these RFLP groups (Fig. 4.4, Table 4.5). The first group contained *P. xylostella* isolates from Mexico, Kenya, Japan, New Zealand, Australia, Taiwan and Philippines. The second group contained *P. xylostella* isolates from Malaysia, and the third group *P. xylostella* isolates from New Zealand and Australia. Groups four to nine had only one representative isolate in each group and were from a range of different countries and hosts but none of them isolated from *P. xylostella* (Fig. 4.4, Table 4.5).

Using the same five restriction enzymes no variability was observed among the *P. blunckii* isolates. Only the restriction enzyme *Taq* I (not used with *Z. radicans* because variability was already found with other enzymes) showed some differences. This difference was the presence of a second faint band in isolates NW385, ARSEF6293 and ARSEF6309 all from *P. xylostella* in Mexico (Fig. 4.5).

4.3.3 Cloning and sequencing of the ITS region of selected *Zoophthora radicans* and *Pandora blunckii* isolates

The ITS gene from seven *Z. radicans* isolates and one *P. blunckii* isolate were successfully cloned. It was not possible to obtain the sequence for the entire ITS region. There was more variability in the region that correspond to the ITS1 (Fig. 4.6) than to the region that correspond to the ITS2 (Fig. 4.7). In addition to base differences among the isolates, there were also some deletions in the sequence such as for isolates ARSEF2411 where seven bases were deleted (from position 120 to 127). At position 215 all isolates except ARSEF2411 were missing one base. ARSEF2411 was also missing bases at the following positions; 358, 502 and 583. At these same positions, isolates NW386 and NW250 also showed a base deletion (Fig. 4.6). No deletions were observed in the sequences that may correspond to the ITS2 region. However, two additional T bases were inserted at positions 534 and 535 in isolate

ARSEF6003, and an additional C base was inserted at position 550 in isolate ARSEF2411 (Fig. 4.7).

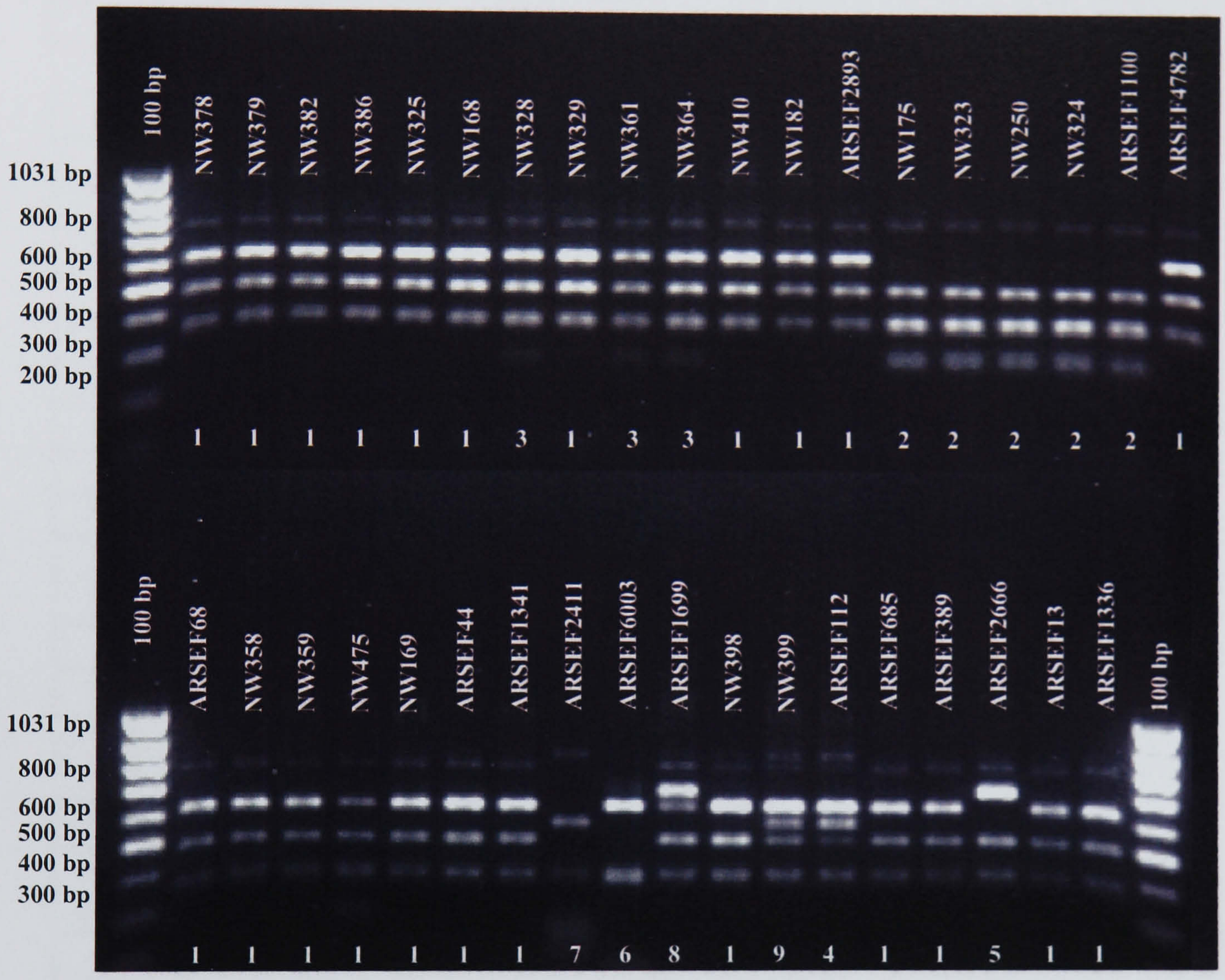


Figure 4.4. ITS-RFLP profiles of *Zoophthora radicans* isolates generated by the restriction enzyme *Dde* I. Isolate designations are given above each lane. RFLP group number are given below each lane. Size marker = 100 bp.

Table 4.5. RFLP groups obtained using the enzyme *Dde* I after the RFLP analysis of the ITS region of all the *Zoophthora radicans* isolates (Table 4.2).

RFLP	ISOLATE	HOST	COUNTRY
1	NW 353	*Plutella xylostella* (Lepidoptera: Plutellidae)	Mexico
1	NW 378	" "	" "
1	NW 379	" "	" "
1	NW 382	" "	" "
1	NW 386	" "	" "
1	NW 325	" "	Kenya
1	NW 168	" "	Japan
1	NW 329	" "	N. Zealand
1	NW 410	" "	Australia
1	NW 182	" "	Taiwan
1	ARSEF 2893	" "	Philippines
1	ARSEF 4782	*Epinotia aporema* (Lepidoptera:Tortricidae)	Uruguay
1	ARSEF 1674	*Ptycholoma aeriferana* (Lepidoptera:Tortricidae)	Japan
1	ARSEF 68	*Tortrix viridana (*Lepidoptera:Tortricidae)	France
1	NW 358	Unidentified Leafroller	N. Zealand
1	NW 359	*Eutorna phaulacosma* (Lepidoptera: Oecophoridae	" "
1	NW 475	*Heliothis sp.* (Lepidoptera:Noctuidae)	" "
1	NW 169	*Choristoneura fumiferana* (Lepidoptera:Tortricidae)	U.S.A.
1	ARSEF 44	*C. fumiferana* (Lepidoptera:Tortricidae)	U.S.A
1	ARSEF 1341	*Pieris brassicae* (Lepidoptera:Pieridae)	Poland
1	NW 398	*Drepanosiphon aceris* (Homopetera:Drepanosiphidae)	Switzerland
1	ARSEF 685	*Nilaparvata lugens* (Homoptera:Delphacidae)	China
1	ARSEF 389	*Dicyphus pallidus* (Hemiptera:Miridae)	Switzerland
1	ARSEF 13	*Neodiprion tsugae* (Hymenoptera:Diprionidae)	Alaska
1	ARSEF 1336	Diptera:Nematocera	Poland
2	NW 175	*Plutella xylostella* (Lepidoptera: Plutellidae)	Malaysia
2	NW 323	" "	" "
2	NW 250	" "	" "
2	NW 324	" "	" "
2	ARSEF 1100	" "	" "
3	NW 328	" "	N. Zealand
3	NW 361	" "	" "
3	NW 364	" "	Australia
4	ARSEF 112	*Therioaphis maculata* (Homoptera:Aphididae)	Israel
5	ARSEF 2666	*Delia radicum* (Diptera:Anthomyiidae)	Denmark
6	ARSEF 6003	*Trichoplusia ni* (Lepidoptera:Noctuidae)	Mexico
7	ARSEF 2411	*Sesamia inferens* (Lepidoptera:Noctuidae)	Java
8	ARSEF 1699	*Cnaphalocrocis medinalis* (Lepidoptera:Pyralidae)	Philippines
9	NW 399	*Metapolophium dirhodum* (Homoptera:Aphididae)	U.S.A.

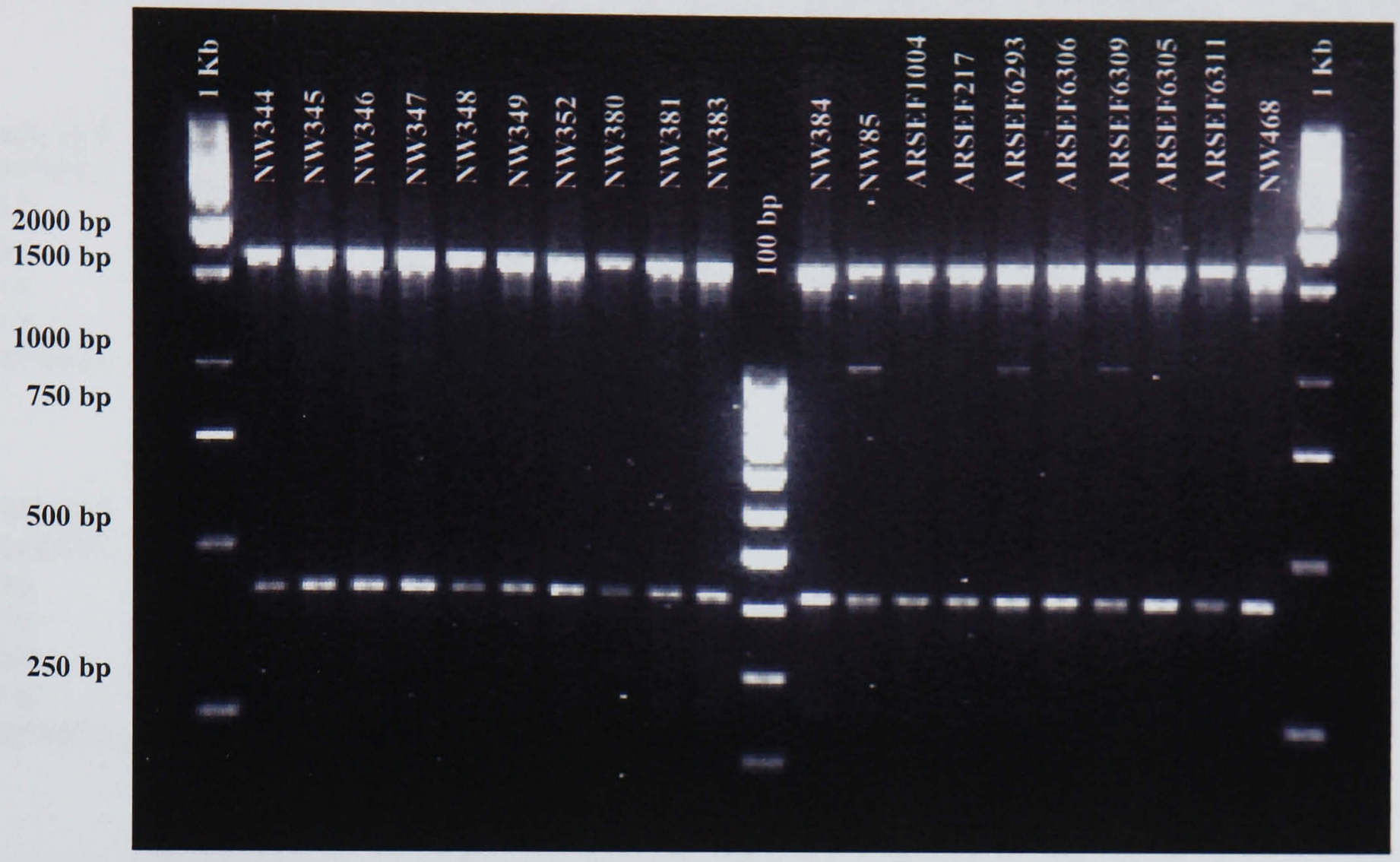


Figure 4.5. ITS-RFLP profiles of *Pandora blunckii* isolates generated by the restriction enzyme *Taq* I. Isolate designations are given above each lane. Size marker on ends = 1 Kb, size marker in the middle = 100 bp.

Figure 4.6. Multiple sequence alignment of partial ITS sequences starting from the 3' end of the small sub-unit (SSU) towards the 5' end of the large sub-unit (LSU) of the ribosomal gene showing 620 bp from *ca* 1510 bp. The sequences shaded in black are identical and those bases without shading are different from the other sequences.

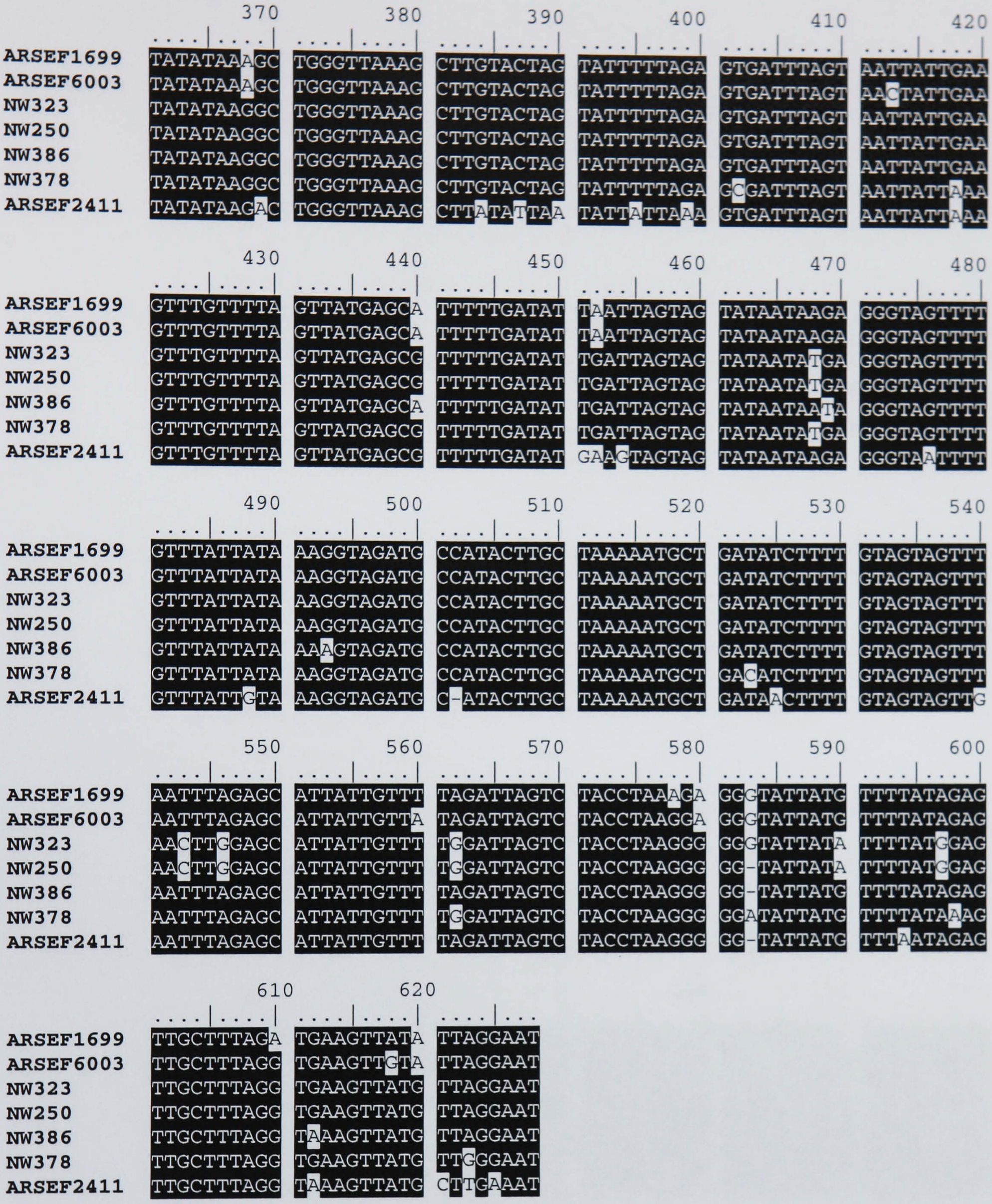


Figure 4.6. Continued.

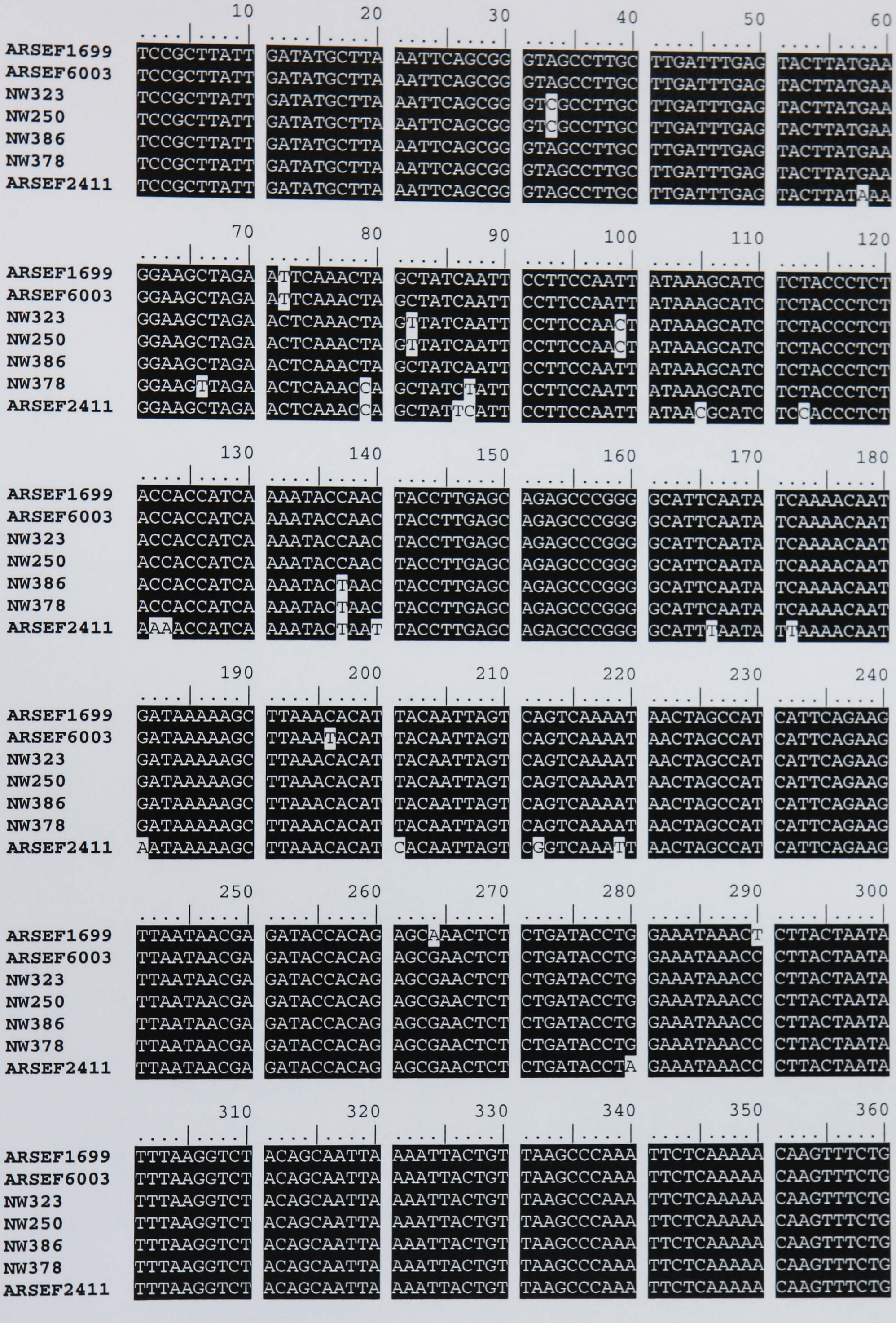


Figure 4.7. Multiple sequence alignment of partial ITS sequences starting from the 3' end of the large sub-unit (LSU) towards the 5' end of the small sub-unit (SSU) of the ribosomal gene showing 640 bp from *ca* 1510 bp. The sequences shaded in black are identical and those bases without shading are different from the other sequences.

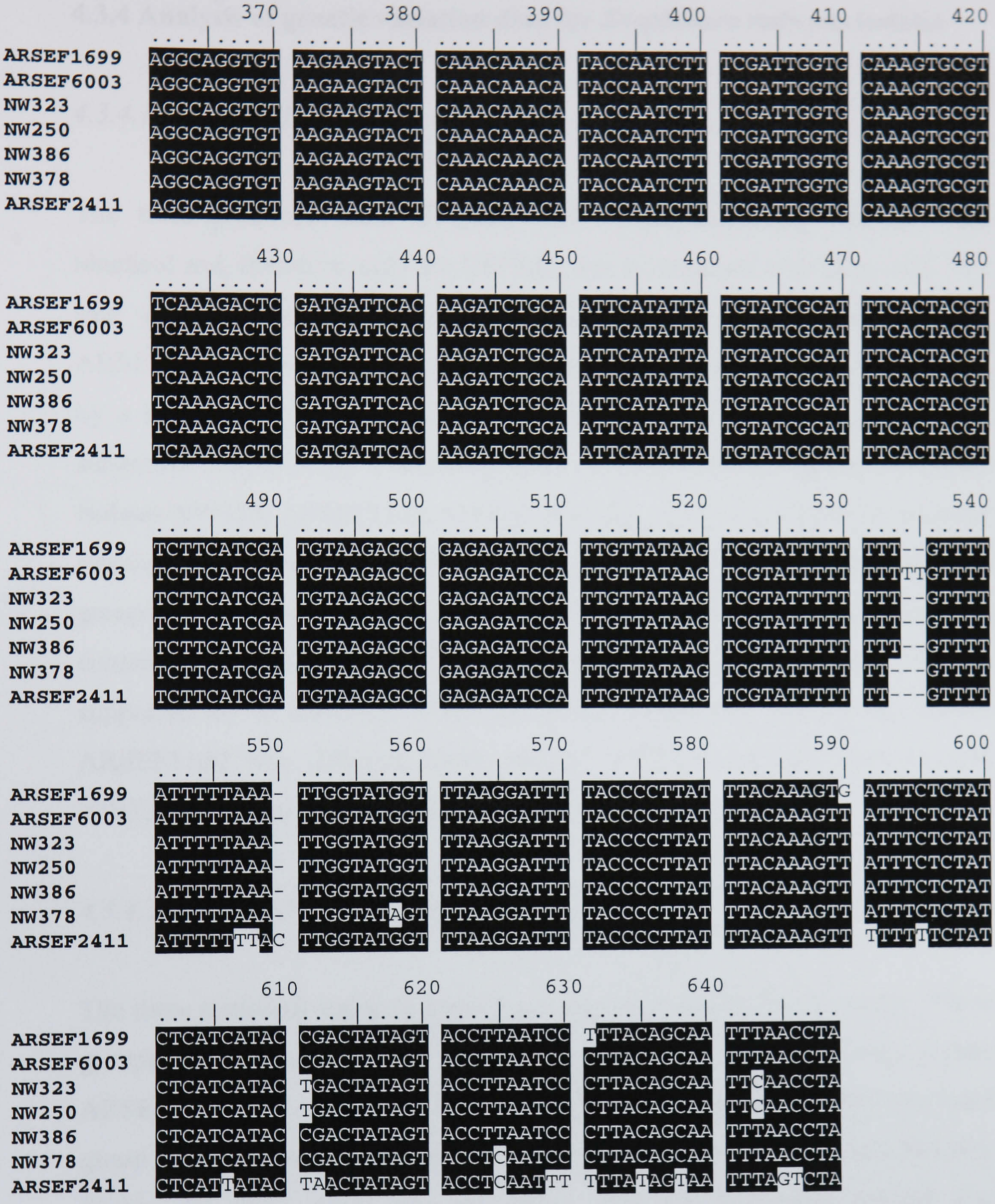


Figure 4.7. Continued.

4.3.4 Analysis of genetic variation data for *Zoophthora radicans* isolates

4.3.4.1 Analysis of RFLP patterns

The trees produced from UPGMA and Neighbour-joining analysis were identical and, therefore, only the UPGMA tree is described here (Fig. 4.8). The tree showed that there were two main groups, one contained isolate ARSEF2411 alone and the second group contained all other isolates supported by a bootstrap value of 100%. Within the second group there are also two subgroups supported by a bootstrap value of 63. The first subgroup contained isolates NW358, ARSEF1100, NW324, NW250, NW175 and NW323, with the remaining isolates in the second subgroup. Within the first subgroup, two groups were found; the first contained only isolate NW358 and the second contained isolates ARSEF1100, NW324, NW250, NW323 and NW175 supported by a bootstrap value of 54%. Within this last group, isolate ARSEF1100 was different from isolates NW324, NW250, NW175 and NW323 supported by a bootstrap value of 81 %.

4.3.4.2 Analysis of ITS sequence data

The three statistical analyses carried out showed a similar tree topology. Three groups were separated (Fig. 4.9). The first group contained only isolate ARSEF1699, the second group contained isolate ARSEF6003 and the third group contained isolates NW323, NW250, NW378, ARSEF2411 and NW386. Within the third group two subgroups were found; isolate NW386 was separated from the other isolates supported by bootstrap values of 95, 92 and 92% using Maximum Likelihood, Maximum Parsimony and Neighbour Joining analysis respectively. Within the second subgroup, isolates ARSEF2411 and NW378 were separated from isolates NW250 and NW323 supported by bootstrap values of 60, 51 and 81 % using Maximum likelihood, Maximum Parsimony and Neighbour joining analysis respectively. Isolates NW378 and ARSEF2411 were considered in the same group but only with one analysis (Neighbour Joining), supported by a bootstrap value of 55%. No separation was found with the other two analyses. Isolates NW323 and NW250

were resolved as separate groups with bootstrap value of 100 % with all three analyses.

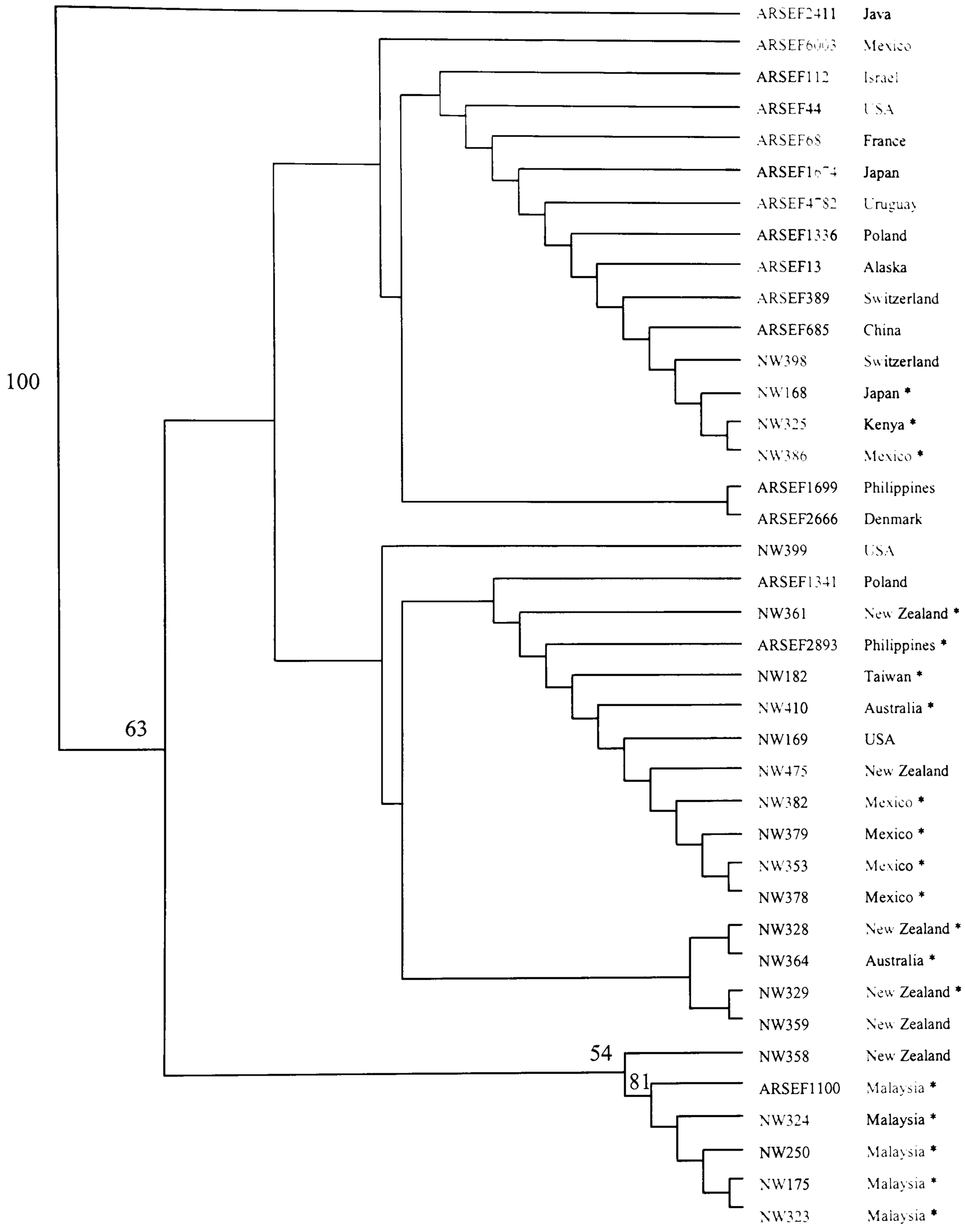


Figure 4.8. Dendogram constructed from ITS-RFLP profiles of *Zoophthora radicans* isolates inferred from the distance matrix by the un-weighted pair group method, arithmetic mean (UPGMA) using Jaccard's co-efficient. Only bootstrap values above 50% are shown. The host of each isolate is given in Table 4.2, but isolates from *Plutella xylostella* are marked with *.

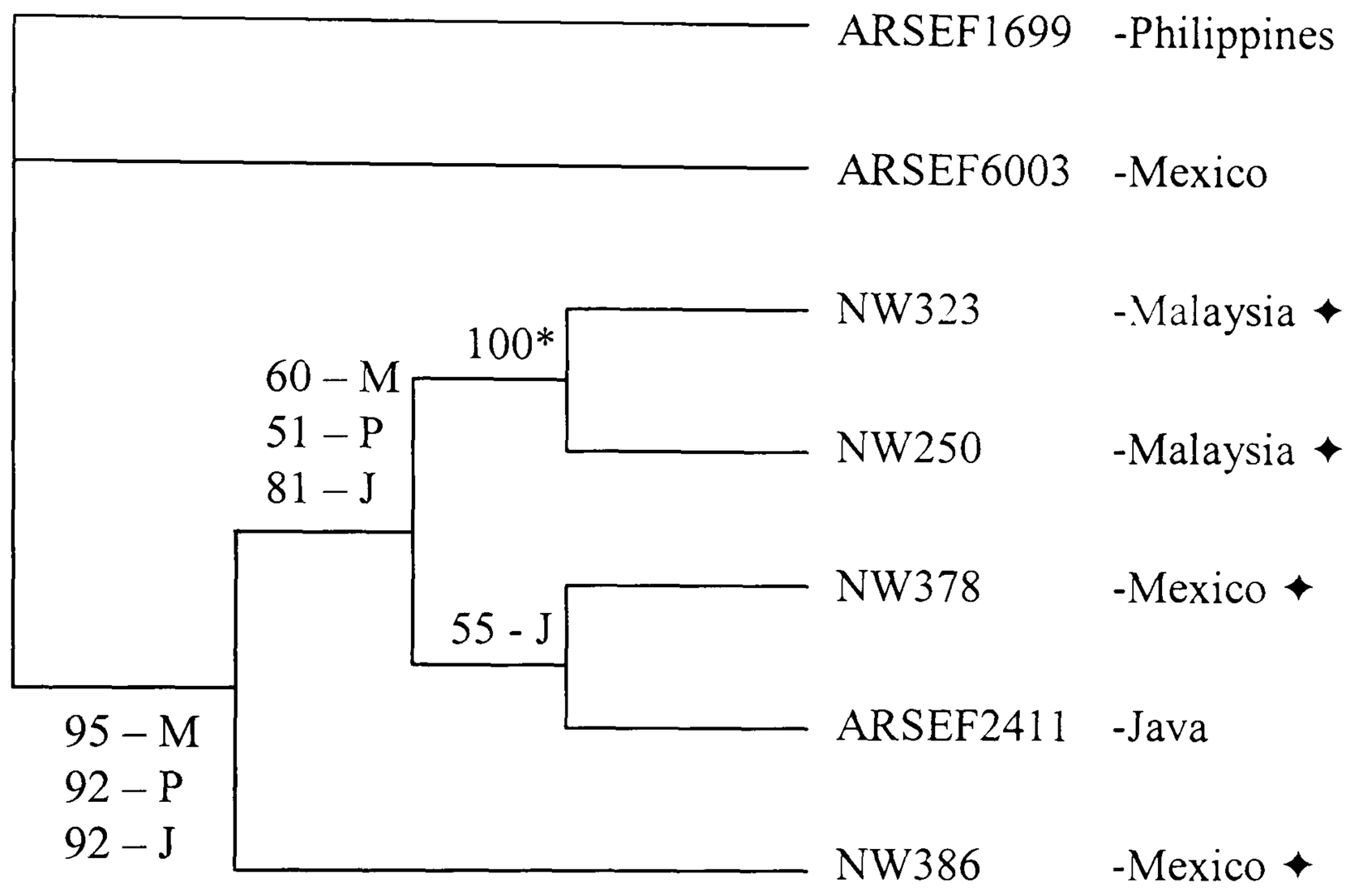


Figure 4.9. Dendogram constructed from ITS sequence data from selected *Zoophthora radicans* isolates using Maximum Likelihood. Maximum Parsimony and Neighbour Joining analysis. Bootstrap values are shown for each type of analysis. M=Maximum Likelihood, P=Maximum Parsimony, J=Neighbour Joining, *=the same value with all analyses. The host of each isolate was given in Table 4.1, but isolates from *Plutella xylostella* are marked with ✦.

4.3.5 Additional molecular techniques used for *Pandora blunckii*

As no large differences were detected using RFLP analysis of the ITS region of *P. blunckii* isolates, some additional analysis were used.

4.3.5.1 Random amplified microsatellites (RAMS) and Enterobacterial repetitive intergenic consensus (ERIC) techniques

For the RAMS analysis, only a few isolates (NW344, NW345, NW346, ARSEF3201 and ARSEF6311) were used to determine whether some variability could be detected. No bands were amplified using the ACA primer (Fig. 4.10) and only one band was obtained with the CCA primer. Using the CCA primer, the band obtained for the *P. blunckii* isolates was *ca* 800bp, and a different size band was obtained for isolate ARSEF3201 (*ca* 1500 bp). Two *Z. radicans* isolates, NW378 and NW250, were also included for comparison and a similar multiple banding patterns were obtained for both isolates (Fig. 4.2).

All *P. blunckii* isolates were evaluated with the ERIC primer set and, although more bands were obtained compared to the RAMS analysis, no differences were detected amongst the isolates (Fig. 4.11).

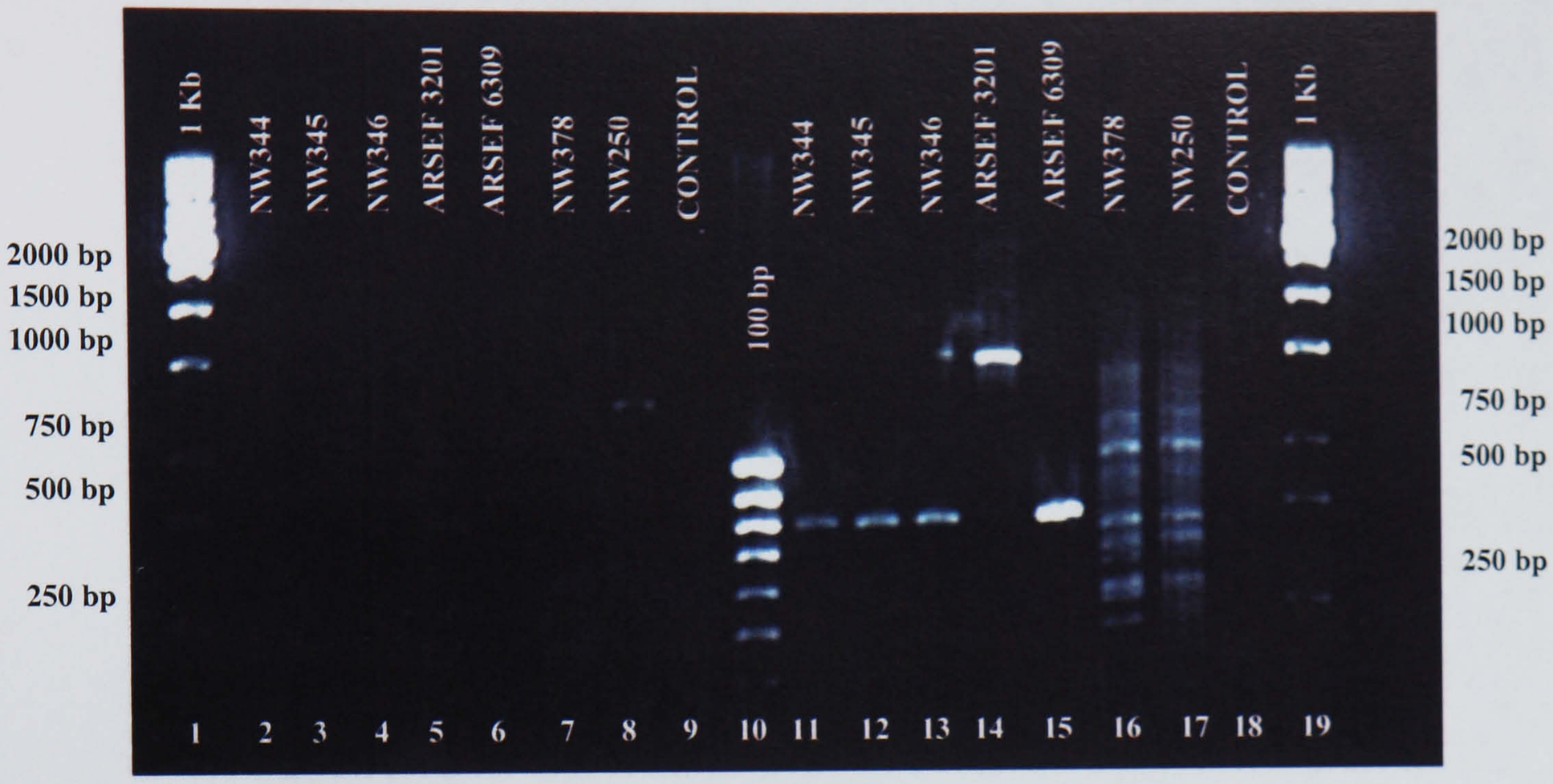


Figure 4.10. Evaluation of some *Pandora blunckii* isolates (Lanes 2-6 and 11-15) and some *Zoophthora radicans* isolates (Lanes 7-8 and 16-17) using the ACA RAMS primer (Lanes 2-9), and the CCA RAMS primer (Lanes 11-18). Isolate designation are given above each lane. Size marker= 1 Kb.

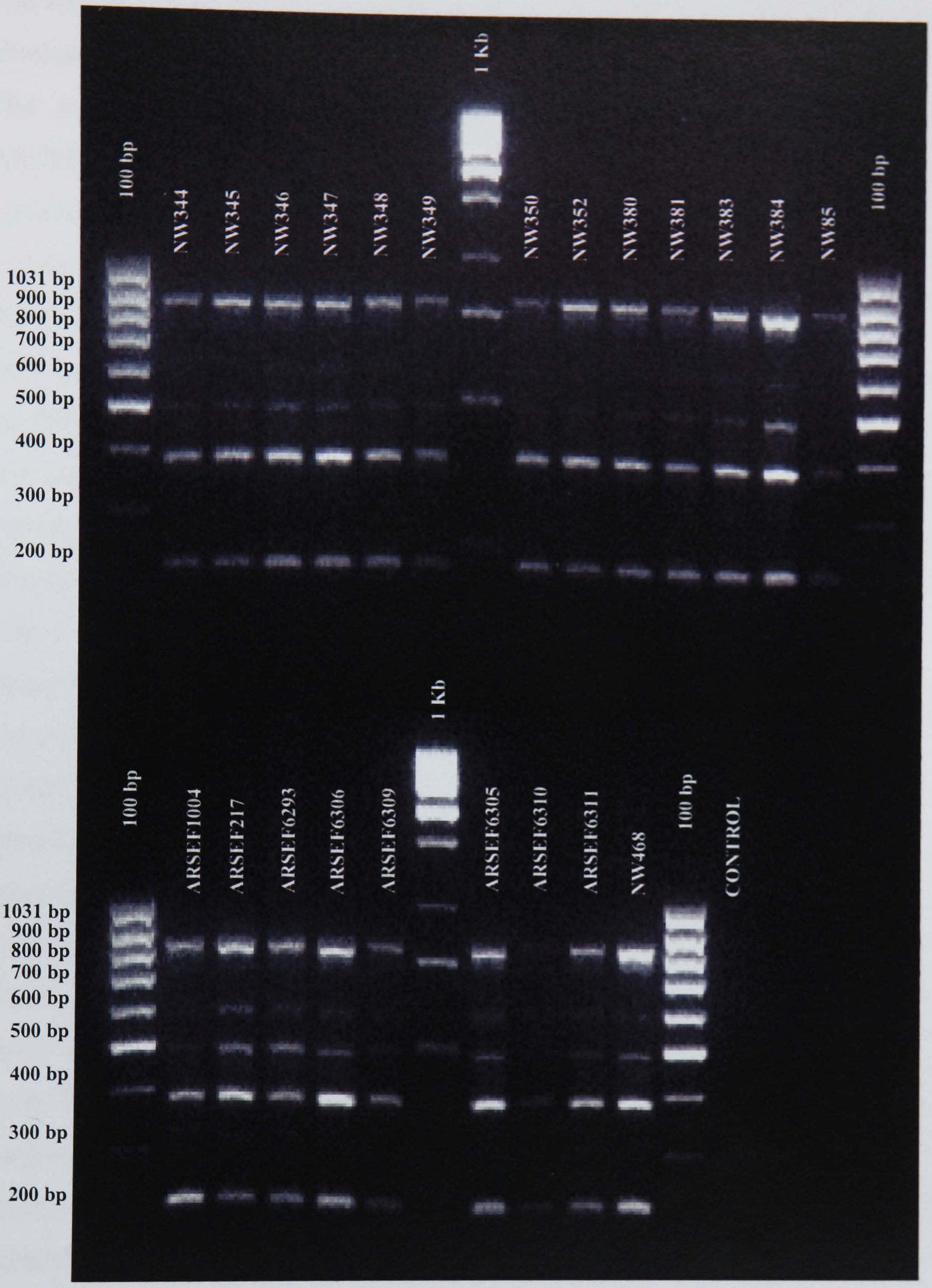


Figure 4.11. ERIC-PCR profiles of *Pandora blunckii* isolates. Isolate designations are given above each lane. Size marker on ends = 100 bb, size marker in the middle = 1Kb.

4.4 DISCUSSION

The size of ITS amplification products for *Z. radicans* and *P. blunckii* isolates obtained in this project have been reported previously (Tymon *et al.*, 2004). The same *Z. radicans* isolates used by Tymon *et al.* (2004) (except ARSEF3737) were used in this project plus 34 additional isolates. No variability was found in the ITS product size for the different *Z. radicans* isolates. However, some variability was found amongst the product sizes from the *P. blunckii* isolates. The majority of *P. blunckii* isolates produced an ITS-PCR product of about 2100 bp except for two isolates ARSEF915 (product size *ca* 800bp) and ARSEF3201 (product size *ca* 750 bp). Isolate ARSEF3201 is the same isolate used in Chapter 3 that was designated as *Pandora* sp. Variability in the ITS region among isolates of the same species has been reported previously (Rohel *et al.*, 1997; Sierotzki *et al.*, 2000; Tymon *et al.*, 2004). However, as Tymon *et al.* (2004) suggested, this was likely to be a result of misidentification and that the isolates with different sized products were different species. Isolate ARSEF951 failed to sporulate under laboratory conditions, so a possible identification from the conidial morphology was not possible. The conidial morphology of isolate ARSEF3201 (personal observation) does suggest that this isolate belongs to the genus *Pandora* and the size of the ITS product is similar to the size of the ITS product reported for *Pandora delphacis* and *Pandora nouryi* (Tymon *et al.*, 2004). Pathogenicity tests carried out against *P. xylostella* larvae showed that this isolate (ARSEF3201) was not pathogenic to this insect compared with *P. blunckii* isolates and *Z. radicans* isolates (data not described). *Pandora delphacis* is mainly reported to infect *Nilaparvata lugens* (Homoptera:Delphacidae) (Balazy, 1993) and, under laboratory conditions, some aphid species. *Pandora nouryi* is also reported to infect aphid species (Balazy, 1993). According to this information it is very likely that ARSEF3201 is a different species attacking a different insect in the cabbage system and not *P. blunckii* as previously considered in the USDA-ARSEF catalogue.

The RFLP analysis did not show variability amongst the *P. blunckii* isolates with all the restriction enzymes evaluated. This suggests that the ITS region of

this species is highly conserved. However, it has to be considered that all the *P. blunckii* isolates, apart from isolates ARSEF217 from Germany and ARSEF1004 from Philippines, came from the same geographical region and the same host, which may be the reason for a lack of variability in the ITS region. Highly conserved ITS regions are not rare in some fungal species including plant pathogens (e.g. Skouboe *et al.*, 1999; De Arruda *et al.*, 2003) and insect pathogenic fungi (Tymon *et al.*, 2004). The use of additional techniques may, therefore, be required. Such techniques include the use of ERIC primers, which identified variability among *P. neoaphidis* isolates (Tymon *et al.*, 2004) and *P. chlamydosporia* isolates (Morton *et al.*, 2003), and primers in the IGS region which have been used to detect variability in plant pathogenic fungi (e.g. De Arruda *et al.*, 2003). However, ERIC and RAMS primers did not yield polymorphisms in the small group of *P. blunckii* isolates tested. The isolates used in this project were highly variable in colony morphology (personal observation) and their response to temperature (Chapter 2), which suggests that they could be different genotypically, but more diverse techniques need to be used in order to identify these differences. When a different restriction enzyme was used (*Taq* I), some differences were detected with the presence of an additional secondary band in some isolates (NW385, ARSEF6293 and ARSEF6309) (Fig. 4.5). The presence of this band was consistent in separate reactions on three different occasions. The rRNA is encoded by a multigene family, the genes are repeated side by side several times (tandem array). It is possible that some of the copies of the ITS genes may be different from the majority of the ITS genes for the same isolate. Multiple rDNA regions in the same fungal species have been reported previously (Fatehi and Bridge, 1998). These differences in individual genes that encode the rRNA may be represented by changes in the sequence but not the size of the region.

Sequencing is one of the most secure ways to identify even small differences in the ITS region. However, in this study, only one *P. blunckii* isolate was successfully cloned and sequenced, which did not allow a comparison amongst isolates. The sequencing of more *P. blunckii* isolates would be highly recommended in order to assess differences.

The ITS region of the *Z. radicans* isolates was variable as seen for two of the five restriction enzymes (*Alu*I and *Dde*I) in the RFLP analysis. In both sets of RFLP patterns there was no clear relationship with host or geographical origin. The only consistent results were for isolates NW175, NW323, NW250, NW324 and ARSEF1100 from Malaysia, isolate ARSEF2411 from Java and isolate ARSEF1699 from Philippines which were always contained in different RFLP groups for both enzymes. This result was confirmed by the Neighbour-joining analysis where isolate ARSEF2411 was placed in a completely separate group from the rest of the isolates (100% bootstrap value), with the Malaysian isolates as another group (63% bootstrap value). All the Malaysian isolates were isolated from *P. xylostella* and it would be interesting to analyse more isolates from the same country but from different insect hosts. It was also interesting that isolates ARSEF2411 and ARSEF1699 from Java and Philippines respectively, were both isolated from lepidopteran insects other than *P. xylostella*, but were placed in different RFLP groups. Originating from islands, they may have evolved separately from the isolates found in the continental countries. However, isolates from Mexico that came from the same host and geographical origin also showed some variability particularly isolate NW386 that was different from the other Mexican isolates from *P. xylostella*. Although this isolate was placed in a different RFLP group, this separation showed a bootstrap value below 50 % which suggests that this may not be a significant separation. Mexican isolate ARSEF6003 from a different lepidopteran insect host showed a different RFLP pattern although there was no significant separation from the other Mexican isolates. There is always the chance that a relationship can be found by using a different method. For example, Morton *et al.* (2003) obtained a host related banding pattern using ERIC analysis for *P. chlamydosporia* populations. The same analysis was done in this study for *P. blunckii* but no differences among isolates were found.

The three analyses used (Maximum Likelihood, Maximum Parsimony and Neighbour Joining) with the sequence information of selected isolates showed that isolates ARSEF6003 (Mexico) and ARSEF1699 (Philippines) were actually different to each other and to the other isolates which were contained in a different group. Isolate ARSEF2411 which, according to the RFLP pattern,

was completely different from the other isolates, showed similarity with isolates NW386, NW378, NW323 and NW250 in its sequence information.

Many authors have found a relationship between genetic variability and host (e.g. Maurer *et al.*, 1997; Glare and Inwood, 1998; Fargues *et al.*, 2002; Morton *et al.*, 2003). Others have found a relationship between geographical origin and genetic variability (Rohel *et al.*, 1997; Nielsen *et al.*, 2001). The *Z. radicans* isolates used in this study did not show a clear relationship with geographical origin or host. One possible reason may be the fact that isolates from *Z. radicans* may infect different insect hosts (Milner and Mahon, 1985) which would allow this fungus to disperse more, particularly if the insect host had a wide distribution as well. It is likely also by using additional molecular techniques that different results could be obtained. For example, Nielsen *et al.* (2001) using RAPD primers found genetic variability and geographical origin relationships for *P. neoaphidis* isolates, although Tymon and Pell (2005), with isolates of the same species and using ERIC primers, found no relationship.

Some of the isolates used in this research were likely to have been isolated from individual insect cadavers found under epizootic conditions which may suggest that they were infecting their primary host, but some of them may originate from single isolated cadavers and not necessarily infecting the primary host. Isolates coming from different hosts but as a result of cross infection can result in no genetic variability among them, or if some variability is detected, no direct relationship would be found.

The compatibility groups formed in the intra-specific mycelial interactions (Chapter 3) was not supported by the RFLP grouping. For example, isolates NW410, NW386 and NW168, each from different compatibility groups, were all in the same RFLP group. Again, the use of other molecular techniques would be necessary in order to assess genetic variability, perhaps using other genes or targeting the whole genomic DNA.

In conclusion, the RFLP analysis demonstrated that *Z. radicans* was genetically more diverse than *P. blunckii,* although no direct relationship was found with

host or geographical origin. The lack of variability amongst the *P. blunckii* isolates may be because all the isolates came from the same host and geographical origin. Unfortunately, it was difficult to obtain isolates from other hosts because it seems that this species has only been found infecting *P. xylostella* (Humber, 1997).

The genetic variability found amongst the *Z. radicans* isolates did not correlate with geographical origin or host. Also, it did not correlate with the mycelial compatibility group found in Chapter 3. However, this variability could be helpful to develop isolate-specific primers, which would be important for intra-specific interactions experiments. Unfortunately, this could not be carried out during this research, but represent an important future objective.

The larger genetic differences found amongst the *Z. radicans* isolates than amongst the *P. blunckii* suggest that the former species can adapt better than *P. blunckii* to other hosts or regions, which is supported by the fact that *P. blunckii* has been reported infecting *P. xylostella* only. From these results, it is still difficult to determine the mechanisms by which these species interact in the same host population (*P. xylostella*). It is possible that *P. blunckii*, because it seems that it only infects *P. xylostella,* has fewer chances to survive in the absence of the primary host. Therefore it may need to be more aggressive against *Z. radicans* in order to ensure infection of the host (as demonstrated *in vitro* in Chapter 3). *Zoophthora radicans* may survive by infecting alternative hosts or developing resting spores to germinate later in the season (Milner and Mahon, 1985; Glare *et al.*, 1989). It is difficult to confirm this because there are no formal studies evaluating the host range for both pathogens. This assumption is based just on the records of hosts for each isolate.

The type of behaviour of both species of entomopathogenic fungi, when infecting the same host at the same time, would provide important information about the type of interactions that may occur between these two species. Based on the results of this and previous chapters, fewer isolates were selected for the *in vivo* interaction experiments, estimating first their relative virulence against *P. xylostella*. These experiments are described in Chapter 5.

CHAPTER 5. VIRULENCE OF SELECTED ISOLATES OF *Zoophthora radicans* AND *Pandora blunckii* AGAINST *Plutella xylostella* AND THEIR *IN VIVO* INTER-SPECIFIC INTERACTIONS

ABSTRACT

There have been reports of *Z. radicans* and *P. blunckii* isolates co-occurring in the same location and time infecting the same population of *P. xylostella* in the field. It is very likely that both fungal species interact when infecting *P. xylostella* larvae. In the experiments reported in this chapter, the possible outcomes of the interactions between these two species of fungal pathogens under laboratory conditions were investigated. As virulence can have an important role in the outcome of an interaction, the relative virulence of the four isolates (two from each species) used in these experiments were first estimated at two different temperatures, 20 and 25 °C. Overall, the *P. blunckii* isolates were more virulent than the *Z. radicans* isolates. Differences between isolates of the same species were also found. Although the results obtained at the different temperature could not be compared statistically, it seems that the four isolates were more virulent at 25 than at 20 °C. Using the same isolates, inter-specific interactions experiments were carried out in Petri dishes and on Chinese cabbage plants at 20 °C. In the Petri dish experiments all possible inter-specific combination were investigated. Both fungal species had the same opportunity to infect the same group of *P. xylostella* larvae (active dual-inoculation), only the dose ratio between both pathogens were manipulated. In the Chinese cabbage plant experiments, different batchs of *P. xylostella* larvae were placed on the plants with fungal inoculum either from both pathogens at the same time, or separately. The probability of a larva becoming infected either by one fungal species or the other depended on how close the larvae were to the source of the inoculum (passive dual-inoculation). In both types of experiment, four possible outcomes were obtained, infected by one or the other species, larvae infected by both species and unknown cause of mortality. There were live insects at the end of the experiment, but these were not considered

for the analysis. From the Petri dish experiment, the outcomes of the interactions suggested that this depends more on the dose of inoculum than virulence. The fungal species with the largest dose out-competes the other species. From the Chinese cabbage plant experiment, as the dose was not manipulated, it was not possible to assess dose effect, but the results were similar to the Petri dish experiment. The ecological implications of these results for the co-existence of both pathogens in the field are discussed.

5.1 INTRODUCTION

The fact that *Z. radicans*, *P. blunckii* and their host *P. xylostella* co-occur in space and time (Riethmacher and Kranz, 1994; Velasco *et al.*, 2001), suggests a strong possibility of an interaction between these two fungal species. Therefore, it is important to assess the possible outcomes that may occur when *Z. radicans* and *P. blunckii* are dual-inoculated on *P. xylostella* larvae.

Interactions between two or more pathogen species or genotypes are common or even the rule (Read and Taylor, 2001). These interactions have the potential to modify the population dynamics of particular insect–pathogen interactions (Thomas *et al.*, 2003). The interaction between two pathogens infecting the same host can result in different outcomes such as the exclusion of one of the pathogens or co-existence; these results may be due to differences in virulence, for example if one of the pathogens is more virulent than the other, the former will exclude the latter. If the virulence is similar for both pathogens, both may co-infect via scramble competition (e.g. Nowak and May, 1994). The outcomes from a dual-inoculation and infection will also be greatly influenced by some abiotic factors such as temperature (e.g. Inglis *et al.*, 1997; Fargues and Bon, 2004) and other biotic factors inherent to the pathogens such as differences in developmental rate, differences in dose ratio (e.g. Koppenhöfer and Kaya, 1996) or host range (e.g. Perlman and Jaenike, 2001).

The effect of temperature on a host-pathogen interaction can be altered via thermal behaviour of the host. For example, active behavioural thermoregulators elevate and maintain their body temperature higher than

ambient, and some thermal generalists are highly mobile and can move over a range of microclimates and as a consequence change their body temperature (Blanford and Thomas, 1999). Lepidopteran larvae are thermal generalists (Blanford and Thomas, 1999), which means that they do not regulate body temperature as a behavioural thermoregulator, but their body temperature follows environmental temperatures and the capacity to use temperature as a defence is minimal (Thomas and Blanford, 2003).

Plutella xylostella larvae cannot regulate body temperature. However, the interactions between this insect and the enthomopthoralean fungi that attack it will still be influenced by the environmental temperature. Although there are no reports demonstrating the effect of temperature on the interaction between *P. xylostella* and entomophthoralean fungi, there are some reports for other insects and fungal species (Tefera and Pringle, 2003; Milner and Luton, 1983; Yeo *et al.*, 2003; Stacey and Fellowes, 2002; Moorhouse *et al.*, 1994; Dimbi *et al.*, 2004).

Another factor that influences the host pathogen interaction is variability in virulence that can be found among fungal species infecting the same host (e.g. Yeo *et al.*, 2003; Tefera and Pringle, 2004), and among isolates of the same fungal species (e.g. Milner and Lutton, 1983; Feng and Johnson, 1991; Pell *et al.*, 1993a; Shah *et al.*, 2004).

It is important to define what is meant by virulence and how it is different to pathogenicity. There has been confusion because definitions have varied depending on the scientific discipline (e.g. Thomas and Elkinton, 2004). Shapiro-Ilan *et al.* (2005) reviewed the definitions and agreed that the most accurate definitions for pathogenicity and virulence are those given by Steinhaus and Martignoni in 1970, where pathogenicity was defined as the quality or the state of being pathogenic (qualitative) and virulence was defined as the degree of pathogenicity (quantitative). Tanada and Kaya (1993) also suggested that pathogenicity is applied to groups or species and virulence to within species. However, both terms can be used on any group or taxonomic

level as long as pathogencity is considered as qualitative and virulence as quantitative (Shapiro-Ilan *et al.*, 2005).

A common starting point in the evaluation of virulence is to estimate the dose-response relationship under constant laboratory conditions. However, the importance of environment conditions, particularly temperature, on virulence should also be considered (e.g. Thomas and Blanford, 2003; Blanford *et al.*, 2003, Yeo *et al.*, 2003; Stacey and Fellowes, 2002; Blanford and Thomas, 1999; Thomas and Jenkins, 1997; Inglis *et al.*, 1996).

Either for selection of potential isolates for microbial control, or to study the ecology of the interactions between host and pathogen, an estimation of the virulence of a pathogen against the insect is important because this can affect the outcome of potential competitive interactions between different species of pathogens competing for the same host.

The following isolates were selected, ARSEF6293 and ARSEF6311 from *P. blunckii*, and isolates NW386 and NW250 from *Z. radicans*. The selections were based on results of previous chapters. Isolate NW386 was selected because it had the greatest *in vitro* growth amongst all the Mexican isolates of *Z. radicans* (Chapter 2). Although it was not aggressive against any *P. blunckii* isolate in the *in vitro* interactions experiment, its growth was not affected by the presence of any *P. blunckii* isolate either (Chapter 3). In preliminary observation in *P. xylostella*, this isolate normally produced cadavers with internal resting spores only. Isolate NW250, although it did not have a particular behaviour (intermediate growth) when grown *in vitro* at different temperatures (Chapter 2), was selected because a large amount of research has been carried using this isolate previously and it served as a reference (e.g. Pell *et al.*, 1993a, b; Yeo *et al.*, 2001; Furlong and Pell, 1997; Furlong and Pell, 2000; Furlong and Pell, 2001). In addition, the presence of *P. blunckii* isolates, particularly ARSEF6293, had a negative effect on its growth during the *in vitro* interactions experiment (Chapter 3). This isolate did not produce cadavers with resting spores, only external sporulation on cadavers to produce many conidia, which confirmed the work of previous authors (Pell *et al.*, 1993a; Yeo *et al.*,

2001). Each isolate could be placed in different mycelial compatibility group (Chapter 3) and also in different RFLP groups (Chapter 4).

Isolate ARSEF6293 was selected because it had the greatest growth *in vitro* of all *P. blunckii* isolates (Chapter 2). In the *in vitro* interactions experiment, it was the most aggressive isolate against the majority of the *Z. radicans* isolates. In preliminary observations in *P. xylostella*, this isolate normally produced cadavers with ephemeral external mycelial growth (few conidia) or non-sporulating cadavers. Isolate ARSEF6311 had an intermediate *in vitro* growth compared to all *P. blunckii* isolates (Chapter 2). It was not aggressive against the *Z. radicans* isolates, in fact it was the isolate with the least effect on isolate NW250 (Chapter 3). This isolate normally produced external sporulation on cadavers to produce many conidia. The mycelial compatibility between both isolates was variable; they were incompatible at 20 °C, but partially compatible at 25 °C.

The relationship between *Z. radicans* and *P. blunckii* with the diamond back moth, *P. xylostella,* is ecologically and practically important, because the more information available about this relationship, the more chances of success there will be using these entomopathogens for the development of microbial control programmes against *P. xylostella*.

The aims of these experiments were first; evaluate the relative virulence, estimated as the lethal concentration of conidia needed to kill 50 % of inoculated larvae (LC_{50}) of selected isolates of *Z. radicans* and *P. blunckii* against *P. xylostella* larvae. Second; determine the possible outcomes when *Z. radicans* and *P. blunckii* isolates were dual-inoculated onto *P. xylostella* larvae at different spatial scales.

5.2. MATERIAL AND METHODS

5.2.1. *Plutella xylostella* culture

The *P. xylostella* colony was reared in Perspex® cages (0.5 m^2 x 50 cm) at 23

°C (16L:8D) in the quarantine area of Rothamsted Research's insectary. The original colony came from the Philippines in the 1980's and has been maintained at Rothamsted Research continuously since then.

Chinese cabbage leaves with eggs were placed onto two week old Chinese cabbage plants in an insectary cage. After the larvae emerged and began feeding, the plants were replaced continuously as required until the majority of larvae had reached late 4th instar. Prior to pupation, remaining plants were placed close to the cage walls and the watering stopped to encourage larvae to pupate on the cage walls. Dried plants were removed before adults emerged. After adult emergence two or three fresh Chinese cabbage leaves were placed inside the cage for oviposition. When the eggs were used to maintain the colony these leaves were left for two or three days but when the eggs were used for an experiment, leaves were left for 24 hours only to ensure the resulting cohort of larvae were the same age. Excess plant and insect material for disposal was frozen first.

5.2.2. Fungal isolates

The *Z. radicans* isolates NW250 and NW386, and the *P. blunckii* isolates ARSEF6293 and ARSEF6311, all coming from *P. xylostella* were used in these experiments. After retrieval from storage and prior to use in experiments all isolates were subcultured on SEMA medium (See 2.2.1.2). The cultures were incubated at 20 °C in darkness for 15 days before use. Cultures were not subcultured more than three times after retrieval from liquid nitrogen.

5.2.3. Estimation of LC_{50} for selected *Zoophthora radicans* and *Pandora blunckii* isolates under two different temperature conditions

The objective of this experiment was to detect differences in the virulence of the selected isolates, and the effect of temperature on the results. The bioassay procedure was modified from the methods described by Wilding, 1976 and Shah *et al.*, 2004 for aphids and from Pell *et al.*, 1993 for *P. xylostella*.

The inoculation was carried out using fungal plugs (9 mm diameter) from the growing edge of 15 day-old cultures. Five plugs were placed in the lid of a 50 mm diameter Petri dish containing damp filter paper. The Petri dish with the fungal plugs was placed in a plastic box containing moistened tissue paper (to ensure a high humidity and encourage conidial production) at 20 °C in darkness for 18 hours prior to experimentation.

Early third instar larvae were placed in groups of 20 on a cabbage leaf disk embedded abaxial side uppermost in 1.5 % water-agar (5 ml) at the bottom of a 50 mm diameter Petri dish. A 10 mm diameter glass cover slip was placed in the centre of the leaf disk. For inoculation, the lids were replaced with the lids containing the fungal plugs. The larvae were feeding on the leaf while they were inoculated and did not come into direct contact with the inoculum.

To obtain a range of doses, different batches of 20 larvae were exposed to the fungus for 1, 5, 10, 25, 35, 45 and 60 minutes. Control larvae were maintained in the same conditions for 60 minutes without any inoculum. During inoculation, the lids containing the fungal plugs were rotated to ensure an even deposition of conidia.

After inoculation, each batch of larvae was divided into two groups of ten larvae and transferred to ventilated transparent polystyrene boxes (124x82x22 mm) (Stewart Group Holdings Ltd., Surrey, England) containing fresh Chinese cabbage leaf. One group of ten larvae from a pair of groups was incubated at 20 and the other at 25 °C in a 12:12 L:D regime. The polystyrene boxes were placed inside a plastic tray (38x24x8 cm) (Stewart Group Holdings Ltd., Surrey, England) inside a plastic bag for the first 24 hours to maintain high humidity and ensure germination of the conidia. Mortality was recorded daily for 5 days. Cadavers were transferred to 50 mm diameter Petri dishes with 1.5 % water-agar (5 ml) at the bottom to encourage sporulation. The Petri dishes with cadavers were maintained at the same temperature as incubation had taken. After inoculation, the coverslips were removed, fixed with 10% cotton blue in lactophenol on glass slides and conidia doses estimated by counting

conidia from 10 fields of view with a 1 mm^2 eyepiece graticule using the 10x microscope objective.

To score larval mortality due to infection by any isolate, evidence of external sporulation to produce conidia was recorded. However, because resting spores are produced by isolate NW386, the presence of resting spores inside all the cadavers from all treatments (including cadavers from the *P. blunckii* treatments) were also recorded microscopically. Those cadavers that did not produce any external sporulation (conidia) or resting spores were classified as non-sporulating cadavers with an unknown cause of mortality.

The bioassays were carried out on four different occasions and with two replicates of each showering time for each isolate on every occasion, except the first occasion when only one replicate was done.

5.2.3.1 Statistical analysis

The data from the bioassays were analysed using probit analysis in the statistical package GenStat v. 8.1. The criterion to decide whether a larva was infected was the observation of external sporulation (conidia) from the cadavers, internal production of resting spores or both. However, the *P. blunckii* isolate ARSEF6293 normally produced cadavers with an ephemeral external sporulation (few conidia) and non-sporulating cadavers. Considering only the cadavers showing external sporulation, the infection due to ARSEF6293 would have been underestimated, therefore, the total sample size was adjusted to allow for control mortality using a procedure similar to Abbot's formula (Abbott, 1925) using absolute numbers rather than percentages. The formulae used to adjust total sample size and responding size are as follows:

To adjust the initial number of larvae on the treatments:

$$AdjNt = Nt * ((1 - (Rc/Nc))$$

To adjust the number of responding larvae on the treatments:

$$AdjRt = Rt - (Nt - AdjNt)$$

Where: AdjNt=Adjusted initial number of larvae on treatment, Nt=Un-adjusted initial number of larvae on treatment, Rc=Number of dead larvae on the control, Nc=Number of alive larvae on the control, AdjRt=Adjusted number of responding larvae on the treatments and Rt=Number of dead larvae on the treatments.

Before comparing among isolates, a parallel model Probit analysis was done to determine whether the results of all replicates could be combined. First, a single probit line was fitted to data from all the replicates. Secondly, intercepts were allowed to vary among all replicates and third, slopes were also allowed to vary among replicates. The best model was chosen in each case on the basis of partial *F*-tests. Concentrations causing 50 % infection (LC_{50}) of larvae were estimated from best fit models and confidence intervals (CI) for LC_{50} were calculated according to Fieller's theorem (Fieller, 1944). Probit analysis assumes the number of infected larvae follow a binomial distribution with sample sizes equal to the number of larvae tested. Where necessary, more variability in the data than expected under the binomial assumptions was allowed for by testing the ratio of the treatment mean deviance to the residual mean deviance against the *F*-distribution, rather than testing the usual treatment deviance against the χ^2 distribution.

The assay results for each temperature were analysed separately. At 25 °C, as the single line model was the best model for all the replicates for each isolate, data from the different replicates for each isolate were combined, and further parallel model analyses were done to compare among isolates. At 20 °C, only individual assays of the two *Z. radicans* isolates were represented by a single line model. Individual assays of the *P. blunckii* isolates showed variation in intercepts and slopes, hence the data of the individual assays for these isolates could not be combined. The results for all four isolates were analysed separately because of the variability in the two *P. blunckii* isolates.

5.2.4. *In vivo* inter-specific interactions between *Zoophthora radicans* and *Pandora blunckii* isolates against *Plutella xylostella* larvae in Petri dishes

The objective of this experiment was to evaluate the interactions that may occur between these two pathogens in *P. xylostella* larvae. The inoculation was done directly onto the larvae, ensuring that each larva received conidia from both pathogens (active inoculation).

For these experiments, dual-inoculations using the isolates from the dose-response experiment (5.2.3.) were made in all possible combinations (Table 5.1).

Table 5.1. Combinations of isolates for dual-inoculation tested against *Plutella xylostella* larvae. Zr = *Zoophthora radicans*, Pb = *Pandora blunckii*.

Combination	Isolates
1	NW250 (Zr) vs. ARSEF6293 (Pb)
2	NW250 (Zr) vs. ARSEF6311 (Pb)
3	NW386 (Zr) vs. ARSEF6293 (Pb)
4	NW386 (Zr) vs. ARSEF6311 (Pb)

Due to the difference in virulence of each isolate against *P. xylostella,* and the difficulty in delivering a predetermined dose for each combination of isolates (Table 5.1), different times of exposure were used in order to obtain different proportions of conidia of each species in nine different treatments (Table 5.2).

Table 5.2. Treatments with different times of exposure used to obtain different proportion of conidia of each species in each combination.

Treatment	Zr (minutes)	Pb (minutes)
1	45	5
2	5	45
3	45	45
4	5	5
5	45	0
6	5	0
7	0	45
8	0	5
9	0	0

Batches of 20 larvae were each inoculated with the 9 different treatments. All the treatments had a maximum inoculation time of 50 minutes, except treatment 3 which had 90 minutes of inoculation time. The controls were maintained under the same conditions for 90 minutes without inoculum.

The dual-inoculations were carried out in 20 cm diameter plastic Petri dishes. Two 30 mm diameter Petri dish lids were stuck in the lid of the large Petri dish using double sided adhesive tape. Each 30 mm diameter lid contained seven sporulating fungal plugs of 9 mm diameter. One 30 mm diameter Petri dish lid contained only *Z. radicans* fungal plugs and the other lid only *P. blunckii* fungal plugs. Two 30 mm diameter Petri dish bases, each with a cabbage leaf disk embedded abaxial side uppermost in 1.5% water-agar (2 ml) were placed on the base of the 20 cm diameter Petri dish which contained 50 ml of 1.5 water-agar to maintain high humidity during inoculation. A 10 mm diameter glass cover slip was placed in the centre of each cabbage leaf for dose estimation.

During the dual-inoculation, the base of the 20 cm diameter Petri dish was maintained stationary, and only the lid of the 20 cm diameter Petri dish,

containing the two 30 mm diameter lids with the fungal plugs, was rotated. With this rotation the same batch of 20 larvae was inoculated with both species of fungi one after the other; the different proportions of conidia from each fungal species were obtained by exposing the larvae for different lengths of time (Fig. 5.1). The estimation of the conidia concentration was made as in 5.2.3.

After inoculation, each batch of larvae was treated as in 5.2.3. All treatments were incubated at 20 °C in a 12:12 L:D regime. Mortality was recorded daily for 6 days.

During scoring, all larvae were allocated to one of the following categories: living, dead due to *Z. radicans*, dead due to *P. blunckii*, dead due to a combination of both pathogens and dead due to unknown mortality factors.

In order to identify the cause of death of the larvae, each larva was individually placed in the base of a 30 mm diameter Petri dish containing 2 ml of 1.5% water agar. The Petri dishes were inverted and incubated at 20 °C for 24 h and 12:12 L:D regime. The inverted position allowed the collection of conidia on the lid of the Petri dish. After the 24 h incubation period, all lids were fixed with 10% cotton blue lactophenol and covered with a coverslip. First, the presence or absence of conidia was recorded, after that, the presence of one or both pathogens were assessed by identifying the morphology of the conidia collected microscopically. No conidia quantification was done. Simultaneously, the presence or absence of resting spores as an indication of infection by isolate NW386 inside each cadaver from all treatments and isolate combinations was recorded microscopically.

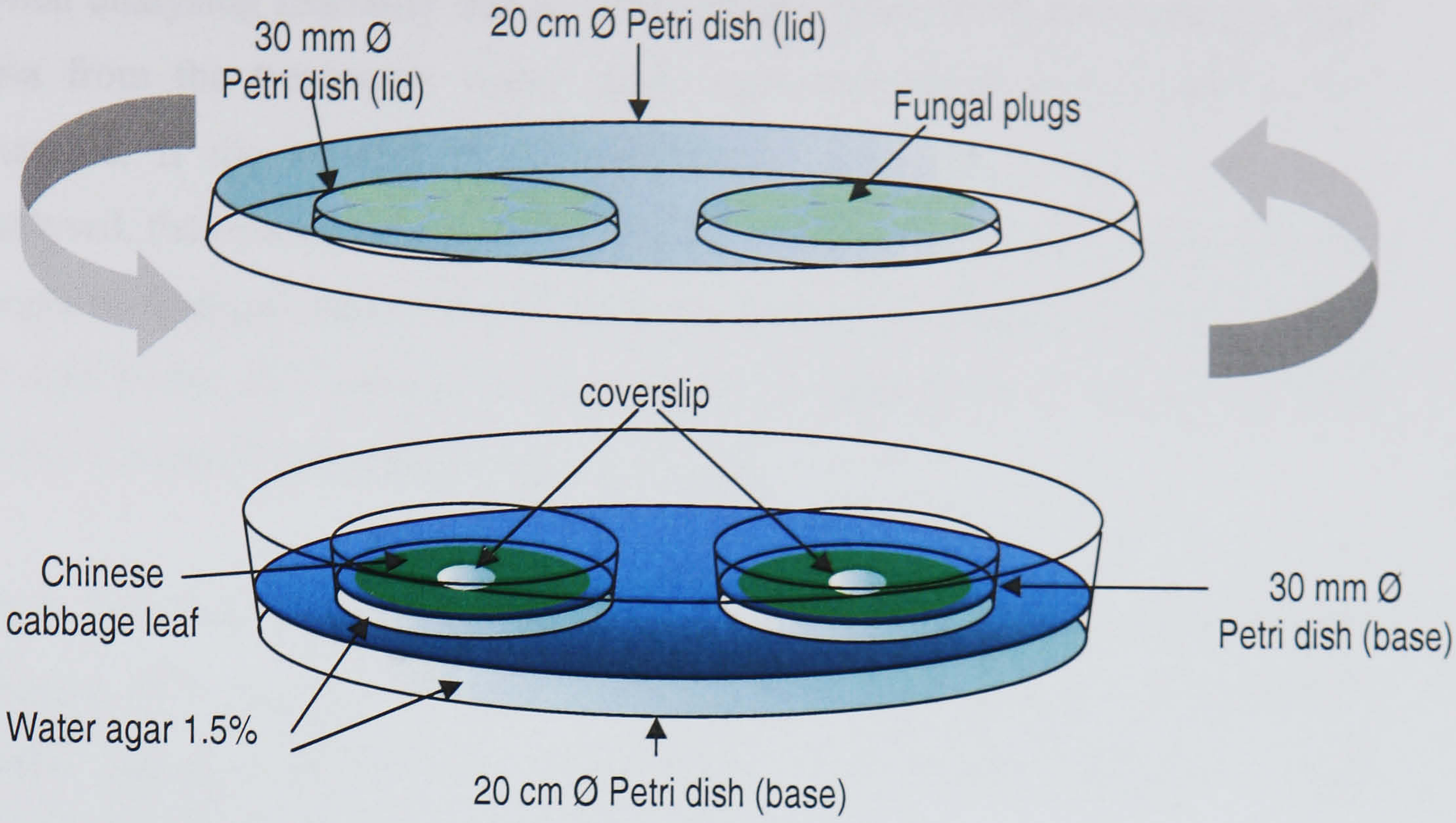


Figure 5.1. Diagram of the Petri dishes used for dual-inoculation in the *in vivo* inter-specific interaction experiments.

The four interactions were carried out on the same day and the whole experiment was repeated on three separate occasions.

5.2.4.1 Statistical analysis

Each isolate combination (Table 5.1), was analysed separately. In each combination, the data were analysed using logistic regression (assumed to follow a binomial distribution) with each cause of death as a variable (either by *P. blunckii*, *Z. radicans*, both pathogens or unknown cause of mortality), and the sample size equal to the number of individuals tested. Each number of deaths was a proportion of the total number of insects tested.

The different concentrations obtained for each isolate in each combination, were allocated into three categories, "No dose" when no fungus was inoculated, "Low dose" with all the concentrations obtained with the least time of inoculation (five minutes), and "High dose" with all the concentrations obtained with longest inoculation time (45 minutes).

When analysing mortality due to either fungus alone or dual-inoculated, only data from the treatments where fungi were inoculated were included. For example, if the number of infected larvae due to *P. blunckii* only were analysed, the treatments where "Low dose" or "High dose" of *P. blunckii* either single or dual-inoculated with *Z. radicans* were used. Hence, the total number of data points and degrees of freedom for treatments terms and the residual differed depending on the variable analysed.

More variability in the data than expected under the binomial assumptions was allowed for by testing the ratio of the treatment mean deviance to the residual mean deviance against the *F*-distribution, rather than testing the usual treatment deviance against the χ^2 distribution.

All the statistical analyses were carried out using the statistical package GenStat v. 8.1.

5.2.5. *In vivo* inter-specific interactions between *Zoophthora radicans* and *Pandora blunckii* isolates against *Plutella xylostella* larvae on Chinese cabbage plants

The objective of this experiment was to estimate the interactions that may occur between these two pathogens in *P. xylostella* populations in which the larvae were not directly inoculated by the fungi but received inoculum passively. In this experiment, the interactions between these two species were quantified in *P. xylostella* larval populations maintained on Chinese cabbage plants.

Only three isolates were used in this experiment, the *Z. radicans* isolates NW250 (Malaysia) and NW386 (Mexico), and the *P. blunckii* isolate ARSEF6293 (Mexico).

Single two week old Chinese cabbage plants were individually placed in an unventilated cylindrical cage made of transparent flexible plastic sheet over an 11"x 8" seed tray with an absorbent mat in the bottom for watering the plant.

Fifteen early third instar larvae were placed on each plant. The larvae were allowed a period of two hours to establish feeding sites and then, according to the treatment, different combinations of fungi were placed on the plants. The cages were without ventilation to avoid cross contamination of fungal conidia among the treatments. Watering was not abundant to avoid excess condensation inside the cages.

The treatments were as follows:

1. NW250+ARSEF6293
2. NW386+ARSEF6293
3. NW250
4. NW386
5. ARSEF6293
6. Control

In treatments one and two, two 9 mm plugs cut from 15 day old growing colonies of each species were placed separately on two of the largest leaves (two plugs of one species on one leaf and two plugs of the other species on a second leaf). In treatments three to five two plugs of only one species were placed together on only one leaf. The control was without either fungus.

The experiment was evaluated on day four. All the cadavers were placed individually in 30 mm diameter Petri dishes on 2 ml of 1.5% water agar. This provided high humidity and encouraged external sporulation and confirmation of infection. The Petri dishes were inverted in order to collect the conidia on the Petri dish lid and determined which species was present in the cadavers if they sporulated externally. At the same time, the presence or absence of resting spores as an indication of infection by isolate NW386 inside each cadaver from all treatments was recorded microscopically. All the remaining living larvae from each treatment were maintained separately in ventilated transparent polystyrene boxes (124x82x22 mm) until day 5 to evaluate any further mortality.

The experiment was run under a completely randomised block design with six treatments and three replicates. The experiment was carried out on two different occasions. The cages were incubated at 20 °C and 12:12 L:D regime.

5.2.5.1 Statistical analysis

The analysis carried out was the same as in 5.2.4 with some modifications. In the previous experiment, each isolate combination was analysed separately, but in this case, all the isolate combinations were analysed together. Each cause of death was analysed separately as in 5.2.4. All the statistical analyses were carried out using the statistical package GenStat ver. 8.1.

5.3. RESULTS

5.3.1. Estimation of LC_{50} for selected *Zoophthora radicans* and *Pandora blunckii* isolates under two different temperature conditions

The variability amongst the individual assays for each isolate was greater at 20 than at 25 °C.

At 25 °C, the different individual assays within each isolate treatment were represented by a single line model because no evidence of different intercepts or slopes was found for the four isolates (Table 5.3.). This justified pooling the data from the individual assays.

Table 5.3. Results of the parallel model probit analysis carried out with the four isolates at 25 °C.

	Zoophthora radicans isolates		*Pandora blunkcii* isolates	
Model	NW386	NW250	ARSEF6293	ARSEF6311
Single line	$F_{1,35}$=16.71, P<0.001	$F_{1,34}$=45.20, P<0.001	$F_{1,29}$=29.88,P<0.001	χ^2_1=79.19, P<0.001
Intercept diff.	$F_{6,35}$=0.61, P=0.724	$F_{6,34}$=1.72, P=0.146	$F_{5,29}$=1.97, P=0.114	χ^2_4=0.52, P=0.720
Slope diff.	$F_{6,35}$=1.49, P=0.209	$F_{6,34}$=0.46, P=0.831	$F_{5,29}$=1.10, P=0.380	χ^2_4=0.87, P=0.483

Using the pooled data of the individual assays within each isolate, a further parallel model probit analysis was performed to compare virulence amongst the four isolates against *P. xylostella*.

The parallel line probit was the best fit for the four isolates tested against *P. xylostella*, because a significant difference was found amongst the intercepts ($F_{3,165}$=9.79, P<0.001) but no evidence of slope differences ($F_{3,165}$=1.06, P=0.366). At 25 °C, *P. blunckii* isolate ARSEF6311 was the most virulent against *P. xylostella* with the smallest LC_{50} value, and the least virulent was *Z. radicans* isolate NW386 with the largest LC_{50} value (Table 5.4). Isolates NW250 (*Z. radicans*) and ARSEF6293 (*P. blunckii*) showed similar LC_{50} values. Their LC_{50} values were 2.7 and 3.8 fold smaller than the LC_{50} value of NW386 respectively, but 3.5 and 2.8 fold larger than the LC_{50} value of ARSEF6311 respectively (Table 5.4).

At 20 °C, only individual assays of the two *Z. radicans* isolates were represented by a single line model. Individual assays of the two *P. blunckii* isolates showed significant non-parallelism in probit models, hence the data from the individual assays for these isolates could not be combined. The results for all four isolates were analysed separately because of the variability in the two *P. blunckii* isolates. A direct comparison amongst the four isolates was possible in five out of six individual assays. The sixth individual assay did not include isolate ARSEF6293 in the analysis because of the large mortality obtained in all the concentrations (Table 5.5.).

Table 5.4. Comparison in virulence of *Zoophthora radicans* and *Pandora blunckii* isolates against *Plutella xylostella* at 25 °C. Number in brackets alongside the isolate code in the first column represents the number of individual assays pooled.

	25 °C		
Isolate	**LC_{50} (95% CI)**	**Intercept (SE)**	**RC (SE)**
NW386 (7)	7.001 (3.758-11.722)	-0.756 (0.158)	0.8946 (0.0838)
NW250 (7)	2.552 (1.239-4.571)	-0.364 (0.149)	" "
ARSEF6923 (6)	2.023 (0.899-3.871)	-0.274 (0.158)	" "
ARSEF6311 (5)	0.716 (1.239-4.571)	0.130 (0.153)	" "

LC_{50} (conidia/mm^2) ± 95% confidence interval (CI).

RC=Regression coefficient (slope).

The first and sixth individual assays at 20 °C were best represented by the single line model, i.e. no differences in intercepts or slopes ($F_{3,20}$=0.34, P=0.799; $F_{3,20}$=2.89, P=0.061) ($F_{2,15}$=0.48, P=0.629; $F_{2,15}$=0.84, P=0.451) were found respectively. This suggests no differences among the isolates in their virulence against *P. xylostella* in these particular assays. Therefore only one LC_{50} value is showed for these particular assays (Table 5.5).

The best model for the other individual assays was a parallel model because there were differences in the intercepts, χ^2_3=4.69, P=0.003; $F_{3,20}$=6.05, P=0.004; $F_{3,20}$=4.61, P=0.013 and $F_{3,15}$=5.84, P=0.008, but not slope differences, χ^2_3=1.93, P=0.122; $F_{3,20}$=0.67, P=0.583; $F_{3,20}$=0.60, P=0.624 and $F_{3,15}$=1.38, P=0.287 from individual assays two to five respectively. In all these four assays, *Z. radicans* isolate NW386 had the largest LC_{50} values (Table 5.5). The smallest LC_{50} values were obtained for isolate ARSEF6311 in two of the assays (assay two and three), ARSEF6293 in assay four and NW250 in assay five. Overall, the *P. blunckii* isolates were more virulent than the *Z. radicans* isolates particularly NW386 (Table 5.5).

Table 5.5. Comparison in virulence of *Zoophthora radicans* and *Pandora blunckii* isolates against *Plutella xylostella* of each individual assay at 20 °C.

20 °C			
Assay 1			
Isolate	**LC_{50} (95% CI)**	**Intercept (SE)**	**RC (SE)**
All isolates combined	13 (3.977-24.28)	-1.375 (0.491)	1.234 (0.308)
Assay 2			
NW386	4.332 (1.5183-9.543)	-0.689 (0.273)	1.083 (0.173)
NW250	3.192 (0.8654-8.125)	-0.546 (0.305)	" "
ARSEF6923	0.587 (0.1495-1.666)	0.251 (0.258)	" "
ARSEF6311	0.387 (0.1353-0.949)	0.447 (0.216)	" "
Assay 3			
NW386	43.04 (11.165-99.88)	-1.994 (0.616)	1.220 (0.274)
NW250	4.54 (1.003-11.36)	-0.802 (0.409)	" "
ARSEF6923	2.00 (0.369-5.51)	-0.368 (0.368)	" "
ARSEF6311	1.28 (0.167-4.07)	-0.130 (0.391)	" "
Assay 4			
NW386	12.044 (3.816-29.41)	-1.147 (0.353)	1.062 (0.195)
NW250	6.266 (2.071-15.80)	-0.846 (0.299)	" "
ARSEF6923	0.768 (0.085-3.16)	0.122 (0.375)	" "
ARSEF6311	2.178 (0.305-8.16)	-0.359 (0.392)	" "
Assay 5			
NW386	89.54 (38.94-203.71)	-3.390 (0.756)	1.737 (0.355)
NW250	6.19 (1.26-22.15)	-1.375 (0.636)	" "
ARSEF6923	15.21 (4.89-34.85)	-2.053 (0.630)	" "
ARSEF6311	39.93 (14.20-118.15)	-2.781 (0.663)	" "
Assay 6			
All isolates combined	7.206 (3.317-12.73)	-1.066 (0.306)	1.243 (0.224)

LC_{50} (conidia/mm^2) ± 95% confidence interval (CI)

SE=standard error

RC= Regression coefficient (slope)

5.3.2. *In vivo* inter-specific interactions between *Zoophthora radicans* and *Pandora blunckii* isolates on *Plutella xylostella* larvae in Petri dishes

In all isolate combinations, different doses in relation to different times of exposure to the sporulating fungal plugs were achieved. The conidia concentrations obtained with the longest exposure time were consistently larger than the concentrations obtained with the shorter exposure time (Table 5.6).

Table 5.6. Mean conidia concentrations (averaged over the three occasions that the experiment was run), achieved in each concentration category and isolate combination. The concentrations are expressed in conidia /mm^2.

Combination 1		NW250	
ARSEF6293	No dose	Low dose	High dose
No dose	0.00	17.60	167.10
Low dose	0.00	17.70	79.83
High dose	0.00	16.23	121.73
		ARSEF6293	
NW250	No dose	Low dose	High dose
No dose	0.00	21.03	91.97
Low dose	0.00	6.27	146.57
High dose	0.00	13.50	73.33
Combination 2		NW250	
ARSEF6311	No dose	Low dose	High dose
No dose	0.00	13.77	111.70
Low dose	0.00	14.97	109.57
High dose	0.00	20.93	142.33
		ARSEF6311	
NW250	No dose	Low dose	High dose
No dose	0.00	8.33	181.33
Low dose	0.00	3.53	35.73
High dose	0.00	18.07	51.79
Combination 3		NW386	
ARSEF6293	No dose	Low dose	High dose
No dose	0.00	12.63	143.37
Low dose	0.00	22.40	192.50
High dose	0.00	8.8	184.73
		ARSEF6293	
NW386	No dose	Low dose	High dose
No dose	0.00	21.13	67.67
Low dose	0.00	20.43	175.10
High dose	0.00	17.03	163.90

Table 5.6. Continued.

Combination 4		NW386	
ARSEF6311	No dose	Low dose	High dose
No dose	0.00	27.23	156.50
Low dose	0.00	34.93	128.13
High dose	0.00	15.03	163.47
		ARSEF6311	
NW386	No dose	Low dose	High dose
No dose	0.00	10.87	65.93
Low dose	0.00	11.67	173.17
High dose	0.00	10.73	115.43

*5.3.2.1 Isolates NW250 (*Z. radicans*) and ARSEF6293 (*P. blunckii*)*

*Proportion of larvae infected **only with ARSEF6293*** (P. blunckii) *in* Plutella xylostella *larvae either inoculated only with ARSEF6293 or dual-inoculated with ARSEF6293 and NW250* (Z. radicans)

No significant effect was found on the interaction between isolates ARSEF6293 and NW250 on the number of infected larvae ($F_{2,10}$=0.45, P=0.648). There is some evidence of differences in the proportion of infected larvae obtained with the low and high doses of ARSEF6293 ($F_{1,10}$=4.73, P=0.055), where the largest proportion of infected larvae were always obtained with the largest dose compared to the smaller dose as expected (Fig. 5.2). The lack of a significant interaction with NW250 showed that the differences on proportion of infected larvae between the low and high doses of ARSEF6293 were independent of the doses of NW250 that were present in the same larval group. A significant effect was found in the proportions of infected larvae obtained with ARSEF6293 in the presence of the three doses of NW250 ($F_{2,10}$=4.74, P=0.036). The largest proportion of infected larvae due to ARSEF6293 occurred when no conidia of NW250 were present, and proportions of infection were greatly reduced in the presence of the low and high doses of NW250 (Fig. 5.2). Again, this effect was independent of the low and high doses of ARSEF6293.

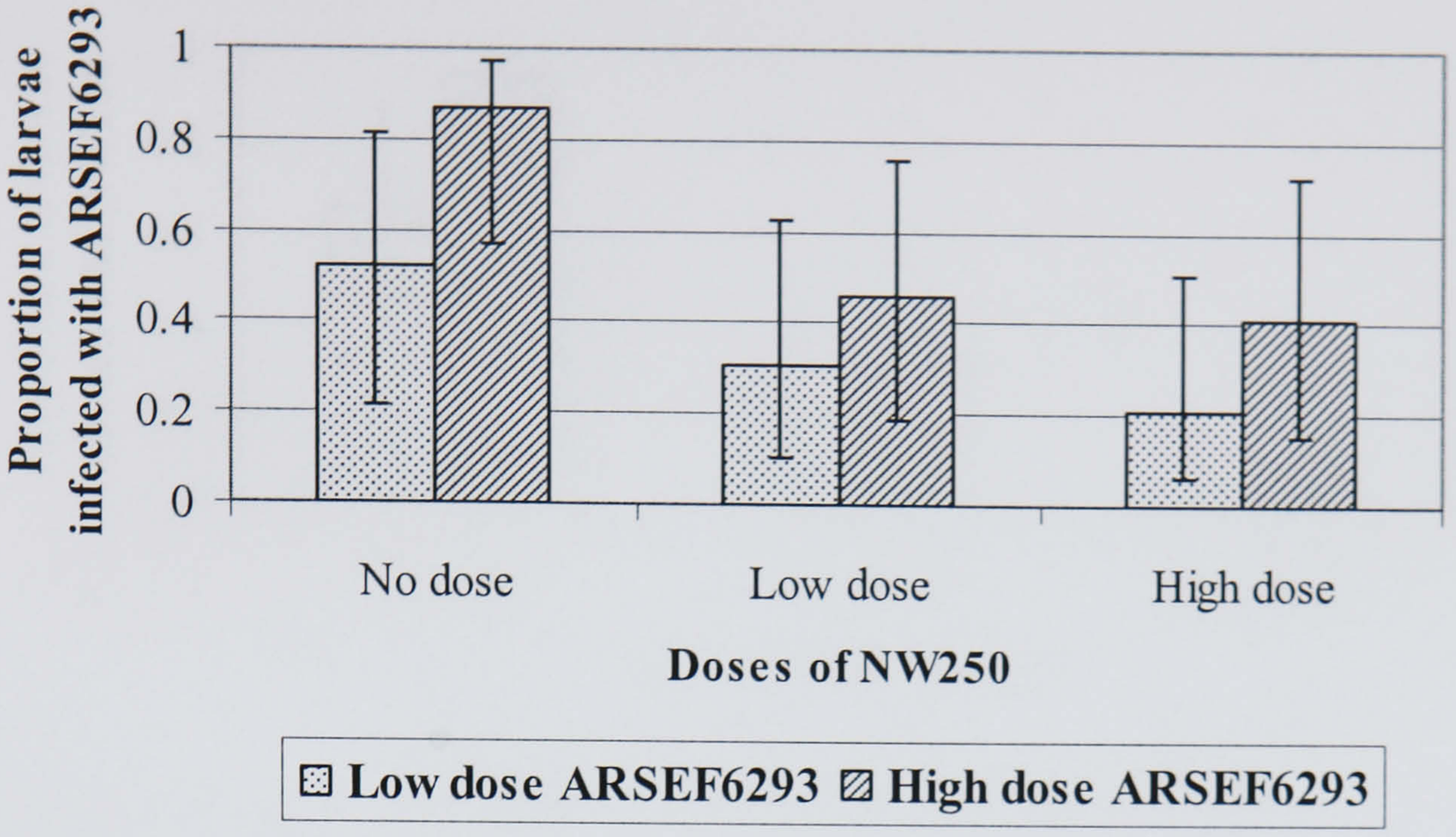


Figure 5.2. Proportion of *Plutella xylostella* larvae infected with ARSEF6293 at the two different dose categories (Low and High) of isolate ARSEF6293, and in the presence of three different dose categories (Low, High and No dose) of isolate NW250 (*Z. radicans*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of larvae infected ***only with NW250*** (Z. radicans) *in* Plutella xylostella *larvae either inoculated only with NW250 or dual-inoculated with NW250 and ARSEF6293* (P. blunckii)

No significant interaction was found between NW250 and ARSEF6293 in the proportion of infected larvae ($F_{2,10}$=1.47, P=0.277). Significant differences were found in the proportions of infected larvae between the low and high dose of NW250 ($F_{1,10}$=5.45, P=0.042). As expected, the largest proportion of infected larvae was obtained with the highest dose of NW250 (Fig. 5.3). Significant differences were found in the proportion of infected larvae obtained with NW250 in the presence of the three different doses of ARSEF6293 ($F_{2,10}$=23.90, P<0.001). The smallest proportion of infected larvae due to NW250 was in the presence of the highest dose of ARSEF6293, and the largest proportion of infected larvae due to NW250 was when no conidia of ARSEF6293 was present (Fig. 5.3).

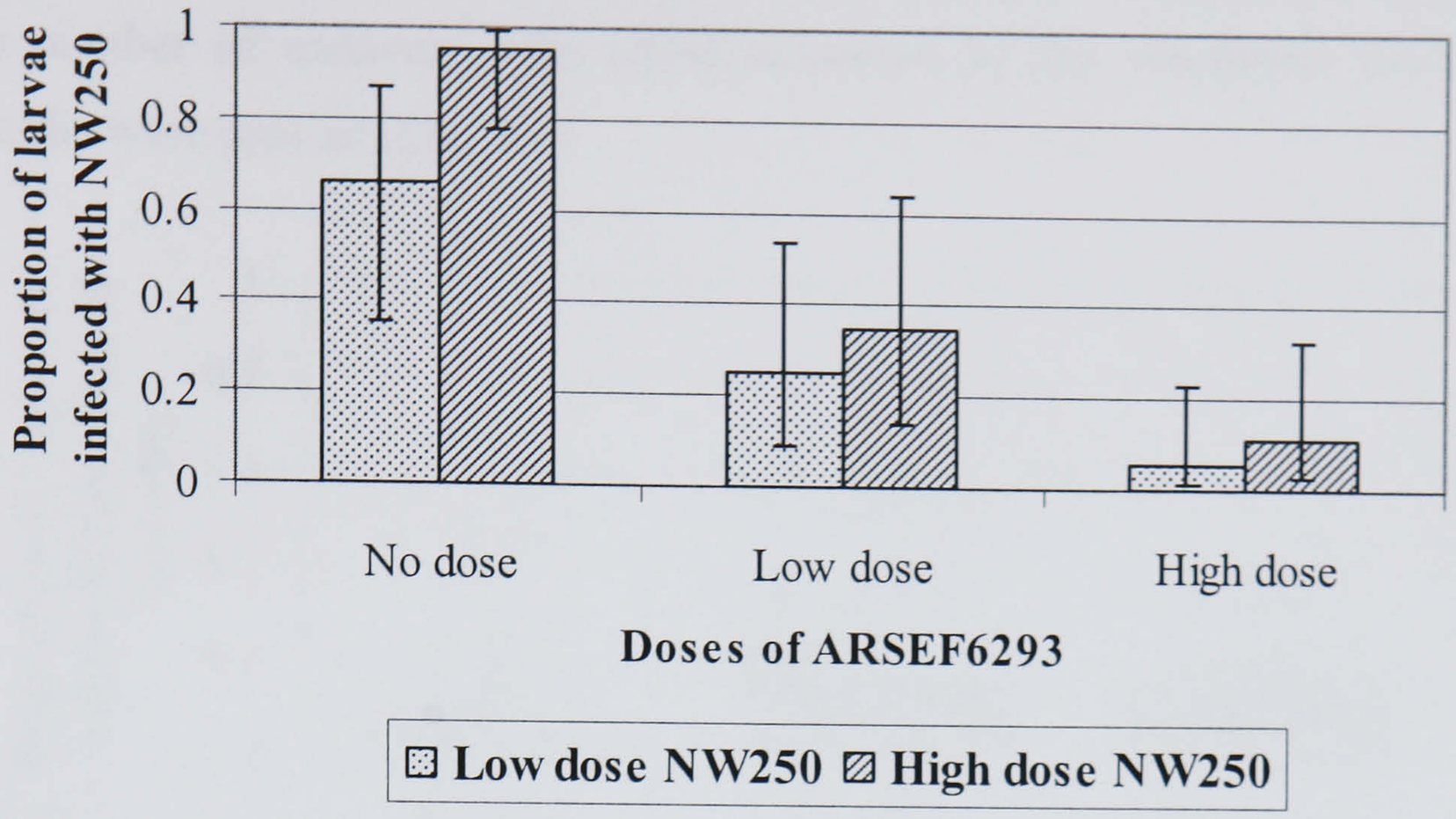


Figure 5.3. Proportion of *Plutella xylostella* larvae infected with the two different dose categories (Low and High dose) of isolate NW250 (*Z. radicans*), in the presence of three dose categories (Low, High and No dose) of ARSEF6293 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *larvae* ***dual-infected*** *with NW250* (Z. radicans) and *ARSEF6293* (P. blunckii)

The proportion of dual-infected larvae was small, less than 0.01 in all treatments. No significant effect by ARSEF6293 ($F_{1,6}$=2.52, P=0.164), NW250 ($F_{1,6}$=0.08, P=0.790) or the interaction between them ($F_{1,6}$=0.36, P=0.572) was found in the proportion of dual-infected larvae.

Proportion of Plutella xylostella *cadavers with* ***unknown cause of mortality (UCM)***

No interaction was found between both pathogens in the proportion of cadavers with unknown cause of mortality (UCM) ($X^2_{4,}$=0.97, P=0.421). Significant differences were found in the proportion of cadavers with UCM amongst the different doses of ARSEF6293 (X^2_2=5.75, P=0.003). The presence of NW250 had no significant effect on the proportion of cadavers with UCM (X^2_2=0.52,

P=0.592). The presence of ARSEF6293 (either in low or high dose) increased the number of cadavers with UCM compared to the treatments where no conidia were present (Fig. 5.4).

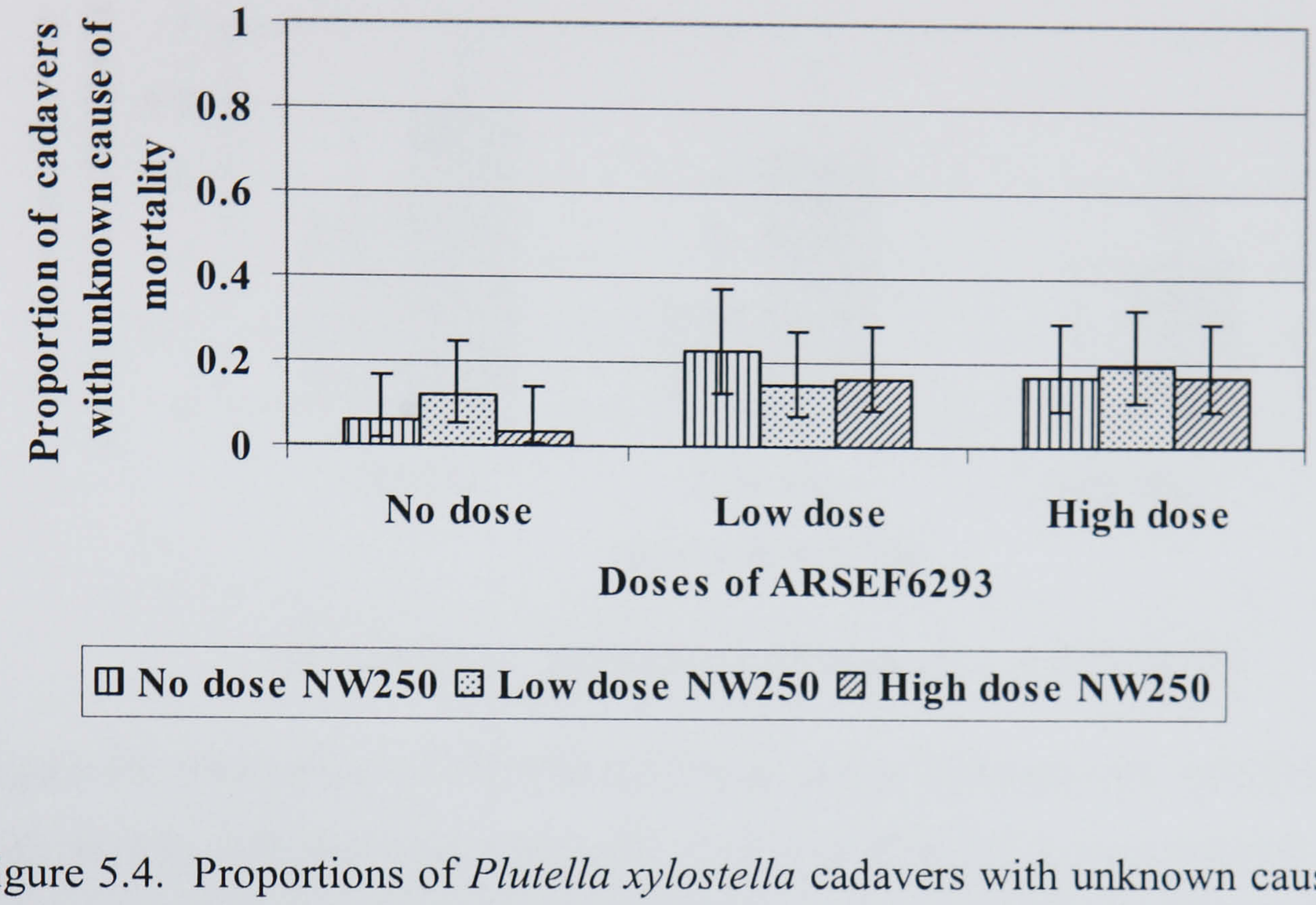


Figure 5.4. Proportions of *Plutella xylostella* cadavers with unknown cause of mortality, obtained under the three dose categories (Low, High and No dose) of both fungal species. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

*5.3.2.2 Isolates NW250 (*Z. radicans*) and ARSEF6311 (*P. blunckii*)*

Proportion of larvae infected ***only with ARSEF6311*** (P. blunckii) *in* Plutella xylostella *larvae either inoculated only with ARSEF6311 or dual-inoculated with ARSEF6311 and NW250* (Z. radicans)

No interaction was found between ARSEF6311 and NW250 on the proportion of infected larvae ($F_{2,10}$=0.28, P=0.761). Significant differences were found between the two doses of ARSEF6311 on the proportion of infected larvae ($F_{1,10}$=9.97, P=0.010). The largest proportion of infected larvae was always obtained with high doses compared to the low doses of ARSEF6311 (Fig. 5.5). Significant differences were also found in the proportion of infected larvae due to ARSEF6311 in the presence of the different doses of NW250 ($F_{2,10}$=5.93, P=0.020). The largest proportion of infected larvae due to ARSEF6311 was

when no conidia of NW250 were present, and the proportion of infected larvae due to ARSEF6311 decreased as the doses of NW250 increased (Fig. 5.5).

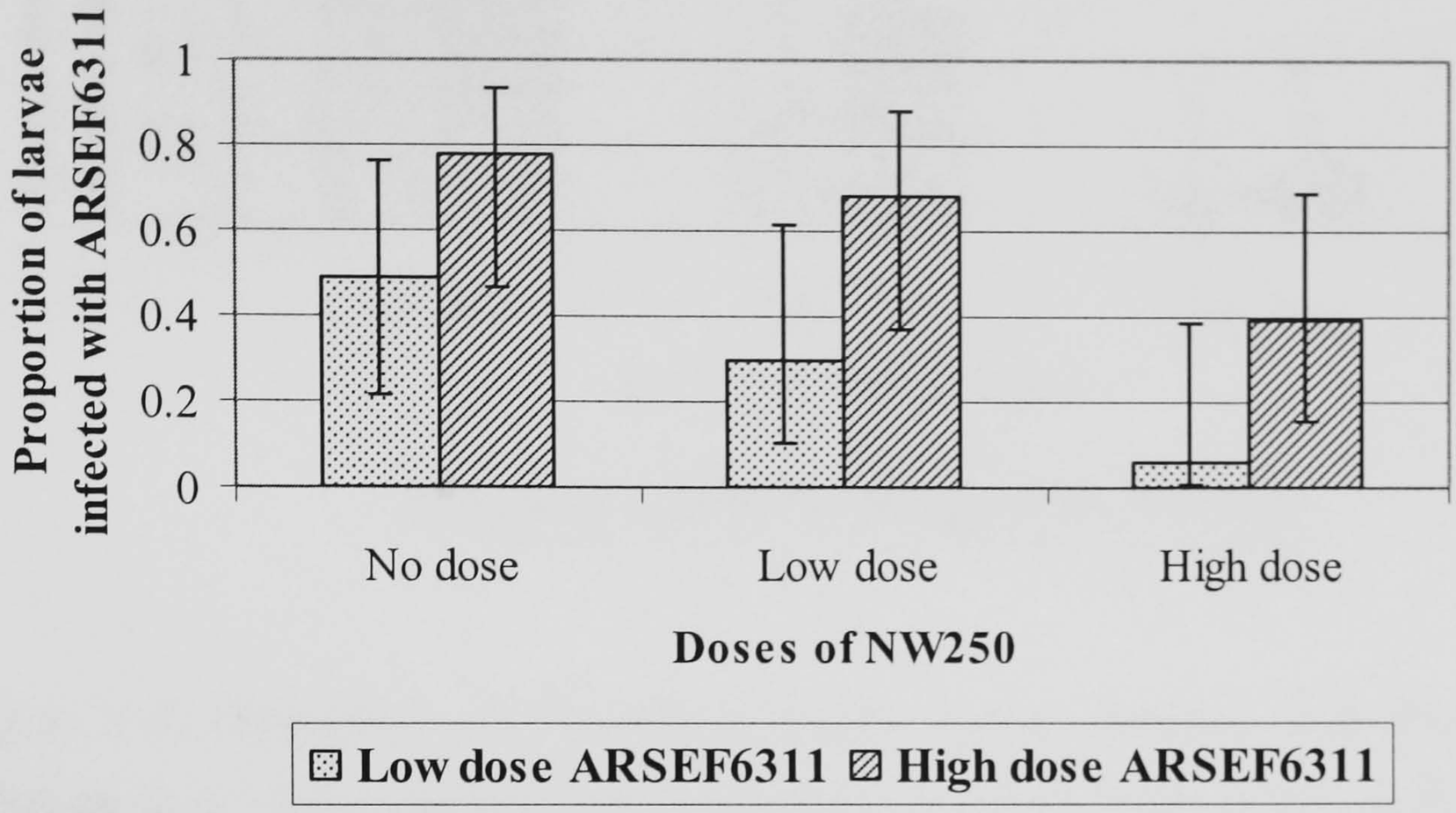


Figure 5.5. Proportion of *Plutella xylostella* larvae infected with ARSEF6311 with the two different dose categories (Low and High) of isolate ARSEF6311, and in the presence of the three different dose categories (Low, High and No dose) of NW250 (*Z. radicans*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of larvae infected ***only with NW250*** (Z. radicans) *in* Plutella xylostella *larvae either inoculated only with NW250 or dual-inoculated with NW250 and ARSEF6311* (P. blunckii)

No significant interaction was found between ARSEF6311 and NW250 in proportions of infected larvae ($F_{2,10}$=0.30, P=0.747). Significant differences were obtained between the low and high doses of NW250 in the proportion of infected larvae ($F_{1,10}$=5.85, P=0.036). The largest proportion of infection by NW250 was obtained with the high doses compared to the low doses (Fig. 5.6). The three different dose categories of ARSEF6311 had a significant effect on the proportion of infected larvae ($F_{2,10}$=17.19, P<0.001). The highest proportion of infected larvae was found when no ARSEF6311 conidia were present, and this proportion decreased as the dose of ARSEF6311 increased (Fig. 5.6).

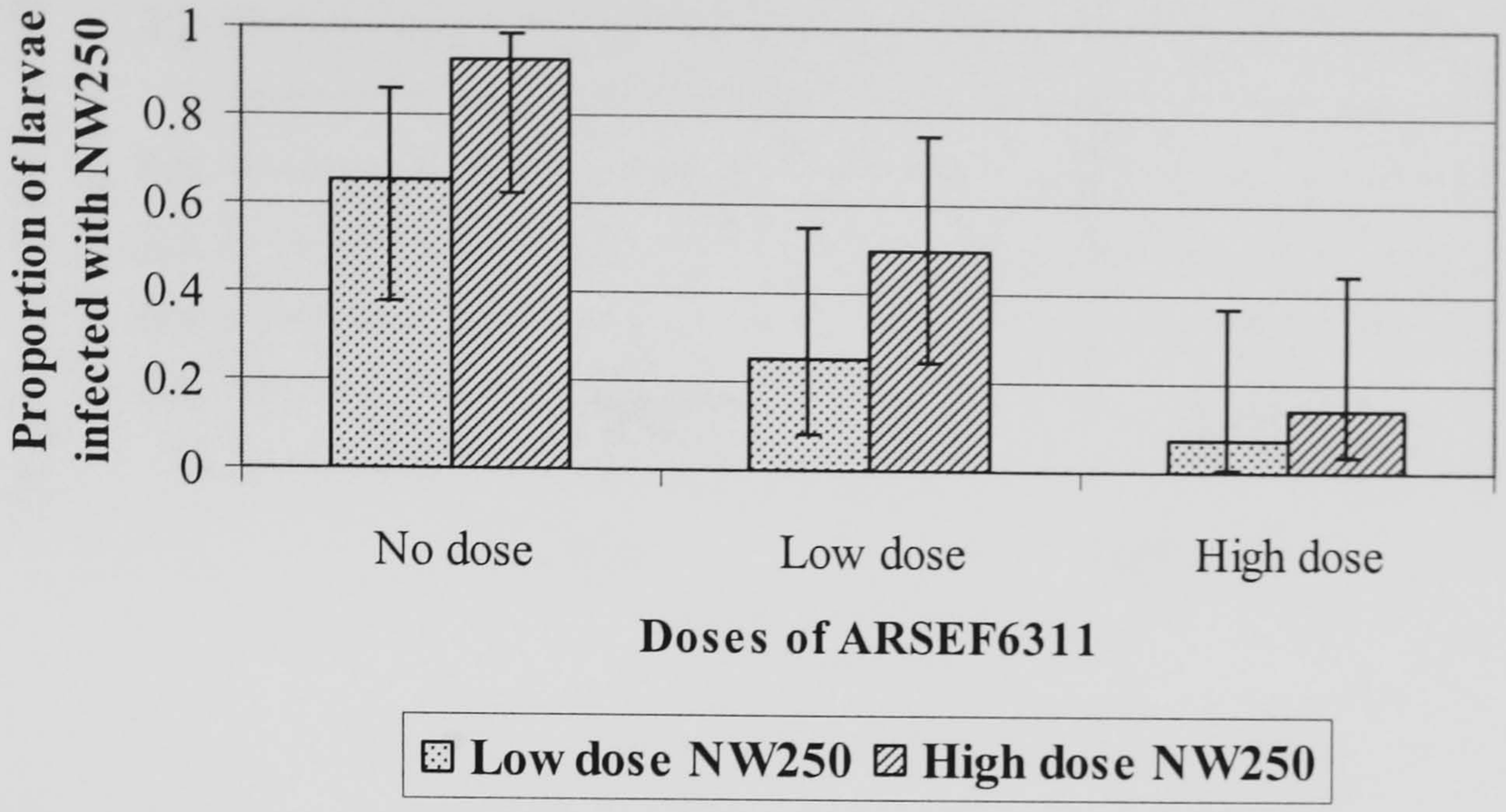


Figure 5.6. Proportion of *Plutella xylostella* larvae infected with the two different dose categories (Low and High dose) of isolate NW250 (*Z. radicans*), and in the presence of three dose categories (Low, High and No dose) of ARSEF6311 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *larvae* ***dual-infected*** *with NW250* (Z. radicans) and *ARSEF6311* (P. blunckii)

There were a larger proportion of dual-infected larvae when high doses of NW250 were inoculated than when low doses were inoculated (X^2_1=18.49, P<0.001). The proportion was greatly reduced in the presence of high doses of ARSEF6311 (X^2_1=5.66, P=0.017). No differences between the high and low doses of ARSEF6311 were found in the proportion of dual-infected larvae (X^2_1=1.82, P=0.178) (Fig.5.7).

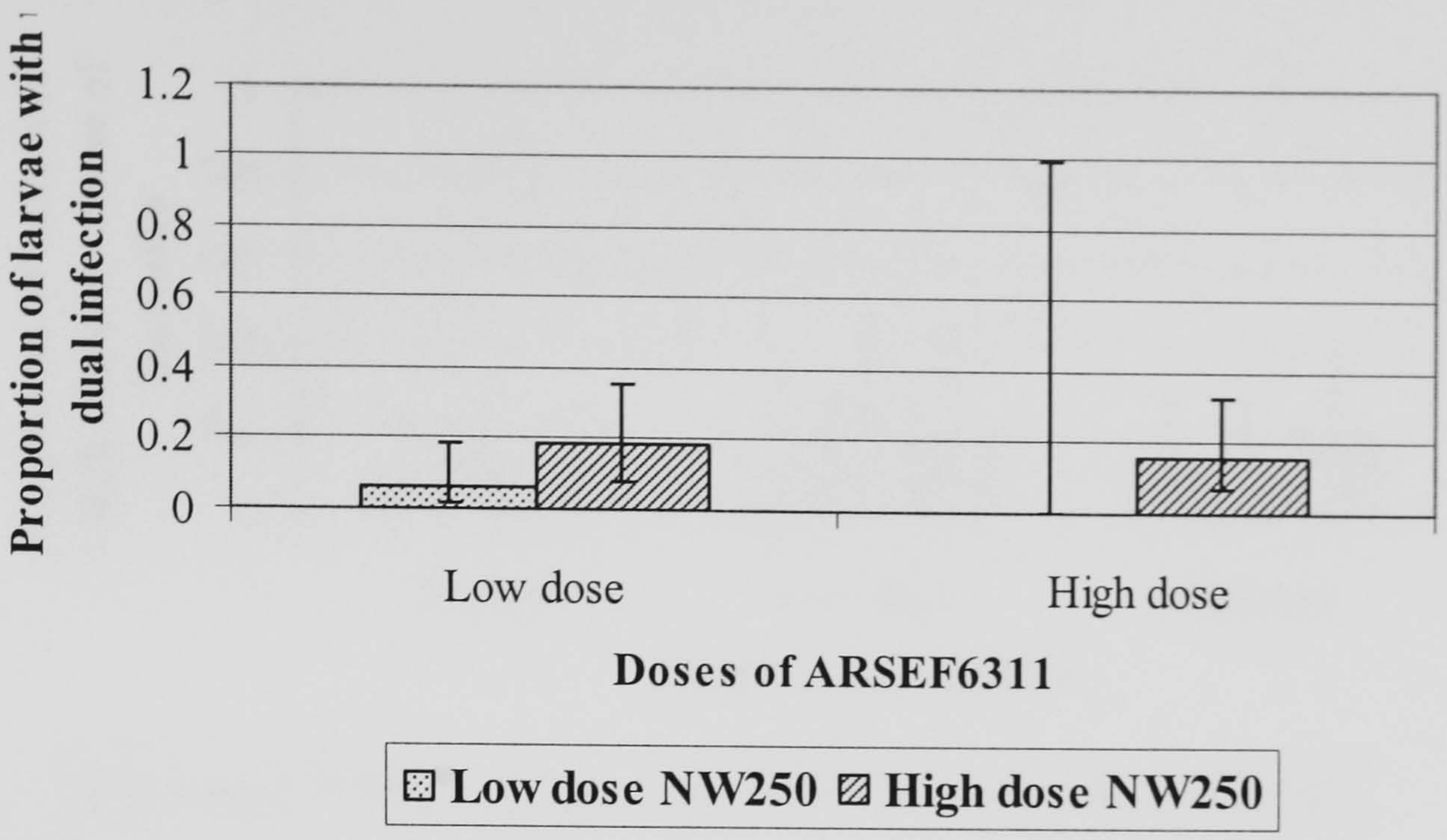


Figure 5.7. Proportions of *Plutella xylostella* larvae with dual-infection caused by NW250 (*Z. radicans*) and ARSEF6311 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

The large confidence interval obtained for the treatment with the low doses of NW250 and high doses of ARSEF6311, was due to a large frequency of zeros in this set of data. This causes problems trying to fit a logistic regression model and as a consequence large estimated errors may be obtained (Hosmer and Lemeshow, 1989).

Proportion of Plutella xylostella *cadavers with* ***unknown cause of mortality (UCM)***

There were no differences amongst the three doses of ARSEF6311 ($F_{2,16}$=2.43, P=0.120), NW250 ($F_{2,16}$=1.37, P=0.282) or an interaction between both pathogens ($F_{4,16}$=0.67, P=0.620) in the proportion of cadavers with UCM. The proportion of cadavers with UCM were statistically the same in the treatments where no fungus was applied compared to the treatments where the different dose categories where applied (Fig. 5.8). The average proportion of cadavers with UCM was 1.33.

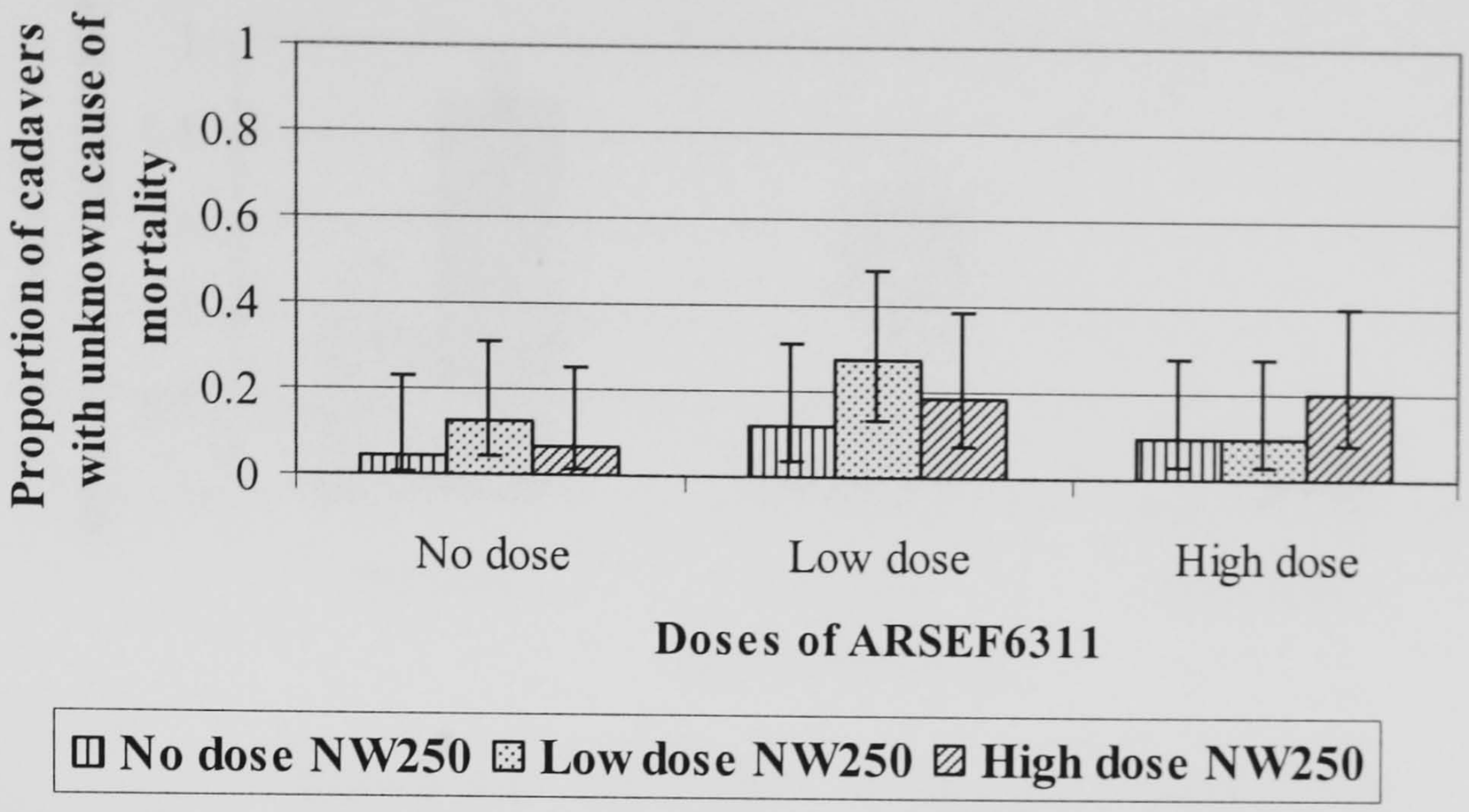


Figure 5.8. Proportions of *Plutella xylostella* larvae cadavers with unknown cause of mortality, obtained under the three dose categories (Low, High and No dose) of both fungal species. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

*5.3.2.3 Isolates NW386 (*Z. radicans*) and ARSEF6293 (*P. blunckii*)*

Proportion of larvae infected ***only with ARSEF6293*** (P. blunckii) *in* Plutella xylostella *larvae either inoculated only with ARSEF6293 or dual-inoculated with ARSEF6293 and NW386* (Z. radicans)

Significant differences were found between the proportion of infected larvae obtained at the two doses of ARSEF6293 (X^2_1=42.40, P<0.001). The largest proportion of infected larvae was normally obtained with the largest dose of ARSEF6293 (Fig. 5.9). However, the proportions obtained with the high and low doses of ARSEF6293 were no longer different in the presence of the high dose of NW386, which suggests and interaction between ARSEF6293 and NW386 (X^2_2=9.44, P<0.001), (Fig. 5.9). There were significant differences amongst the three dose categories of NW386 in the proportion of infected larvae (X^2_2=32.91, P<0.001), where the largest proportion of infected larvae due to ARSEF6293 was in the absence of NW386 conidia, and this proportion decreased as the dose of NW836 increased (Fig. 5.9).

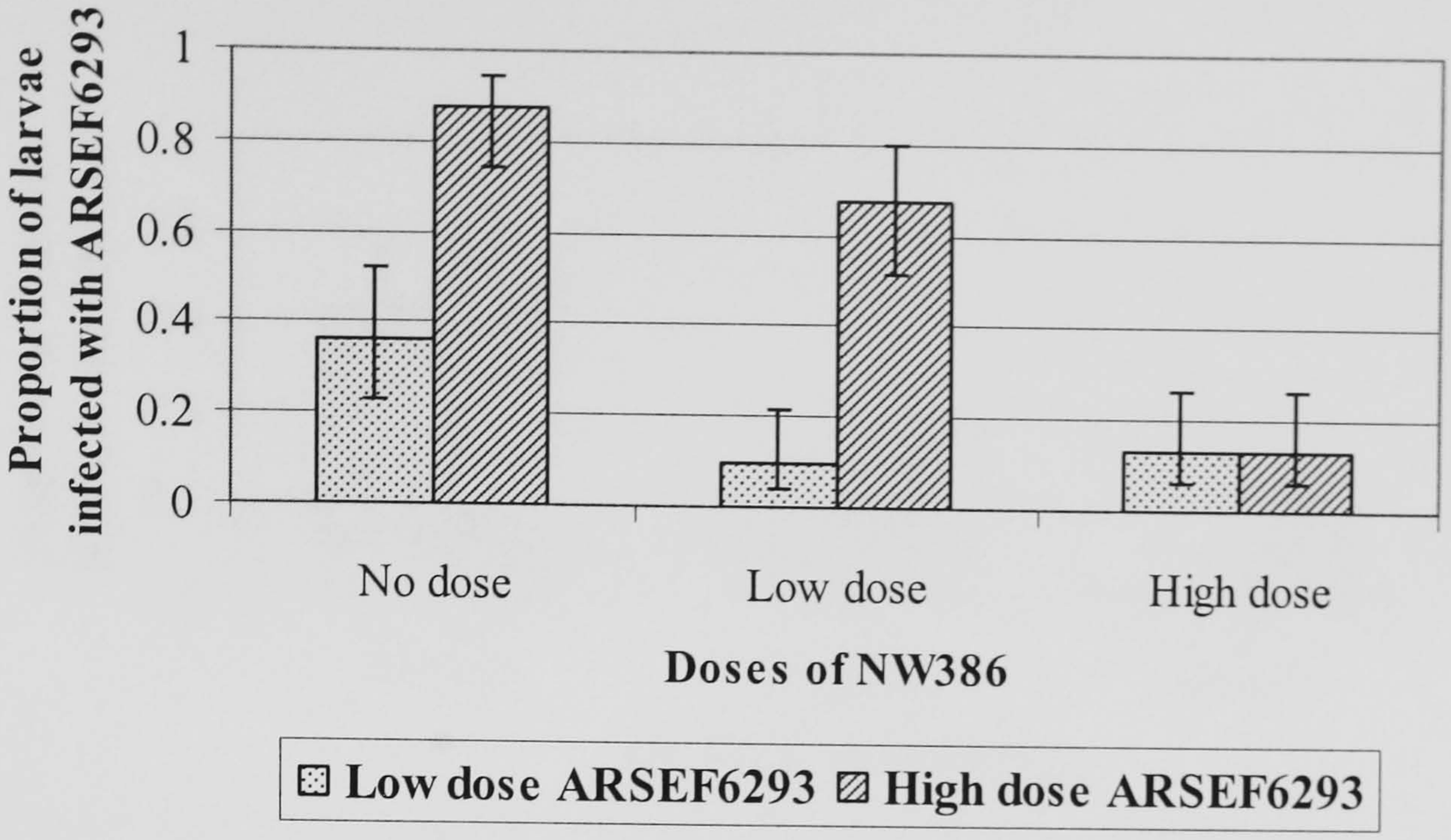


Figure 5.9. Proportion of *Plutella xylostella* larvae infected with ARSEF6293 (*P. blunckii*) at the two different dose categories (Low and High) of isolate ARSEF6293, and in the presence of three different dose categories (Low, High and No dose) of NW386 (*Z. radicans*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of larvae infected ***only with NW386*** (Z. radicans) *in* Plutella xylostella *larvae either inoculated only with NW386 or dual-inoculated with NW386 and ARSEF6293* (P. blunckii)

Significant differences were found between the two doses of NW386 in the proportion of larvae infected due to NW386 (X^2_1=18.20, P<0.001). The largest proportion of infected larvae normally occurred with the high dose of NW386. However, this difference seems to be even larger in the presence of the high doses of ARSEF6293 (Fig. 5.10), hence the borderline effect for the interaction between NW386 and ARSEF6293 (X^2_2=2.83, P=0.059). A significant effect was also found amongst the proportion of infected larvae due to NW386 in the presence of the three dose categories of ARSEF6293 (X^2_2=34.33, P<0.001). The largest proportion of infected larvae due to NW386 were when no conidia of ARSEF6293 were inoculated onto the larvae, and the smallest proportion of infected larvae was in the presence of the high doses of ARSEF6293 (Fig. 5.10).

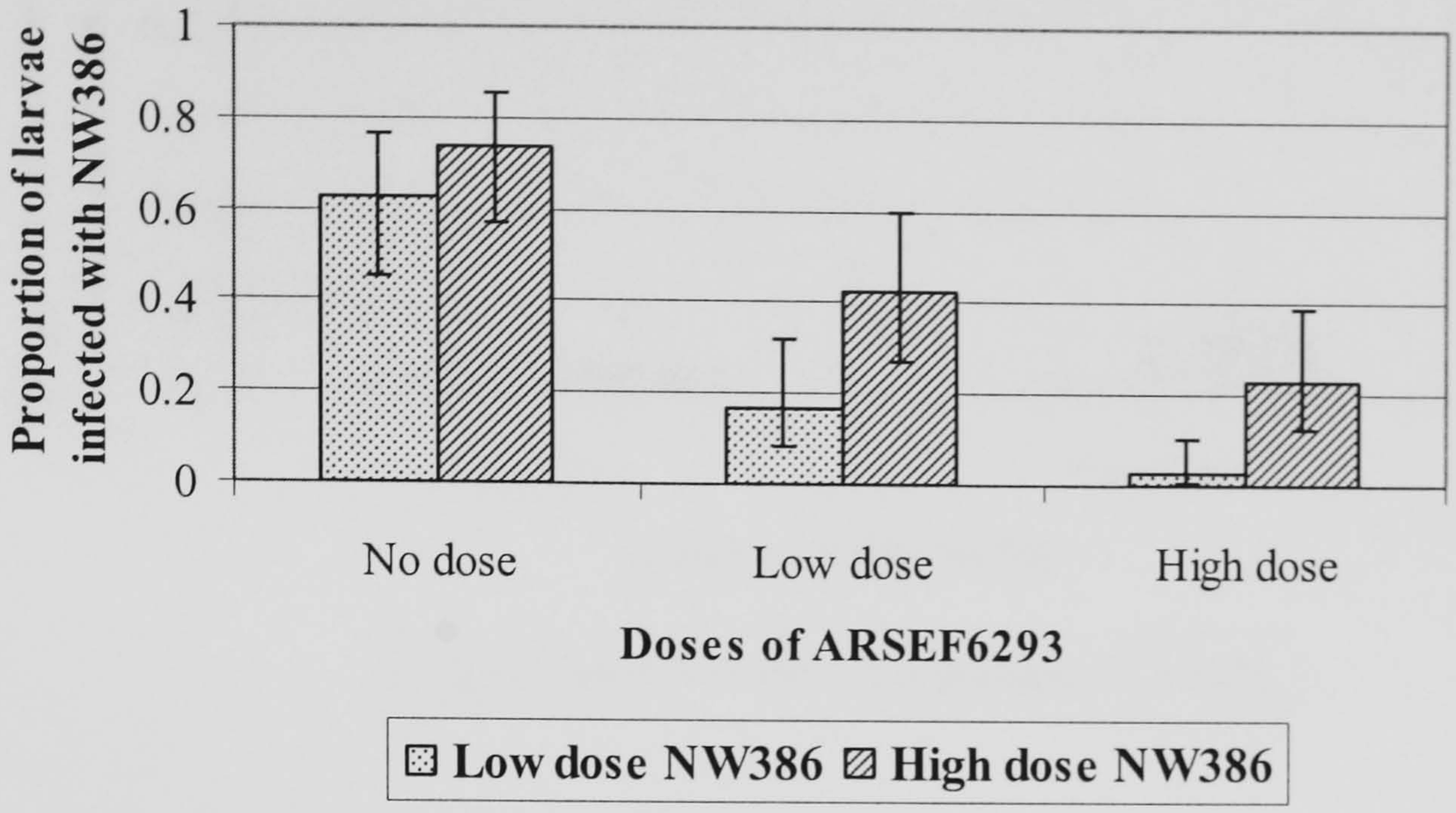


Figure 5.10. Proportion of *Plutella xylostella* larvae infected with NW386 (*Z. radicans*) at two different dose categories (Low and High) of isolate NW386, and in the presence of three different dose categories (Low, High and No dose) of ARSEF6293 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *larvae* ***dual-infected*** *with NW386* (Z. radicans) and *ARSEF6293* (P. blunckii)

No significant effect was found in the interactions between NW386 and ARSEF6293 on the proportion of dual-infected larvae (X^2_1=1.89, P=0.170). Therefore, the effect of each isolate was independent of each other. The two doses of ARSEF6293 had no significant effect on the proportion of dual-infected larvae (X^2_1=1.38, P=0.239). However, the two doses of NW386 had a significant effect on the proportion of dual-infected larvae (X^2_1=4.83, P=0.028). The largest proportion of dual-infected larvae was obtained with high doses of NW386 (Fig. 5.11).

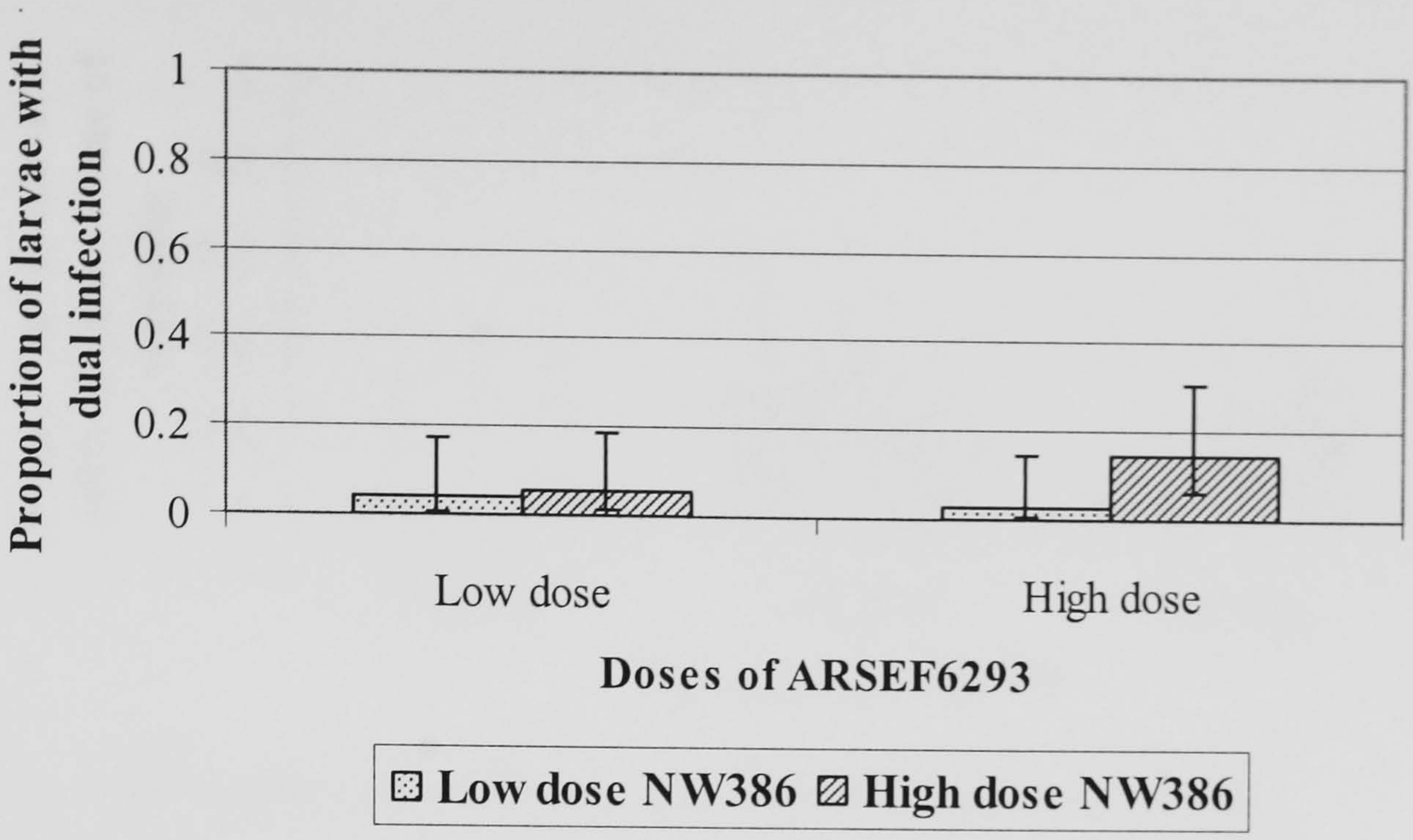


Figure 5.11. Proportions of *Plutella xylostella* larvae with dual-infection caused by NW386 (*Z. radicans*) and ARSEF6293 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *cadavers with* ***unknown cause of mortality*** *(UCM)*

No significant effect was found in the interaction between NW386 and ARSEF6293 on the proportion of cadavers with UCM ($F_{4,16}$=0.79, P=0.548). The differences amongst the proportions of cadavers with UCM obtained at the three dose categories of NW386 was not significant ($F_{2,16}$=0.79, P=0.548). However, the effect of the three dose categories of ARSEF6293 had a significant effect of the proportion of cadavers with UCM ($F_{2,16}$=7.54, P=0.005). The proportion of cadavers with UCM was larger in the treatments where ARSEF6293 conidia were applied (Fig. 5.12).

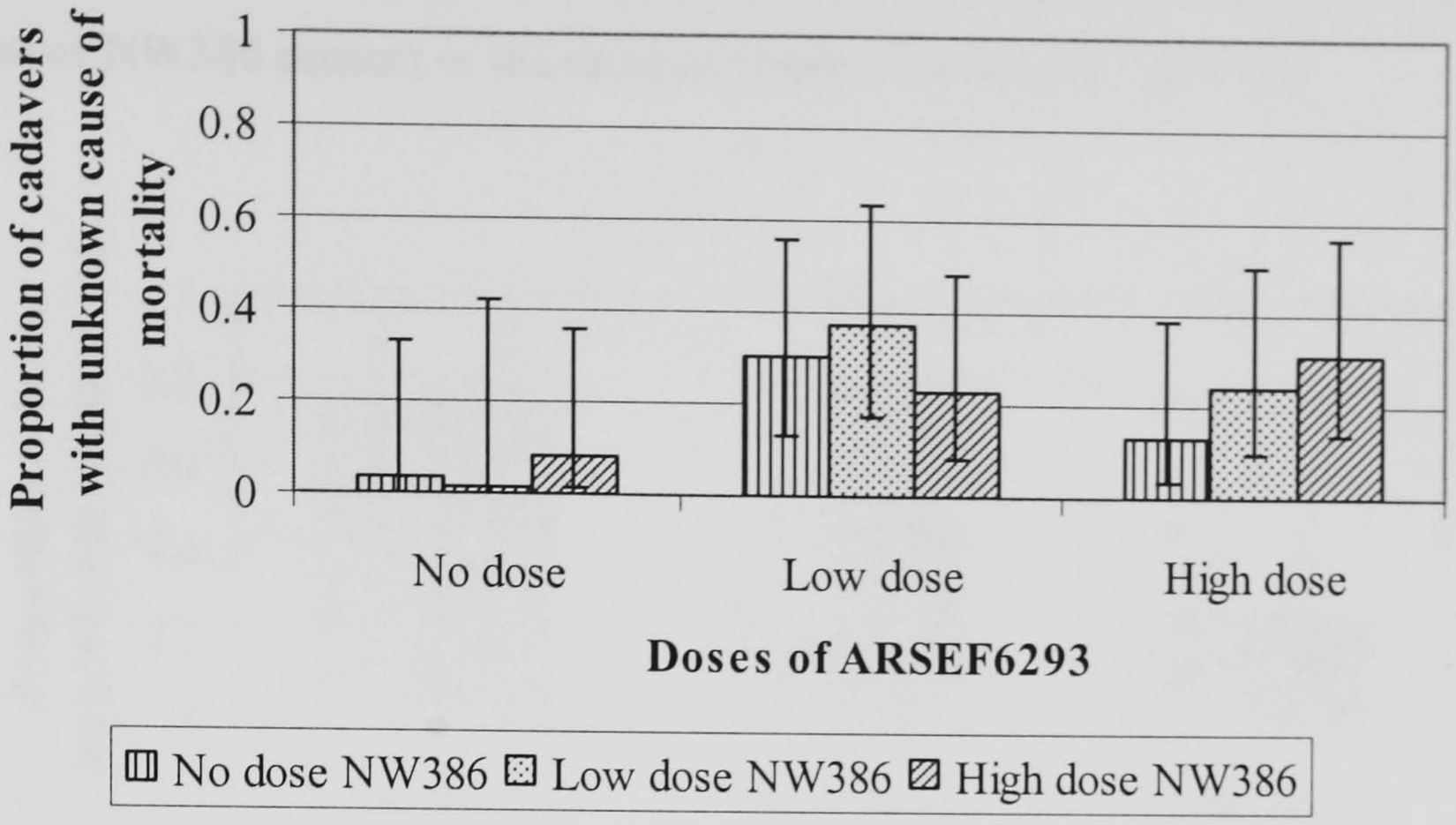


Figure 5.12. Proportions of *Plutella xylostella* larvae cadavers with unknown cause of mortality, obtained under the three dose categories (Low, High and No dose) of both fungal species. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

*5.3.2.4 Isolates NW386 (*Z. radicans*) and ARSEF6311 (*P. blunckii*)*

Proportion of larvae infected ***only with ARSEF6311*** (P. blunckii) *in* Plutella xylostella *larvae either inoculated only with ARSEF6311 or dual-inoculated with ARSEF6311 and NW386* (Z. radicans)

No significant effect was found on the interaction between NW386 and ARSEF6311 in the proportion of infected larvae ($F_{2,10}$=2.85, P=0.105). There is evidence of some effect of the two doses of ARSEF6311 on the proportion of infected larvae ($F_{1,10}$=4.79, P=0.054). It is possible that this borderline effect was due mainly to the large difference in the proportion of infected larvae obtained in the presence of low doses of NW386, because the difference in proportion in the presence of the other two dose categories (High and No dose) was minimal (Fig. 5.13). Significant differences were obtained amongst the proportion of infected larvae due to ARSEF6311 obtained in the presence of the three dose categories of NW386 ($F_{2,10}$=11.24, P=0.003). The largest proportion of infected larvae due to ARSEF6311 was when no conidia of

NW386 were present in the treatment, and this proportion decreased as the dose of NW386 present in the same treatment increased (Fig. 5.13).

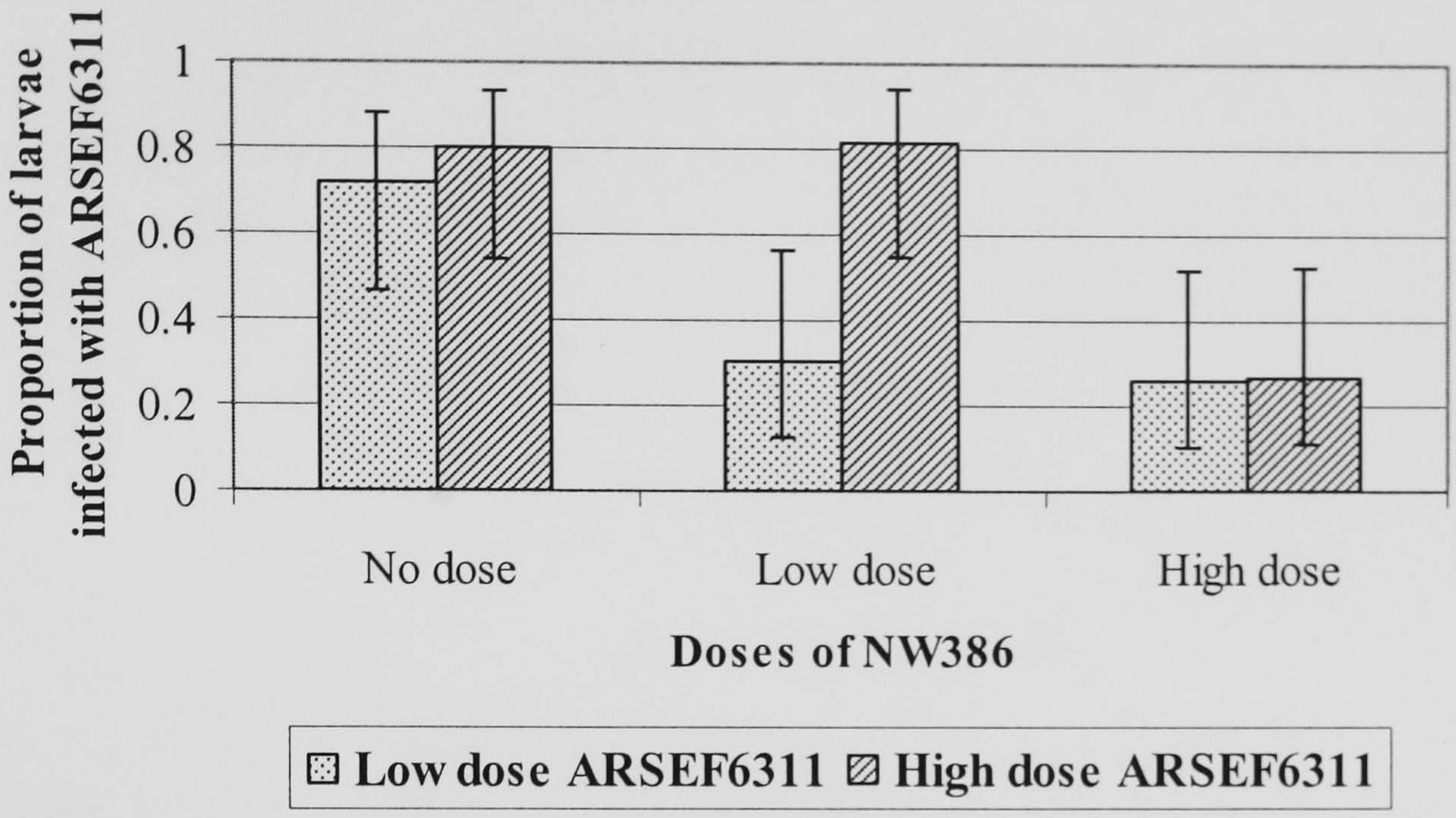


Figure 5.13. Proportion of *Plutella xylostella* larvae infected with ARSEF6311 (*P. blunckii*) at the two different dose categories (Low and High) of isolate ARSEF6311, and in the presence of three different dose categories (Low, High and No dose) of NW386 (*Z. radicans*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI)

Proportion of larvae infected ***only with NW386*** (Z. radicans) *in* Plutella xylostella *larvae either inoculated only with NW386 or dual-inoculated with NW386 and ARSEF6311* (P. blunckii)

A significant effect was found on the interaction between NW386 and ARSEF6311 on the proportion of larvae infected (X^2_2=5.15, P=0.006). A significant difference was found between the high and low dose of NW386 (X^2_1=14.73, P<0.001). The largest proportion of infected larvae was normally obtained with high doses of NW386, but the difference in proportions obtained with the high and low doses of NW386 was even larger in presence of high doses of ARSEF6311 (Fig. 5.14). Significant differences were found amongst the proportions obtained at the three dose categories of ARSFE6311 (X^2_2=34.39, P<0.001). The largest proportion of infected larvae due to NW386

was obtained when no ARSEF6311 conidia were present, and the smallest proportion in the presence of high doses of ARSEF6311 (Fig. 5.14).

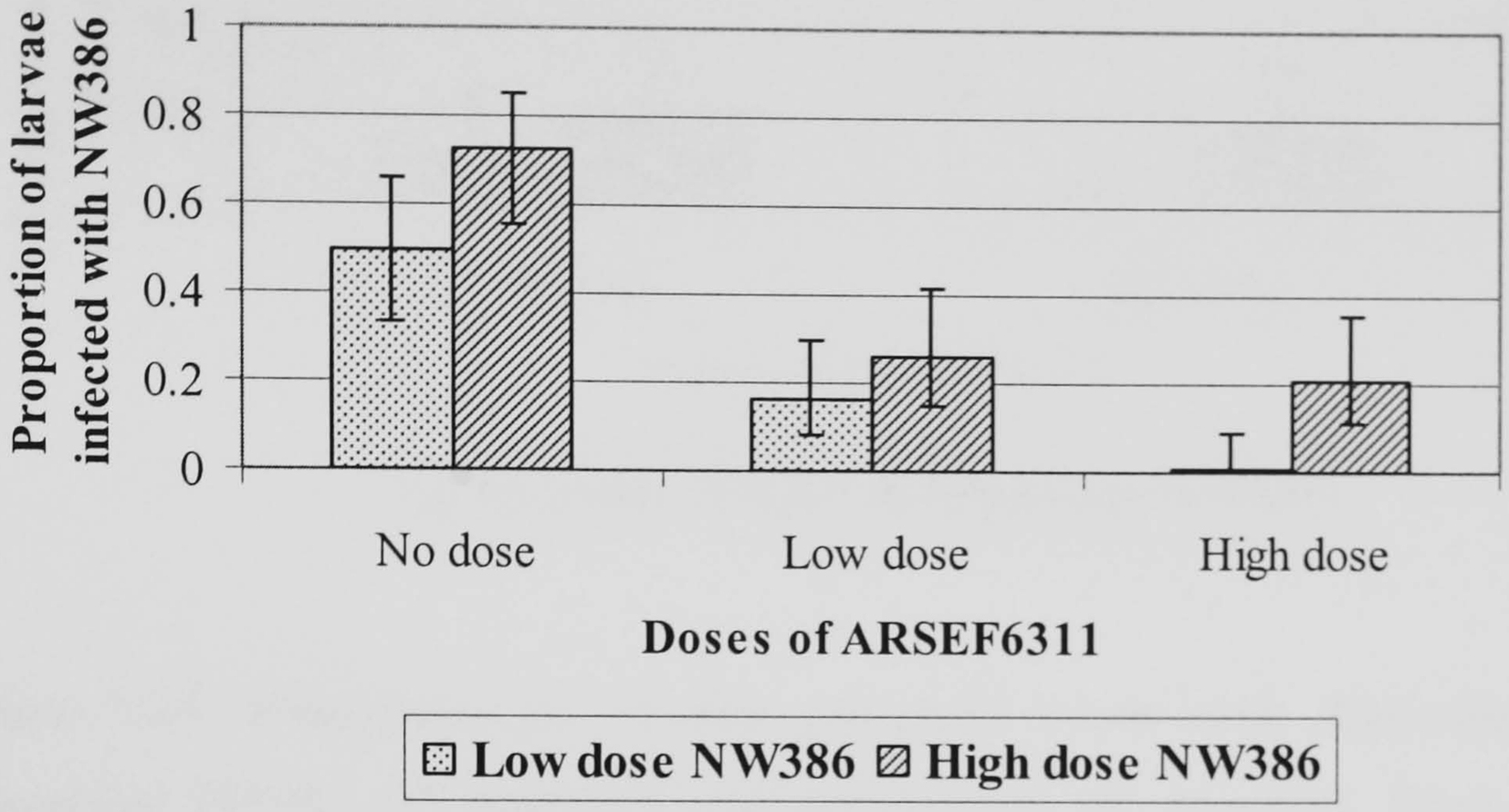


Figure 5.14. Proportion of *Plutella xylostella* larvae infected with NW386 (*Z. radicans*) at the two different dose categories (Low and High) of isolate NW386, and in the presence of three different dose categories (Low, High and No dose) of ARSEF6311 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *larvae* ***dual-infected*** *with NW386* (Z. radicans) and *ARSEF6311* (P. blunckii)

No significant effect was found on the interaction between NW386 and ARSEF6311 on the proportion of dual-infected larvae (X^2_1=2.64, P=0.104). No significant difference was found between the high and low dose of ARSEF6311 on the proportion of dual-infected larvae (X^2_1=2.45, P=0.117). A significant difference between high and low doses of NW386 on the proportion of dual-infected larvae was found (X^2_1=8.58, P=0.003), where the largest proportion of dual-infected larvae was found in the treatment with high doses of NW386 (Fig. 5.15).

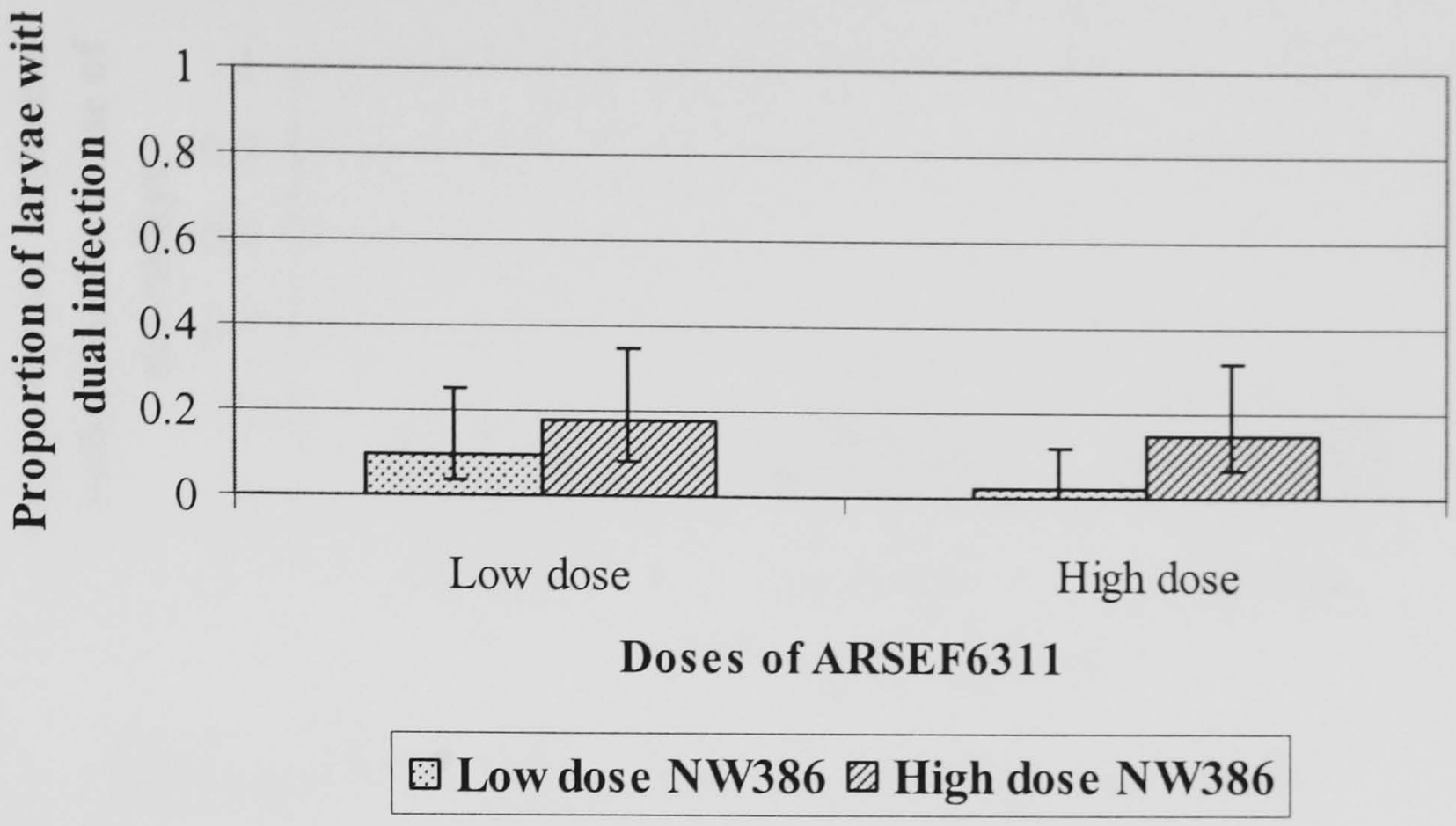


Figure 5.15. Proportions of *Plutella xylostella* larvae with dual-infection caused by NW386 (*Z. radicans*) and ARSEF6311 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

Proportion of Plutella xylostella *cadavers with* ***unknown cause of mortality*** *(UCM)*

Significant differences were found amongst the proportion of cadavers with UCM obtained with the three doses of ARSEF6311 (X^2_2=5.78, P=0.003), where the smallest proportion was obtained when no conidia of ARSEF6311 were applied compared to the other two doses (Fig. 5.16). This effect was independent of the presence of the three dose categories of NW386 (X^2_4=1.58, P=0.176). Also, no significant differences were found amongst the three dose categories of NW386 (X^2_2=1.86, P=0.156).

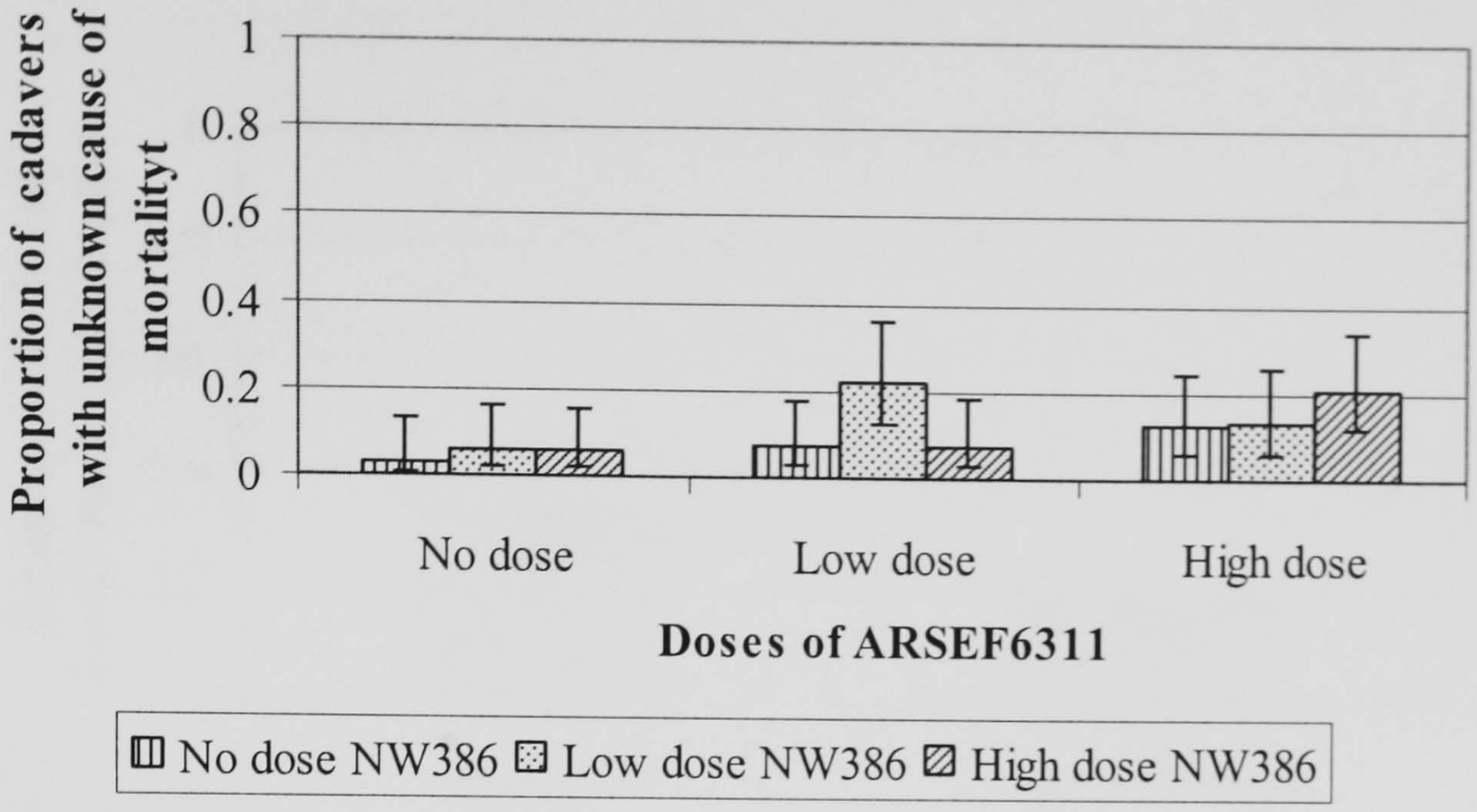


Figure 5.16. Proportions of *Plutella xylostella* larvae cadavers with unknown cause of mortality, obtained under the three dose categories (Low, High and No dose) of NW386 (*Z. radicans*) and ARSEF6311 (*P. blunckii*). Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

5.3.3. *In vivo* inter-specific interactions between *Zoophthora radicans* and *Pandora blunckii* isolates on *Plutella xylostella* larvae on Chinese cabbage plants

5.3.3.1 Proportion of larvae infected ***only with ARSEF6293*** *(*P. blunckii*)*

Significant differences were found in the proportion of larvae infected only with ARSEF6293 between populations on plants inoculated only with ARSEF6293 and populations on plants inoculated with ARSEF6293 and NW250 or NW386 ($F_{1,10}$=6.14, P=0.033). The proportion of larvae infected only with ARSEF6293 was greater on plants inoculated only with ARSEF6293 compared to plants where ARSEF6293 was co-inoculated either with NW250 or NW386 (Fig. 5.17). Within the dual-inoculated plants only, no significant difference was obtained in the proportions of infected larvae from populations on plants inoculated with ARSEF6293 and either NW250 or NW386 separately ($F_{1,10}$=1.59, P=0.236).

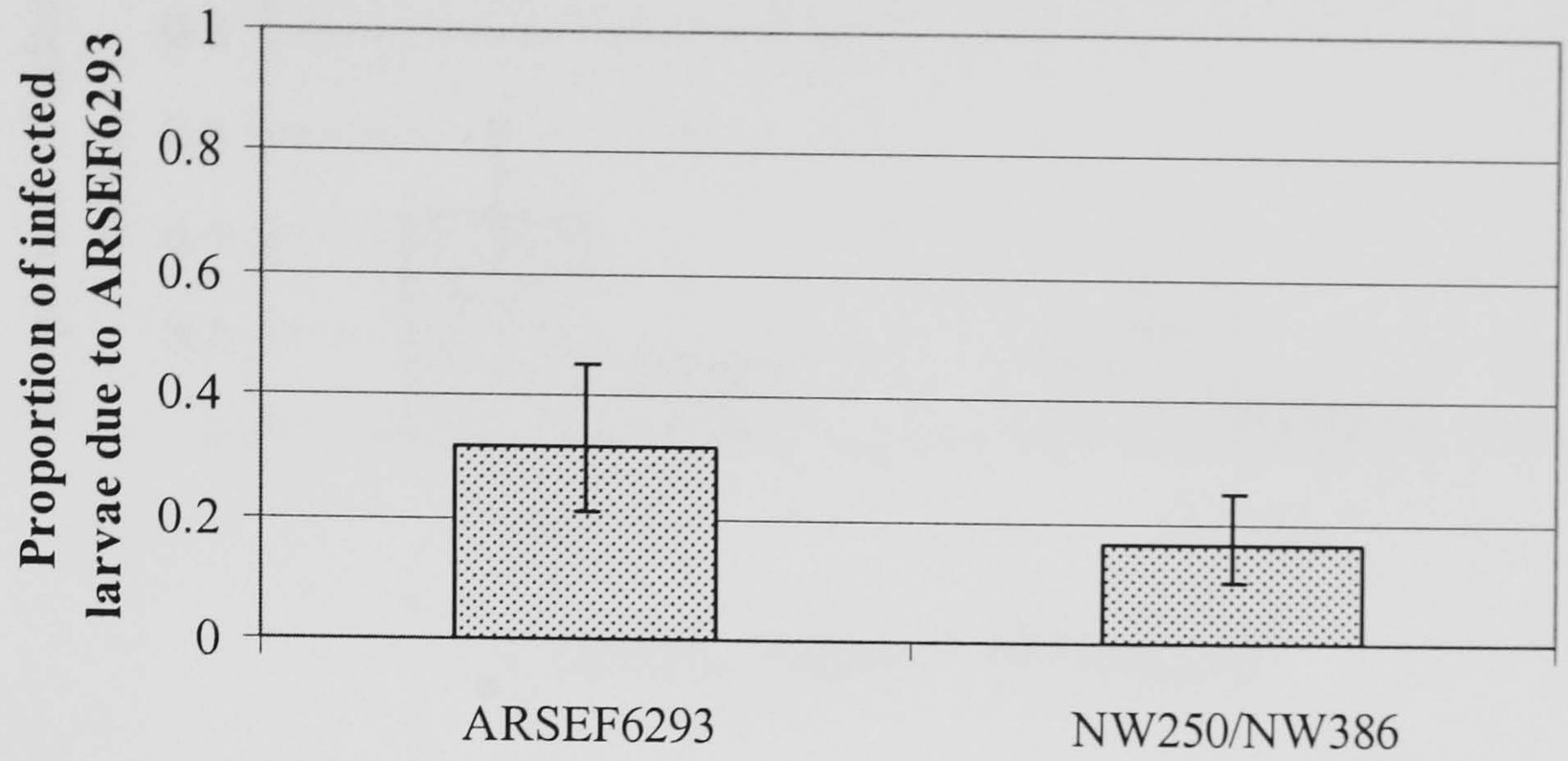


Figure 5.17. Proportion of larvae infected only with ARSEF6293 (*P. blunckii*) obtained from larvae on plants inoculated only with ARSEF6293 and the average proportion obtained from plants inoculated with ARSEF6293 and NW250 or NW386. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

5.3.3.2 Proportion of larvae infected ***only with NW250 or NW386*** *(Z. radicans)*

The average proportion of the larval population infected with NW250 from single or dual-inoculated (with ARSEF6293) plants were not different from the average proportion of the larval population infected with NW386 ($F_{1,15}$=3.45, P=0.083). However, the presence of ARSEF6293 had a significant effect on the proportion of larvae infected with either of the two *Z. radicans* isolates ($F_{1,15}$=11.88, P=0.004), where the largest proportion of larvae infected with both *Z. radicans* isolates was always in the absence of ARSEF6293 (Fig. 5.18). The differences in proportion of larval population infected with *Z. radicans* isolates in the presence and absence of ARSEF6293 was similar for both *Z. radicans* isolates ($F_{1,15}$=0.14, P=0.712).

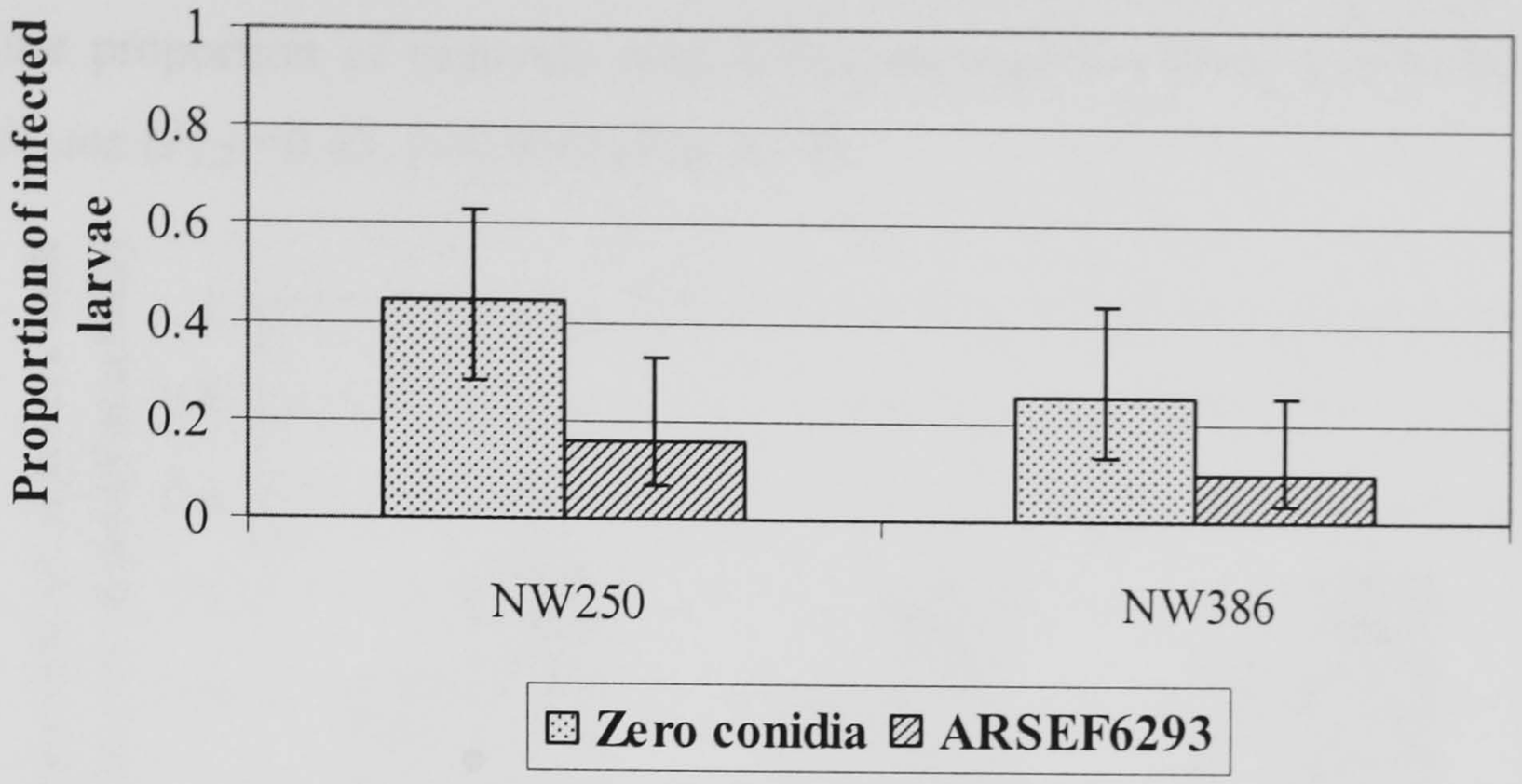


Figure 5.18. Proportion of larval population infected with *Zoophthora radicans* on plants inoculated either with NW250 or NW386 only (*Z. radicans*) and plants dual- inoculated with ARSEF6293. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

5.3.3.3 Proportion of ***dual-infected*** *larvae either with NW250 or NW386 (*Z. radicans*) and ARSEF6293 (*P. blunckii*)*

The proportion of dual-infected larvae obtained from larval populations on plants inoculated with NW250 and ARSEF6293 was no different from the proportion obtained from populations on plants inoculated with NW386 and ARSEF6293 ($F_{1,5}$=1.83, P=0.234). The overall proportion of dual-infected larvae was generally very small, with 0.02 and 0.009 proportions of dual-infected larvae for the combination NW250-ARSEF6293 and NW386-ARSEF6293 respectively.

5.3.3.4 Proportion of cadavers with ***unknown cause of mortality*** *(UCM)*

Only the presence of isolate ARSEF6293 had a significant effect on the proportion of cadavers with UCM ($F_{1,25}$=18.18, P<0.001). In all the treatments where ARSEF6293 was inoculated (either single or dual-inoculated with any of the *Z. radicans* isolates), the proportion was always larger than in the absence of ARSEF6293 (Fig. 5.19), and this was independent of the *Z. radicans* isolate

that was present ($F_{2,25}$=0.12, P=0.887). No significant differences were found in the proportion of cadavers with UCM amongst the three treatments of *Z. radicans* ($F_{2,25}$=0.43, P=0.656) (Fig. 5.19).

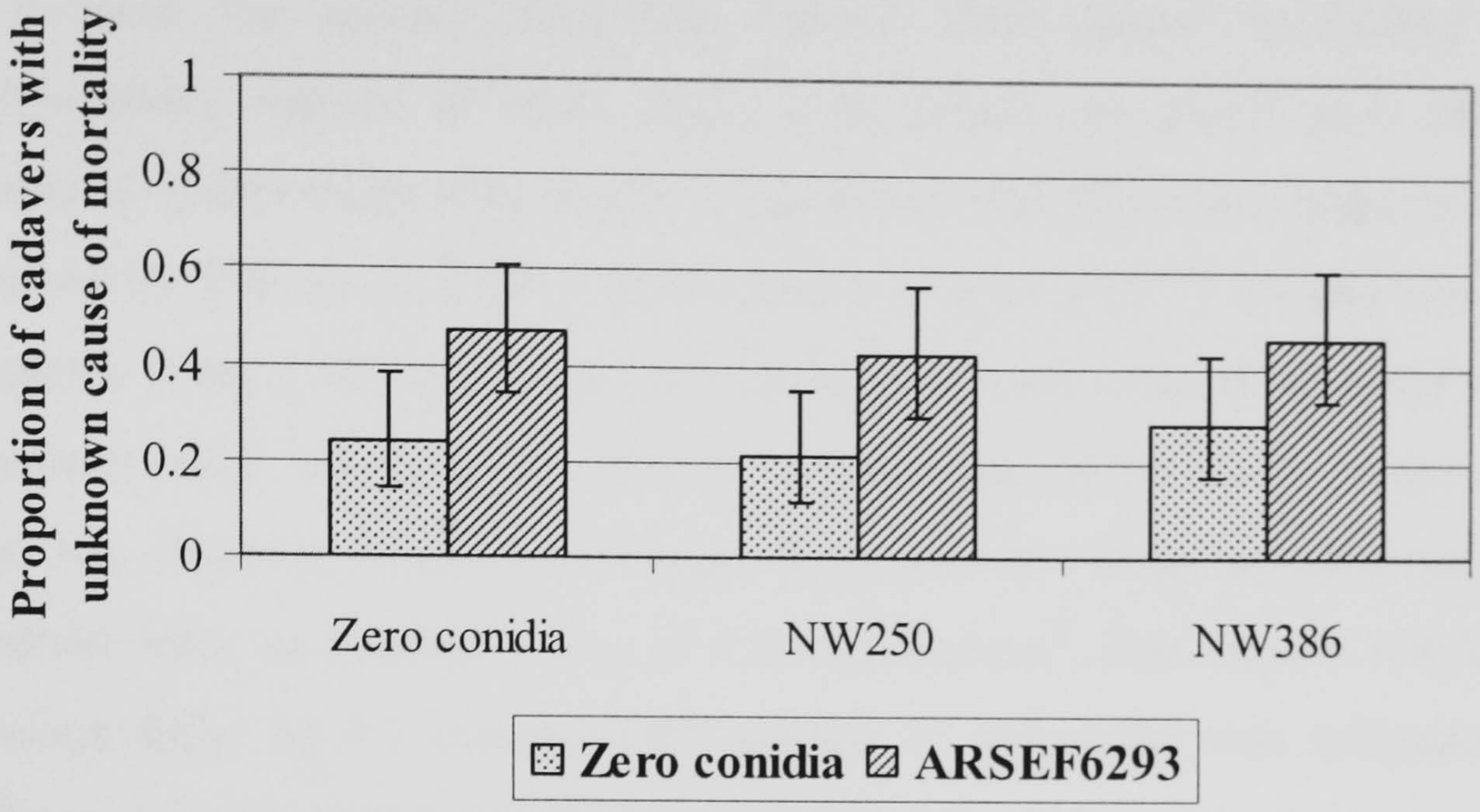


Figure 5.19. Proportion of cadavers with unknown cause of mortality obtained with all the isolates single or dual-inoculated. Error bars represent confidence interval (95%) back-transformed from the logistic scale (CI).

5.4 DISCUSSION

5.4.1 Estimation of LC_{50} values

Overall, the four isolates showed high virulence against *P. xylostella,* and the *P. blunckii* isolates were more virulent than the *Z. radicans* isolates. The *Z. radicans* isolate NW250 was more virulent than the *Z. radicans* isolate NW386 at both temperatures. The *P. blunckii* isolates showed a similar virulence, although at 25 °C, ARSEF6311 was more virulent than ARSEF6293. At 20 °C, there was a large variability amongst the individual assays for each isolate which made it difficult to determine which isolate was the most virulent. Large variability amongst individual assays using the same isolate and host was previously reported for *Z. radicans* against *P. xylostella* (Pell *et al.*, 1993a), and for *Pandora neoaphidis* against aphids (Shah *et al.*, 2004). It was difficult to find a reason, but the results suggest that temperature had a strong effect on this variability, because the larvae incubated at 20 and 25 °C received the same doses, from the same sporulating fungal plugs.

There are previous reports of LC_{50} values for isolate NW250 obtained at 20 °C (Pell *et al.*, 1993a; Yeo *et al.*, 2001). In the former one, smaller LC_{50} values were reported (0.02-0.52 conidia/mm^2) than the current study, and this might be because the authors used late second instar larvae inoculated with capilliconidia, instead of early third instar larvae inoculated with primary conidia as in this study. Our results using isolate NW250 were similar to those obtained by Yeo *et al.* (2001) where an LC_{50} value of 6.97 conidia/mm^2 was estimated using a mixed population of late second and early third instar larvae inoculated with capilliconidia. In our experiments, at the same temperature, from six different bioassays a range from 3.19 to 15.46 conidia/mm^2 was obtained with an average LC_{50} of 6.8 conidia/mm^2. For isolate NW386, a previous LC_{50} of 3.1 conidia/mm^2 against *P. xylostella* was estimated by Velasco, J. L. (personal communication). The LC_{50} values at both temperatures estimated in this experiment were larger than those obtained by Velasco J. L., the difference may be a result of using different populations of *P. xylostella*. Velasco J.L. used a Mexican population of *P. xylostella* and in this experiment the population was from the Philippines. Differences in susceptibility amongst different populations of the same insect species to the same fungal pathogen species have been previously demonstrated using an isolate of *Pandora neoaphidis* against different clones of the aphid *Acyrthosiphon pisum* (Ferrari *et al.*, 2001), and this variation in susceptibility amongst different populations of the same species can also have a strong interaction with temperature (Blanford *et al.*, 2003; Stacey *et al.*, 2003).

Considering only the *Z. radicans* isolates, NW250 was more virulent than NW386. When these isolates were grown at different temperatures (Chapter 2), isolate NW250 had greater *in vitro* growth than NW386 at all temperatures except 30 °C, where NW250 did not grow at all and NW386 showed the greatest growth of all *Z. radicans* isolates. The difference in virulence may be related to growth rate. Under the same assumption, the *P. blunckii* isolate ARSEF6293 would have been more virulent than ARSEF6311 because it always had the greatest *in vitro* growth. However, at 25 °C ARSEF6311 was more virulent than ARSEF6293 and, at 20 °C, the LC_{50} values were variable and it was difficult to determine which was the most virulent. It is difficult to

explain why a relationship between *in vitro* growth and virulence for the *P. blunckii* isolates was not found. However, ARSEF6293 was sometimes faster to kill than ARSEF6311, starting to cause mortality one day earlier, unfortunately this was not experimentally determined.

When isolate ARSEF6293 was inoculated in a larval population either in Petri dish or on a plant, a larger number of cadavers with unknown cause of mortality than the control were normally obtained, which suggests that this isolate is efficient at infecting and killing larvae, but was then unable to sporulate externally and produce conidia on cadavers. This was confirmed in the *in vivo* inter-specific interactions experiments. Insect pathogens, as well as other pathogens in general, have developed different strategies to survive. The ability of the pathogen to be transmitted is one of the most important as without an efficient means of dispersal to other hosts, the pathogen cannot persist (Andreadis, 1987). The survival of a pathogen has two components, one is its ability to transmit to other hosts, and the other is its ability to exploit the host efficiently to reproduce and produce infective transmission stages (Ebert and Weisser, 1997). Many pathogens, including the entomopathogens, must kill the host before they are able to transmit. These pathogens grow inside the host and transform the host biomass into pathogen transmission stages (Ebert and Weisser, 1997). If the number of transmission stages (or conidia in our case) is used as a measure of pathogen fitness (Ebert and Weisser, 1997), then isolate ARSEF6293 was not using the host efficiently because the production of non-sporulating cadavers meant no production of transmission stages. This isolate was probably killing the host before all the host biomass has converted into fungus biomass and, therefore, transmission stages. Premature killing and low conidia production has been reported for *B. bassiana* infecting *Triatoma infestans* (Klug) (Luz *et al.,* 1999), the production of conidia was reduced when the infection took place at 30 °C, being optimal at 20 °C. They also found that higher doses killed the host faster but the actual fungal growth on the cadaver was delayed. An effect of temperature on external sporulation was also observed in our experiments, especially for isolate NW250 where at 25 °C, the number of cadavers with unknown cause of mortality or cadavers with ephemeral external sporulation was more frequent than at 20 °C. Isolate

ARSEF6293 produced non-sporulating cadavers regardless of the temperature. The effect of temperature on sporulation of isolate NW386 could not be observed because this isolate always produced resting spores regardless of the temperature. Isolate ARSEF6293 killed its host faster than the other isolates, which may be the result of high doses (Luz *et al.*, 1999). Although this was not investigated, it is possible that having greater growth *in vitro* than other *P. blunckii* isolates, it may also have a faster germination of conidia which would lead to a faster time to kill. If, as demonstrated by Luz *et al.* (1999), premature host death before fungal growth was complete, this suggests that between both events (host death and mycelial growth), there may be sufficient time for saprophytic organisms to take over the cadaver and inhibit growth and therefore external sporulation of ARSEF6293. Non-sporulating cadavers have also been reported in another entomophthoralean fungus. Nielsen *et al.* (2005) found an *Entomophaga maimaiga* isolate that produced non-sporulating cadavers of its host *Lymantria dispar.* The authors suggest that this can be result of a negative trade-off between virulence and conidial production, although that isolate was not the most virulent of all tested. In the case of ARSEF6293, it is possible that this negative trade off could have happened, but this could not be confirmed. However, as hypothesised by Ebert and Weisser (1997), the existence of external mortality factors of the host can also have an effect on the speed of kill of the pathogen. The higher the mortality rate of the host due to other factors, the earlier the pathogen should kill the host. The region where this isolate was found is a very intensive broccoli production area, which means that the populations of *P. xylostella* are under pressure from many mortality factors such as chemical insecticide application, bioinsecticides (e.g. *Bacillus thurigiensis*) and the presence of other competitors such as *Z. radicans*, which may have selected for faster kill by this particular isolate. Unfortunately, the reasons for a lack of or reduced external sporulation remain unclear.

5.4.2 In vivo *inter-specific interactions in Petri dishes*

During simultaneous dual-inoculation, the probability of either of the two species used in this experiment infecting a group of larvae (*P. xylostella*) was

negatively affected by the presence of the other species. The results suggest that the larger the dose the stronger the effect. Although the isolates had different virulence against *P. xylostella*, this difference was not sufficient to have an effect on the final outcomes of the interactions.

Interactions between pathogens can take place within the host or between hosts (Furlong and Pell, 2005). In within host interactions, the competition has been described as a continuous effect between two extremes, one called "superinfection", where a highly virulent pathogen takes over a host already infected by a less virulent pathogen, and only the most virulent pathogen is transmitted, and "coinfection" where multiple pathogens with similar virulence (closely grouped around a maximum virulence level) infect the same host, all strains eventually transmit infection with rates independent to the other pathogens present (Nowak and May, 1994; May and Nowak, 1995). Coinfection can be considered as scramble competition and superinfection as contest in ecological terms (May and Nowak, 1995). However, although ecologically these terms refer to intra-specific competitions only (Begon *et al.*, 1996), different authors have used these terms to describe the interactions between different species of pathogens or genetically different between isolates of the same species of pathogen (Cox, 2001; May and Nowak, 1995).

In our experiment, only intra-host competition was determined. In direct dual-inoculation, both isolates had the same opportunity to infect the host, without consideration for differences in transmission abilities. When isolate NW250 was co-inoculated either with ARSEF6293 or ARSEF6311, all three isolates with similar virulence levels, the main result of the intra-host competition was larvae infected only with one isolate, which suggests that one isolate excluded the other completely, although, as demonstrated in Chapter 6, even if a cadaver produces conidia from only one species, it is possible to have unidentifiable mycelia of the other species within the host as well. However, the isolate that was ultimately unable to produce conidia can be considered as excluded, if the numbers of transmission stages (or conidia) are used as a measure of pathogen fitness (Ebert and Weisser, 1997; Solter *et al.*, 2002). The similarity in virulence of these isolates (NW250 and ARSEF6293-6311) suggests that the

results were more dose ratio related, which means that the one with the larger number of conidia outcompeted the other one regardless of the virulence of each isolate. This may be because larger doses of conidia infecting a host will produce a larger pool of mycelium more quickly (Hughes *et al.*, 2004), which may lead to competition by exploitation, where the most abundant pathogen will use the resources faster, and will deprive the other isolate of them (Rayner and Weber, 1984). This was also found by Koppenhöfer *et al.* (1995), where after dual-inoculation with the entomopathogenic nematodes *Steinernema carpocapasae* and *Steinernema glaseri* in *Galleria mellonella* larvae, both species performed better if they outnumbered their competitor. They concluded that the outcome of interspecific competition depends on inoculum size, numbers of each species penetrating and time between infections.

Some dual-infected larvae were observed, although the proportions were always very low (always below 0.2). Dual infection is likely to have occurred as a result of a different kind of interaction taking place, where both isolates were inoculated at the same concentration of conidia and shared the host equally. It is also possible that each species had different tissue preferences. Although under normal circumstances both pathogens eventually invade the whole larva, it is possible that some preferences might exist at the beginning of infection, and once an isolate took possession of that host tissue or tissues, it could only use those tissues and not invade another tissue already occupied by the other species. A host can be an environment with substantial spatial and temporal heterogeneity in resource availability, mortality factors for the pathogen and indigenous microflora (Smith and Holt, 1996). Massey *et al.* (2004) found that when two species of virulent entomopathogenic bacteria (*Photorhabdus asymbiotica* and *Xenorhabdus nematophilus* were inoculated simultaneously onto *Galleria mellonella* larvae, they did not exclude each other, which seemed to be as a result of the spatial structure within the larvae, allowing different species to dominate different patches thereby avoiding competitive exclusion. The dual-infected *P. xylostella* larvae showed very ephemeral external fungal growth and sporulation on the host body if any. Sometimes sporulation only occurred in small patches and did not cover the whole larva (personal observation) and very few conidia were produced. This

external sporulation in patches may suggest an effect of the internal spatial structure of the larvae.

The presence of non-sporulating cadavers was also found, which may be as a result of within host competition. As mentioned earlier, fungal outgrowth, after host death, was limited in many dual-inoculated cadavers. It is possible that in the non-sporulating cadavers a high dose of each pathogen would be present leading to mutual antagonisms or scramble competition, where none of them survived. Similar results but with other pathogens (microsporidia) have also been described as a result of scramble competition (Solter *et al.*, 2002). Fargues and Bon (2004) also found non-sporulating cadavers, during dual-inoculation with two isolates of *Paecilomyces fumosoroseus* on *Galleria mellonella* larvae, but their results were more related to a temperature effect than a dose effect. Krauss *et al.* (2004) also found non-sporulating cadavers as a result of dual- inoculation of one entomopathogenic fungus (either *B. bassiana* or *M. anisopliae*) and one mycoparasite (either *Clonostachys* spp. or *Trichoderma harzianum* Tul.). They found more non-sporulating cadavers in small insects (*Bemisia tabaci* (Gennadius)) than in large insects (*Sitophilus oryzae* (Linnaeus)) which suggested that larger insects may have provided more protection against negative competition in internal habitats which were not accessible to the mycoparasite. The effect of temperature is discarded in our experiment because, in previous dose response assays, where the incubation from inoculation until sporulation were carried out either at constant 20 or 25 °C, isolate ARSEF6293 produced non-sporulating cadavers at both temperatures, and the other isolates particularly NW250, produced more non-sporulating cadavers at 25 °C. For this reason, 20 °C was used in all *in vivo* competition studies. At 20 °C, negative effects of temperature on sporulation were avoided because conidia production was the parameter measured to assess the outcome of possible interactions. Our results suggest that the non-sporulating cadavers were more a result of strong scramble competition between both species, possibly because of large doses of both isolates in those larvae, and also due to the characteristic of isolate ARSEF6293 to not use the host efficiently in converting host biomass into fungal biomass and therefore

conidia. As mentioned earlier, isolate ARSEF6293 normally produced cadavers with an ephemeral external sporulation and non-sporulationg cadavers.

Although isolate NW386 was the least virulent of all the four isolates, the relationship between NW386 and the two *P. blunckii* isolates was similar to that described for NW250, which supported the hypothesis that interactions between *Z. radicans* and *P. blunckii* are related to the initial dose ratio of each pathogen. However, a significant relationship was found between NW386 and the proportion of dual-infected larvae. This suggests that this isolate may have stronger competitive abilities than NW250, because NW250 can only compete effectively with both *P. blunckii* isolates if its initial conidia inoculation concentration is larger than its competitor. However, NW386 had a greater ability to survive even when it was outnumbered in conidia at inoculation because it produced identifiable resting spores in cadavers. All the isolates except NW250 were from Mexico, isolated from the same population of *P. xylostella* and from the same geographical area. The better ability of NW386 to survive dual-inoculations with the more virulent isolate ARSEF6293 may be the result of co-evolution between these isolates. In addition, NW386 normally produces resting spores inside the dead host. Although the factors responsible of the formation of resting spores is not well understood, adverse environmental conditions are considered one of the main factors responsible (Glare *et al.*, 1988, Hajek and St. Leger, 1994). Strong competitors such as *P. blunckii* isolates can also be a component of those adverse conditions. It is possible, therefore, that the *P. blunckii* isolates, especially ARSEF6293 being more virulent than NW386, are better intra-host competitors, and use that ability to survive, and that NW386 being the least virulent, is a better inter-host competitor producing resting spores that survive long term (Nowak and May, 1994). If there is a trade-off between virulence and transmissibility, then co-existence of two species is possible (Pugliese, 2000). The combination of these two attributes is part of the mechanism that maintains the diversity of entomophthoralean fungi in the *P. xylostella*-crucifers system. All the *Z. radicans* isolates isolated from the same area and host as NW386 produced resting spores (personal observation).

Whether the final results of any of the combinations used in this experiment were additive or synergistic with respect to host mortality was difficult to determine. The main reason was the difficulty in obtaining predetermined doses with entomophthoralean fungi. A range of doses can be obtained by manipulating the length of time that a group of larvae is exposed to sporulating fungi, but whether these doses are the ones that were initially aimed for, or if it can be replicated again, is entirely at random. Additionally, these isolates were very virulent, with small LC_{50} values (e.g. 2.5 and 2.0 conidia/mm^2 for NW250 and ARSEF6293 respectively). If only low mortalities are desired to measure synergism or additive interactions, lower doses would be needed, which was difficult to achieve. Therefore, in this experiment, we aimed for smaller and larger doses using extreme exposure times, five and 45 minutes respectively. Nevertheless, the variability amongst the doses within each dose category was high.

5.4.3 In vivo *inter-specific interactions on Chinese cabbage plants*

When the dual-inoculation of *P. xylostella* larvae was carried out in Petri dishes, both pathogens had the same opportunity to infect the larvae. However, when the experiment was done on cabbage plants, inoculation was passive, which means that the larvae were infected as a consequence of being closer to the inoculum. The results obtained showed a very similar pattern to that obtained in Petri dishes. The presence of isolate ARSEF6293 reduced the possibility of both *Z. radicans* isolates infecting *P. xylostella* larvae and *vice versa*. This may be because one cabbage plant does not represent sufficient spatial heterogeneity to allow each fungus species to perform differently. The overall proportions of infections were smaller than in the Petri dish experiment, which suggests that not all the larvae were in contact with the fungi (either primary or secondary conidia), and did not receive a sufficient dose to initiate infection (Regoes *et al.*, 2002).

This experiment was carried out in a larger spatial scale than the Petri dish experiment, and the way that the larvae could be infected was also similar to the way that they may be infected in the field if we consider a sporulating plug

as a simulation of a sporulating cadaver. Although the experimental conditions were optimal for the fungi, the results were important because it gave an insight into what may be happening in the field. In the field, conditions are not favourable for the fungi all the time, but there are always some periods of optimal conditions for both fungal species, otherwise no infected cadavers would be found in the field. During the periods of optimal conditions, some of the results obtained on the cabbage plant in these experiments could be found on a cabbage plant in the field. This suggests that if both species can co-exist on a plant, they have better opportunities to co-exist and survive at larger spatial scales (brassica crop) and may even avoid competition. Under field conditions, there are many factors that will have an effect on the outcomes of an interaction. Temperature is one of the most important (e.g. Inglis *et al.*, 1996; Fargues and Bon, 2004), and also the presence of other microoganisms that may not be direct competitors for the host but can actually interfere with the infection and survival of the pathogen (Hughes and Boomsma, 2004; Krauss *et al.*, 2004; Rostás and Hilker, 2003). Under field conditions, it has been suggested that two different pathogens may survive in the field by having different distributions or alternate hosts (Perlman and Jaenike, 2001; Koppenhöfer and Kaya, 1996). It is known that *Z. radicans* is likely to be a generalist pathogen (e.g. Pell *et al.*, 2001; Glare *et al.*, 1987; 1989), which suggests that it is possible that isolate NW386 may infect other insect species than *P. xylostella* to survive and avoid direct competition with *P. blunckii* which is thought to be a specialist pathogen.

The proportion of cadavers with unknown cause of mortality was larger than in the Petri dish experiment, but again it showed a significant interaction with the presence of ARSEF6293. However, these high proportions of cadavers with unknown cause of mortality were also present in the controls. This might be the result of the high humidity conditions that the experiment was carried out under because a cage with no ventilation was used. This was to avoid any primary or secondary conidia contaminating other treatments.

In conclusion, it could not be determined whether a single isolate was dominant in these interaction experiments. Our results suggest that isolates of

both species can co-exist in the same geographical area, and that outcomes will depend more on the relative doses rather than on relative virulence of the isolates. It is possible that the ability of NW386 to produce resting spores gives this isolate an ecological advantage in the long term, and that it can survive direct competition with *P. blunckii* isolates. The results also suggest that *P. blunckii* isolates, particularly ARSEF6293, are better intra-host competitors. and the fact that isolate ARSEF6293 did not produce sporulating cadavers, or if the cadavers sporulated, they did not produced many conidia, suggests there is a negative trade-off between virulence and transmission.

CHAPTER 6. REAL-TIME PCR DETECTION AND QUANTIFICATION OF *Zoophthora radicans* ON SINGLE AND DUAL-INOCULATED *Plutella xylostella* LARVAE

ABSTRACT

The interactions between isolates of *Z. radicans* and *P. blunckii* infecting the same individual larva during the time course of infection were investigated. As molecular detection was required for this experiment, species-specific primers were first developed based on the rDNA-ITS sequence information obtained for *Z. radicans* and *P. blunckii* isolates. Using conventional PCR, each pair of species-specific primers successfully detected each species from DNA extracted from infected larvae either single or dual inoculated with both fungal pathogens. However, for the interaction within a larva, quantitative real-time PCR techniques were required. Only the *Z. radicans* species-specific primer could be optimized for use in quantitative PCR. Therefore, the effect of the presence of *P. blunckii* in a larva inoculated with *Z. radicans* was evaluated by comparing the quantity of *Z. radicans* DNA in larvae either inoculated only with *Z. radicans* or dual inoculated with *Z. radicans* and *P. blunckii*. The dose of both fungal species was manipulated in the dual-inoculation treatments. The differences in the DNA quantities from both treatments were measured at different times after inoculation. Overall, the presence of *P. blunckii* (isolate ARSEF6293) had a negative effect on the *in vivo* development of *Z. radicans* isolates (NW386 and NW250) within the host. In larvae inoculated only with *Z. radicans*, there was an increase in the amount of *Z. radicans* DNA throughout the time course of infection. However, in dual-inoculated larvae, there was only an increase in the amount of *Z. radicans* DNA within the larvae on days three and four after inoculation, compared to the single inoculated larvae. Although *Z. radicans* isolates showed an overproduction of fungal biomass after days three and four of infection, possibly as a defence against ARSEF6293, eventually *P. blunckii* almost excluded both *Z. radicans* isolates, particularly when ARSEF6293 had a larger inoculation dose than the *Z. radicans* isolates. The role of the ability of one of the *Z. radicans* isolates

(NW386) to produce resting spores and the implications of the results for co-existence of these fungal pathogens infecting *P. xylostella* larvae in the field are discussed.

6.1 INTRODUCTION

During this research, the interactions between *Z. radicans* and *P. blunckii* attacking *P. xylostella* larvae have been investigated *in vivo* under different conditions and spatial scales. First, both pathogens were directly inoculated (active inoculation) onto the larvae at the same time and only dose was manipulated so that both pathogens had the same opportunity to infect *P. xylostella* larvae. Secondly, groups of larvae were placed on Chinese cabbage plants, allowed to establish feeding sites, and both pathogens were introduced as spatially separated sporulating fungal plugs with the anticipation that larvae would self inoculate as they foraged on the plant (passive inoculation).

In both cases the total percent infection was evaluated, and also, most importantly, the proportion of infection caused by each species determined. Although, mortality caused by *Z. radicans* or *P. blunckii* alone was expected, dual-infected larvae were found in both experiments. This suggested that the interactions between these two pathogens should also be investigated within an individual larva.

To evaluate within-host interactions between fungi, the use of molecular techniques becomes important. These techniques can detect the presence of one or both pathogens inside an individual larva when morphologically they are indistinguishable. One of the techniques widely used for this is the amplification of a specific DNA segment that is unique to a species or even isolate using PCR techniques. Species-specific sequence information from the ITS region of the gene that encodes the ribosomal RNA is useful in this respect (White *et al.*, 1990; Bridge and Arora, 1998).

To investigate the possible interactions between these two species of fungus within a *P. xylostella* larva, detection of one or both of them throughout the

time period of the infection process was necessary. Unfortunately, using conventional PCR methods, only the presence or absence of a particular species is possible. Quantitative changes in the amount of fungal DNA of each species inside the larva are only detectable using quantitative real-time PCR.

6.1.1 Quantitative real-time PCR

Quantitative real-time PCR uses the same principle as the conventional PCR, which is the exponential amplification *in vitro* of a nucleic acid sequence using a procedure called polymerise chain reaction (PCR) (Mullis and Faloona, 1987). Although conventional PCR is simple and inexpensive, it has some disadvantages such as an inability to distinguish between products of the same molecular weight but different sequence (Saunders, 2004). Another disadvantage for gel electrophoresis is that the analysis is made on the final product of the PCR, which is not quantitative because it does not depend on the initial amount of DNA (Saunders, 2004). Real-time PCR can simplify the recognition of the desired product by different methods. Basically, real-time PCR machines measure the increase during the amplification progress by monitoring changes in fluorescence inside the PCR tube, which are related to product accumulation (Saunders, 2004).

There are two main classes of real-time fluorescent chemistries: generic and strand-specific detection. Generic detection methods include fluorescent dyes that bind to any double stranded DNA molecule. Strand-specific methods use small sequences (probes) that anneal specifically to a sequence in the area between two primers (Lee *et al.*, 2004). The strand-specific method has the advantage that the production of unwanted PCR products (primer-dimers), does not influence the final fluorescence value (Lee *et al.*, 2004).

6.1.1.1 Generic detection

Generic detection uses fluorescent dyes such as SYBR® Green I (Morrisson *et al.*, 1998) which binds to all double-stranded DNA. This technique does not require high specialisation; its specificity relies completely on the good design

of the primers to be used. It does not have the limitations of primer and probe design that TaqMan® does and current primers designed for PCR assays can be utilised. Optimisation of PCR conditions is necessary for this process. It is less expensive than the strand-specific methods and does not have problems when the target DNA has some sequence modification which would greatly affect strand-specific detection methods (Mackay *et al.*, 2002). Binding to the double stranded DNA causes a conformational change in the SYBR® Green I molecule causing it to emit fluorescence. During denaturation, the SYBR® Green I molecules are free in solution, although it shows some fluorescence, this is not high enough to be detected by the real-time PCR machine. During annealing, some SYBR® Green I molecules bind to the small double stranded DNA formed when the primers anneal to the target sequence. This produces enough fluorescence to be detected by the real-time PCR machine. During extension, more SYBR® Green I molecules bind to the new double stranded DNA formed, and again, the fluorescence can be detected by the real-time PCR machine. At denaturation, the dye molecules are released again and the fluorescence decreases again (Bustin, 2000). During this process the real-time PCR machine quantifies fluorescence at the annealing and extension stages of the PCR reaction.

As the SYBR® Green I can bind to any double stranded DNA, it can also bind to primer-dimers which can confuse interpretation of the results (Mackay *et al.*, 2002). The problem of primer-dimers can be solved by the use of software that can perform melting curve analysis (Ririe *et al.*, 1997). This analysis shows the temperature at which all the PCR products are separated (denaturation), normally the shorter primer-dimer has a lower denaturation temperature compared to the desired product. In this way, the fluorescent signal from primer-dimers can be differentiated from the desired product (Ririe *et al.*, 1997). This information can also be used to monitor fluorescence at a temperature below the denaturation temperature of the desired product but higher than the denaturation temperature of the primer-dimers (Lee *et al.*, 2004).

6.1.1.2 Strand-specific detection

In general, this technique uses a labelled probe that binds specifically to the target DNA. There are different modes of action for different type of probes (Lee *et al.*, 2004). The main factor affecting the efficacy of the use of probes is the sequence of the target DNA itself. If this sequence is variable between samples, the designed probe may not bind to it, and, therefore no signal is produced (Lee *et al.*, 2004). The most important methods are described briefly.

6.1.1.2.1 The 5' nuclease assay

This assay is also called the TaqMan® assay (Bustin, 2000). This involves the annealing of two primers and one probe to the target DNA. The primers anneal to the target DNA and this is considered the first specificity level. More specificity is achieved when the probe binds to the target sequence between the primers (Bustin, 2000). The probe is labelled with two fluorescent dyes. One of the fluorescent dyes is called the reporter (such as FAM e.g. 6-carboxy-fluoroscein) and is attached to the 5' end of the probe, and its fluorescence is suppressed by the second dye (the quencher) (such as TAMRA e.g. 6-carboxy-tetramethyl-rhodamine) attached to its 3' end (Heid *et al.*, 1996). After denaturation, the dual-labelled probe and primers anneal to the target DNA. The extension step is carried out at the same temperature as annealing to ensure the probe remains attached to the target DNA. During extension (when the polymerase is creating the reverse complement of the target DNA), the polymerase removes the dual-labelled probe. This action separates the reporter from the quencher, and fluorescence is produced (Heid *et al.*, 1996; Bustin, 2000). The real-time PCR machine measures the fluorescence of all wells of a PCR plate continuously during the PCR amplification and, therefore, the reactions are monitored in real-time (Heid *et al.*, 1996). The dual-labelled probe is protected at the 3' end to prevent it being considered as target DNA and extended by the *Taq* polymerase (Lee *et al.*, 2004).

Some background fluorescence can be produced that is not related to the actual PCR reaction and can influence the final readings. This background

fluorescence can be eliminated or "normalised" using a non-participating or "passive" internal reference fluorophore (ROX: 6-carboxy-*N,N,N',N'*-tetramethylrhodamine) (Mackay *et al.*, 2002). Some of the sources of non-PCR related fluorescence can be volume differences amongst the wells due to pipetting errors or changes that may occur because of evaporation as a consequence of the high temperatures used in some steps of the PCR. There is also the possibility of non-PCR related fluorescence due to very small differences in the optical properties of the tubes or wells used (Lee *et al.*, 2004).

6.1.1.2.2 Linear hybridisation probes

This system uses two probes instead of one for more specificity, each labelled with different dyes. One of the probes carries at its 3' end a fluorescent donor (fluorescein phosphoramidite), the other probe carries an acceptor fluorophore (Cy5™ phosphoramidite) attached to the 5' end (Wittwer *et al.*, 1997). The detectable fluorescence comes when the donor is excited (by the light source of the real-time PCR machine) and energy is transferred to the acceptor (both probes must be close together) and the emission of red fluorescent light is detected. During denaturation, both probes are free in the PCR solution and remain separated. During annealing, the probes bind to the target DNA and fluorescence is produced and detected. When extension takes place, both probes are released into the solution and separated, therefore no detectable fluorescence is produced (Bustin, 2000).

Some limitations of this technique are that, in addition to the primers, two probes need to be designed. The selection of these two probes may be difficult if the target area has little or no conserved area in its sequence (Lee *et al.*, 2004).

6.1.1.2.3 Molecular beacons

This technique uses a probe similar to the dual-labelled probe (TaqMan®) (Mackay *et al.*, 2002). The probe is called a "molecular beacon" because it

emits fluorescence only when it is bound to the target DNA (Tyagi and Kramer, 1996). The probe is single stranded and has a stem-loop structure. The loop part of the probe is a sequence that is complementary to a sequence in the target DNA. The stem is formed by the annealing of two arms located on both extremes of the molecular beacon; both arms must have a different sequence to the target DNA. One of the arms contains a fluorogenic label (fluorophore) and the other arm a non-fluorescent quenching label (quencher) (Tyagi and Kramer, 1996; Mackay *et al.*, 2002). When both labels are close together, quenching of the fluorophore takes place and, therefore, no fluorescence is produced (Mackay *et al.*, 2002). When the molecular beacon finds its complementary sequence on the target DNA, it binds to it and forms a small section of double stranded DNA that is more stable than the stem part of the molecular beacon. As the target DNA is linear, the loop part of the molecular beacon has to adopt the same shape as the target DNA when it is bound to the target DNA. This change in the shape of the loop part of the molecular beacon facilitates separation of the fluorophore and the quencher and, therefore, fluorescence is produced (Tyagy and Kramer, 1996).

It is suggested that this type of probe is more specific than the linear probes because the structure of the molecular beacon is a more stable alternate conformation than the linear structure. Therefore, any mismatch between the molecular beacon and the target DNA is more destabilising than a mismatch between a linear probe and the target DNA (Mackay *et al.*, 2002).

One disadvantage of the molecular beacon is that an optimal design is needed, especially at the stem part. If the design is not optimal the two arms of the stem may adopt different conformations, which can result in the quencher not being close enough to the fluorophore and unwanted fluorescence being produced without being bound to the target DNA (Bustin, 2000).

6.1.1.2.4 Self-fluorescing PCR products

The main difference in these techniques compared to the others described earlier is that in the other techniques the labelled probes attach to the target

DNA temporarily (at annealing and/or the beginning of extension), therefore the fluorescence is measured only at those stages. The self-fluorescing methods generate fluorescence only when the probes are incorporated into the amplification product definitively (Nazarenko *et al.*, 1997; Lee *et al.*, 2004). A stem-loop structure (as in molecular beacons) is attached to the 5' end of a specific primer, and at the end of the stem part, a fluorophore and quencher are placed in each arm (Nazarenko *et al.*, 1997). There are two approaches described, sunrise primers and Scorpion® primers (Mackay *et al.*, 2002).

In sunrise primers, a stem-loop structure is attached to a primer. During the first PCR cycle, after the extension step, the primer and the probe (stem-loop structure) form part of the new DNA sequence, the probe maintains their original structure (fluorophore and quencher together) therefore no florescence is produced. In the following cycle, the polymerase creates the reverse complement of this new DNA sequence. When the polymerase reaches the probe this separates the stem arms and copies the sequence. As a consequence, the loop part of the probe is linearised, the fluorophore and the quencher are now separated and fluorescence is produced (Nazarenko *et al.*, 1997). In Scorpion® primers, the structure is similar to the sunrise primer (Whitcombe *et al.*, 1999). The probe also contains a fluorophore and a quencher. In this approach the primer has a molecule that blocks the probe from being copied and included in the new DNA strand as in sunrise primers. In the initial PCR cycles, the primer binds to the target sequence and extension occurs due to the action of the polymerase. After one cycle of PCR and extension completes, there is a newly synthesised target region and the probe attached to it. In the second PCR cycle, during the denaturation step, the stem part of the probe separates as a consequence of the high temperature during annealing. With the stem part separated, the loop part binds to its complement on the target DNA. This results in the separation of the fluorophore from the quencher and causes emission of fluorescence (Whitcombe *et al.*, 1999).

The main disadvantage of these techniques is that the synthesis of the probes is more complex and expensive, and not many companies can produce them (Lee *et al.*, 2004).

6.1.2 Threshold cycle (C_t)

The threshold cycle (C_t) is the most important value for quantification using real-time PCR (Higuchi *et al.*, 1993). These values are recorded during each cycle and represent the amount of PCR product amplified at each cycle (Bustin, 2000). The relationship is simple; the more DNA present in the reaction, the fewer are the number of cycles needed to detect fluorescence (Bustin, 2000).

Data are plotted placing fluorescence on the Y axis and cycle number on the X axis. Using this amplification plot, the C_t value of each reaction is the cycle number at which the amplification plot crosses the detection threshold (Heid *et al.*, 1996). The detection threshold can be set automatically by the software of the real-time PCR machine, which determines the standard deviation of the readings of the base line. For example, this can be set as three standard deviations above the baseline mean. Another method called "second derivative maximum" selects the cycle number when the fastest change in fluorescence is detected and selected as the detection threshold. However, in practice the baseline method is more accurate (Saunders *et al.*, 2004). The C_t value is then converted into quantitative data based on a standard curve.

6.1.3 Standard curve

Determination of how much DNA there is in a sample can be performed in two ways: as a relative or as an absolute quantification. Relative quantification only describes changes in the amount compared with other samples, e.g. when comparing the differences in DNA production between two samples (Mackay *et al.*, 2002). Absolute quantification determines the number of target DNA copies present within a sample (Saunders *et al.*, 2004). Relative quantification sometimes provides enough information and is simpler to develop than the absolute quantification. However, when a process is being followed, e.g. the progress of infection, absolute quantification is more appropriate (Mackay *et al.*, 2002). The accuracy of quantification by real-time PCR depends on the quality of the standard curve (Saunders *et al.*, 2004).

A standard curve is generated by plotting the C_t values against known quantities of DNA from serial dilutions obtained after a real-time PCR reaction. The quantities of DNA in the unknown samples can be calculated from the linear regression equation of the standard curve (Bustin, 2000). The maximum increment between quantitative standards should be $\log_{10}$ with a minimum of four points to construct the curve (Saunders *et al.*, 2004). The software in the latest real-time PCR machines can perform all the calculations required.

6.1.4 Applications of real-time PCR

Since their first introduction in the 1990s, the use of this technique has increased considerably (Saunders, 2004). It has been used in many fields especially biomedical research and molecular diagnostics (Valasek and Repa, 2005). An important application of this technique is the measurement of RNA levels to determine the expression of a particular gene, which is important for the understanding of host pathogen interactions during infection (Saunders, 2004). There is only a little information about the use of this technique in biological control of agricultural pests (e.g. Atkins *et al.*, 2003; 2005) There are few examples using real-time PCR to investigate interactions between two pathogens in the same host and only in human medicine (De Roode *et al.*, 2005).

There is some information about the interactions between two entomopathogenic fungi infecting the same insect population (e.g. Inglis *et al.*, 1997; Thomas *et al.*, 2003; Krauss *et al.*, 2004; Fargues and Bon, 2004). However, no work has been done to investigate the possible interactions between two entomopathogenic fungi in individual hosts during the time course of infection.

The aim of this experiment was to investigate the possible interactions that may occur between two isolates of *Z. radicans* and one isolate of *P. blunckii* during the time course of infection in dual-inoculated *P. xylostella* larvae. To do this, quantitative real-time PCR by the generic detection method using the

fluorescent dye SYBR® Green I was used. Strand-specific detection was not appropriate in this case because of the variability in sequence between the two isolates of *Z. radicans* which would have made design of labelled probe more difficult.

6.2 MATERIALS AND METHODS

The interactions between *Z. radicans* and *P. blunckii* inside *P. xylostella* larvae were analysed by comparing the quantity of *Z. radicans* DNA in *P. xylostella* larvae at different times after dual-inoculation of larvae. Species-specific diagnostic primers for both fungi and real-time PCR assays were developed for this purpose.

6.2.1 Development of species-specific diagnostics primers for *Zoophthora radicans* and *Pandora blunckii*

Based on the sequence information of the ITS region of seven isolates of *Z. radicans* and one isolate of *P. blunckii* obtained in Chapter 4, species-specific diagnostic primers were designed. Primers were compared to other sequences using BLAST and FASTA searches against the GenEMBL database to confirm specificity. Specificity was also tested against DNA samples.

PCR reactions were carried out using DNA extracted from pure cultures *in vitro* from both species to confirm specificity of the primers for each species. The PCR reactions were done as in 4.2.3.1. The optimised PCR cycling conditions for the species-specific primer set for *Z. radicans* was 95 °C followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 59 °C for 1 minute and extension at 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. For the species-specific primer set for *P. blunckii*, all the conditions were the same except the annealing temperature which was set at 63 °C.

The PCR products were analysed on 1.5 % agarose-gels in 1X TBE and photographed.

6.2.2 Specific detection of *Pandora blunckii* and *Zoophthora radicans* in laboratory infected *Plutella xylostella* larvae using conventional PCR

In order to confirm that the insect DNA did not interfere with the specificity of the primers, the primers were tested using DNA extracted from fungus-infected larvae.

6.2.2.1 Infection of larvae

These bioassays were carried out on *P. xylostella* larvae using isolates ARSEF6293 (*P. blunckii*) and NW378 (*Z. radicans*). Isolates were selected randomly.

Four groups of 15 early third instar larvae of *P. xylostella* were inoculated in 50 mm diameter Petri dishes. The first group was inoculated with *P. blunckii*, the second with *Z. radicans*, the third with both species together. A fourth group of 15 larvae was the control with no fungi. The inoculation method was the same as described in 5.2.3. Larvae were inoculated for 1 hour with each of the pathogens during which time the lid with the sporulating plugs was rotated every 10 minutes in order to achieve an even deposition of conidia. In the dual-inoculation treatment, the batch of 15 larvae was exposed for 30 minutes to *Z. radicans* followed by 30 minutes by *P. blunckii*.

Dose estimation was performed as in section 5.2.3. The cabbage leaf and larvae from every Petri dish were transferred to Blackman boxes with ventilation and additional food plant. All the Blackman boxes were held inside a sealed plastic box at approximately 100 % RH for 24 hours, after which time the plastic box was removed to allow more ventilation. All Blackman boxes were incubated at 20 °C in darkness throughout.

Five or six larvae were sampled randomly from each treatment after 72 and 120 hours post inoculation and frozen individually in eppendorf tubes at -20 °C for later DNA extraction. Larvae sampled 72 hours post inoculation were still alive. Larvae sampled after 120h of inoculation were dead with evidence of

external sporulation. However, there were some exceptions in which larvae were dead but with no visible sporulation after 120 h and these individuals were recorded.

6.2.2.2 DNA extraction from infected Plutella xylostella *larvae*

DNA was extracted from individual larvae using DNAzol ™ (Helena BioSciences, Sunderland, UK) using the following method:

Each larva was ground in liquid nitrogen inside a 1.5 ml Eppendorf tube, then suspended in 200 µl of DNAzol and mixed using a vortex for 5 seconds. The tubes were allowed to stand at room temperature for 10 minutes, and then centrifuged at 13K r.p.m. for 10 minutes. All the supernatant was transferred to a clean tube, and the pellet discarded. One hundred µl of absolute ethanol was added to each tube containing the supernatant. The tubes were mixed gently by inverting them, and maintained at room temperature for a further three minutes. The tubes were centrifuged again for five minutes at 6K r.p.m. The supernatants were removed carefully without touching the pellet and the tubes air dried. The pellet was washed twice in 200µl of 70% ethanol. All the ethanol was discarded and the tubes dried for about 10 minutes with the lid opened. After that, 50 µl of elution buffer was added to dissolve the pellets and the DNA stored at -20 °C until required. The elution buffer used to dissolve the pellet was from the QIAquick gel extraction kit (10 mM Tris pH 8 - Qiagen, Crawley, UK). DNA from healthy larvae was also extracted and used as a negative control. All the tubes used were sterilised by autoclaving prior to use.

PCR amplifications were performed as in 6.2.1 for each species. The PCR products were analysed on 1.5% agarose gels in 1X TBE and a 100bp size marker (GeneRuler ™, Helena Biosciences, Sunderland, UK) was used. Gels were stained with ethidium bromide (0.1 µg/µl) and photographed.

6.2.3 Development of quantitative real-time PCR assays

6.2.3.1 Primer concentration optimisation for Zoophthora radicans *species-specific primers*

The optimal primer concentration was determined using a primer optimisation matrix (Table 6.1). Stratagene Brilliant® SYBR® Green I QPCR Master Mix kit was used (Stratagene, Amsterdam, Netherlands). All the components for the reactions are shown in Table 6.1. Real-time PCR was done in a Thermo-Fast 96 x 0.2 ml detection plate (ABGene, Epsom, UK) and Ultra Clear Cap Strips (ABGene) with the automated ABI Prism 7700 sequence detector (PE Applied Biosystems). The cycle threshold (C_t) and the detection threshold were automatically calculated by the ABI Prism sequence detection software (version 1.5). The thermal conditions were one cycle of two minutes at 50 °C, one cycle of ten minutes at 95 °C, followed by 50 cycles of 30 seconds at 95 °C, 1 minute at 60 °C and 1 minute at 72 °C.

After amplification, a melting curve was acquired by running all the samples for 15 seconds at 95 °C, followed by one minute at 60 °C and increasing the temperature slowly for 20 minutes up to 95 °C. The fluorescence was recorded at the annealing step in each cycle of the PCR reaction. During the melting curve, fluorescence was recorded continuously for the entire 20 minutes. The optimal combination selected was the gradient with the lowest C_t value. The DNA used was from isolate NW250 (*Z. radicans*) extracted from a pure culture *in vitro* as described in 4.2.2.1, and diluted 1 in 50 using 1x TE (10mM Tris-HCL, 1mM EDTA, pH 8).

Table 6.1. The primer optimisation matrix shows all the different quantities and concentrations of primers in a range of 10 gradients. The quantities were enough to run each sample in triplicate with a final volume reaction of 25 µl.

Gradient No	Primer	Final conc. µM	µl reaction (x pM)	Stock pM/µl	Primer added µl	DNA	2 x Buffer	Dye	SdH_2O
1	F	0.05	1.25	1	4.375	3.5	43.75	1.31	30.19
	R	0.05	1.25	1	4.375				
2	F	0.05	1.25	1	4.375	3.5	43.75	1.31	31.94
	R	0.30	0.75	10	2.625				
3	F	0.05	1.25	1	4.375	3.5	43.75	1.31	26.69
	R	0.90	2.25	10	7.875				
4	F	0.30	0.75	10	2.625	3.5	43.75	1.31	31.94
	R	0.05	1.25	1	4.375				
5	F	0.30	0.75	10	2.625	3.5	43.75	1.31	33.69
	R	0.30	0.75	10	2.625				
6	F	0.30	0.75	10	2.625	3.5	43.75	1.31	28.44
	R	0.90	2.25	10	7.875				
7	F	0.90	2.25	10	7.875	3.5	43.75	1.31	26.69
	R	0.05	1.25	1	4.375				
8	F	0.90	2.25	10	7.875	3.5	43.75	1.31	28.44
	R	0.30	0.75	10	2.625				
9	F	0.90	2.25	10	7.875	3.5	43.75	1.31	23.19
	R	0.90	2.25	10	7.875				
10	F	0.90	2.25	10	7.875	0	43.75	1.31	26.69
	R	0.90	2.25	10	7.875				

6.2.3.2 Standard curve

For the standard curve, the two *Z. radicans* isolates NW250 and NW386 were grown and the mycelium obtained as described in 4.2.2. For DNA extraction, two samples were taken from freeze dried material of isolate NW250 (24.12 and 23.72 mg) and three samples from isolate NW386 (23.11, 22.73 and 19.4 mg). The DNA was extracted using DNAzol ™ (Helena BioSciences, Sunderland, UK) as described in 6.2.2.2, except that all the volumes used were increased five times and 100 µl of elution buffer was used to dissolve the final pellet.

The DNA was quantified using a Cary 50 Series Spectrophotometer (Varian, Inc. Palo Alto, CA. USA). Although five samples in total were obtained for *Z. radicans* from both isolates (NW250 and NW386), only one sample was used for development of the standard curve.

The concentrated sample used had 320 ng/µl of DNA. This was diluted logarithmically from 32 ng/µl to 32^{-9} ng/µl. The experimental DNA (unknown samples) used in this assay came from the inoculated larvae used in 6.2.2. The dilutions for the standard curve and the unknown samples were allocated in a real-time PCR detection plate as shown in Table 6.2. The real-time PCR reactions were done in a Thermo-Fast 96 x 0.2 ml detection plate (ABGene) capped with Ultra Clear Cap Strips (ABGene) using the automated ABI Prism 7700 sequence detector (PE Applied Biosystems).

Table 6.2. Typical real-time PCR detection plate. The table shows the allocation of all the unknown samples as well as the dilutions used to develop the standard curve on the detection plate. Cells A1 to F4, contain the 10 dilutions used for the standard curve for *Z. radicans* (NW250) DNA (ng/µl); NTC=No Template Control (No DNA); TC=Template Control (DNA from *Z. radicans* isolate NW250 extracted from pure *in vitro* culture); PxZ1=DNA extracted from a larva infected with *Z. radicans* (NW378); PxZ2=DNA extracted from a different larva infected with *Z. radicans* (NW378); PxP1=DNA extracted from a larva infected with *P. blunckii* (ARSEF6293); PxP2=DNA extracted from a different larva infected with *P. blunckii* (ARSEF6293); PZ1=DNA extracted from a larva dual-inoculated with *Z. radicans* (NW378) and *P. blunckii* (ARSEF6293); PZ2=DNA extracted from a different larva dual-inoculated with *Z. radicans* (NW378) and *P. blunckii* (ARSEF6293); Px=DNA extracted from a healthy larva. Note that each sample was run in triplicate. The shaded area in the plate was not used.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	32	32^{-2}	32^{-5}	32^{-8}	NTC	PxZ2	PZ1	Px				
B	32	32^{-3}	32^{-5}	32^{-8}	TC	PxZ2	PZ1					
C	32	32^{-3}	32^{-6}	32^{-8}	TC	PxP1	PZ1					
D	32^{-1}	32^{-3}	32^{-6}	32^{-9}	TC	PxP1	PZ2					
E	32^{-1}	32^{-4}	32^{-6}	32^{-9}	PxZ1	PxP1	PZ2					
F	32^{-1}	32^{-4}	32^{-7}	32^{-9}	PxZ1	PxP2	PZ2					
G	32^{-2}	32^{-4}	32^{-7}	NTC	PxZ1	PxP2	Px					
H	32^{-2}	32^{-5}	32^{-7}	NTC	PxZ2	PxP2	Px					

This preliminary real-time PCR reaction was performed to determine the range of dilutions in which detectable amounts of DNA were present and second, to determine if the quantification of DNA extracted from infected larvae were in the same range of dilutions. For the real-time reaction Stratagene Brilliant® SYBR® Green QPCR Master Mix Kit was used (Stratagene, Amsterdam, Netherlands) and the primer concentration was the optimum determined in 6.2.3.4.1. Each reaction contained 12.5 µl of 2X Brilliant® SYBR® Green QPCR Master Mix, 0.05µM of the forward primer, 0.90µM of the reverse primer, 0.38 µl of the reference dye diluted 1/50, 2 µl of diluted DNA and the reaction was made up to 25 µl using sterile distilled water. The thermal cycle conditions were: one cycle of 2 minutes at 50 °C, one cycle of 10 minutes at 95 °C and 50 cycles of 95 °C for 15 seconds, 60 °C for one minute and 72 °C for one minute. The melting curve was also acquired at the end of the PCR as described in 6.2.3.4. The fluorescence during the PCR reaction and the melting curve was recorded as in 6.2.3.4. Reactions with the same sample were run by replicate in three different tubes. The C_t values and the detection threshold were automatically calculated by the ABI Prism 7700 sequence detector software (SDS ver. 1.5). To obtain the linear correlation coefficient as well as the linear equation, a linear regression analysis were carried out using the spread sheet EXCEL (Microsoft Corp.). The linear equation was used to convert the C_t values from the experimental DNA into quantities of *Z. radicans* DNA.

6.2.4 Use of quantitative real-time PCR assays to investigate the interactions between *Zoophthora radicans* and *Pandora blunckii* during the time course of an infection

The species-specific primers for *P. blunckii* could not be optimised to use in real time PCR assays. Therefore, only *Z. radicans* was detected and quantified in single and dual-infected larvae. The two *Z. radicans* isolates used had different biological attributes such as different LD_{50} values, resting spore production (only seen in NW386) (Chapter 5), different temperature growth profiles (Chapter 2) and from different RFLP grouping (Chapter 4). By

quantifying the DNA from each *Z. radicans* isolate either single or dual-inoculated with *P. blunckii* within the host, valuable information could be obtained about the types of responses that isolates with different biological attributes make when a competitor is present inside the same host.

Two combinations were evaluated, NW250 (*Z. radicans*) with ARSEF6293 (*P. blunckii*) and NW386 (*Z. radicans*) with ARSEF6293 (*P. blunckii*). The methods described were the same for both fungal combinations.

6.2.4.1 Bioassay method

Different batches of early third instar larvae of *P. xylostella* were inoculated with six different treatments:

1. Inoculated for 30 minutes with *Z. radicans*.
2. Inoculated with *Z. radicans* (30 minutes) and *P. bluncki* (10 minutes).
3. Inoculated with *Z. radicans* (30 minutes) and *P. blunckii* (50 minutes)
4. Inoculated for 10 minutes with *P. blunckii*
5. Inoculated for 50 minutes with *P. blunckii*
6. Control group, where the larvae were kept under the same conditions as if they inoculated but without any fungus.

The inoculation was carried out using fungal plugs (9 mm diameter) from the growing edge of 15 day-old cultures. Thirteen plugs were placed on the lid of a 90 mm diameter Petri dish containing damp filter paper. The Petri dish with the fungal plugs was placed in a plastic box containing moistened tissue paper (to ensure a high humidity and encourage sporulation) at 20 °C in darkness for 18 hours.

Early third instar larvae were placed in groups of 44 on a 73 mm diameter cabbage leaf disk which was itself placed abaxial side uppermost in the bottom of a 90 mm diameter Petri dish containing a 20 ml layer of 1.5 % water-agar. Five 10 mm diameter glass cover slips were placed on the leaf (Fig. 6.1). For

the inoculation, the lids were replaced with lids containing the fungal plugs. The larvae were feeding on the leaf while they were inoculated.

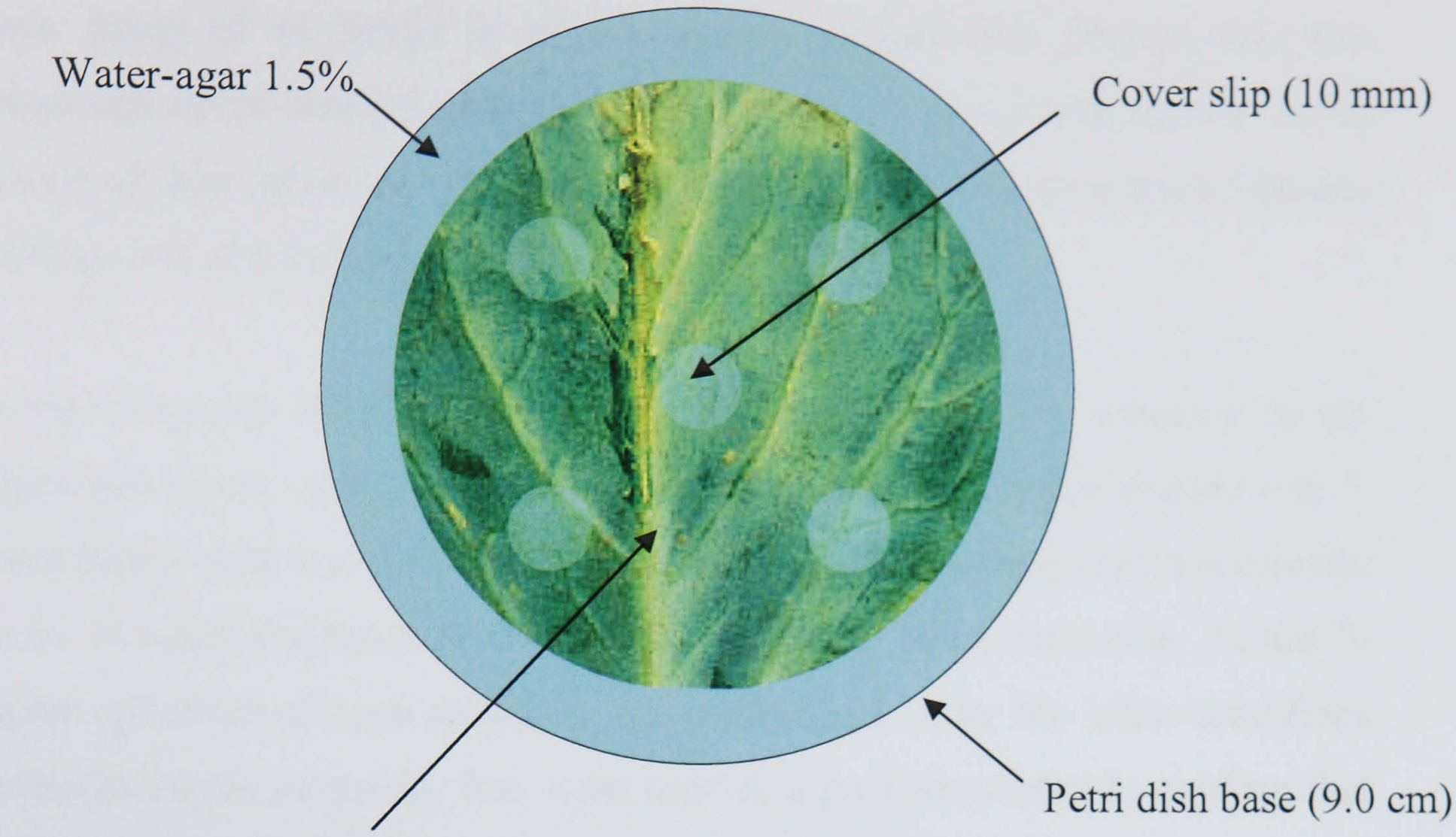


Figure 6.1. Distribution of the five cover-slips on the cabbage leaf disk used to estimate dose of *Zoophthora radicans* and/or *Pandora blunckii* conidia during the larvae inoculation.

Three groups of 44 larvae were exposed to *Z. radicans* for 30 minutes. To avoid differences in dose amongst the three larvae batches, the lids containing the fungal plugs were alternated amongst the three batches of larvae.

The first group was considered as treatment one of the experiment (only *Z. radicans*). The second and third group were further inoculated with *P. blunckii* conidia for 10 and 50 minutes respectively and considered as the second and third treatments. At the same time, two further groups of 44 larvae were inoculated only with *P. blunckii* conidia for 10 and 50 minutes respectively and these were considered as the fourth and fifth treatment. One last group of 44 larvae was maintained under the same inoculation conditions but with no

fungal conidia for 80 minutes (control treatment). Conidia concentration for each species were estimated as in 5.2.3

Each group of 44 larvae in all the treatments was then divided into two subgroups of 24 and 20 larvae. Each subgroup of larvae was transferred in groups of four larvae to ventilated Blackman boxes containing fresh Chinese cabbage leaf and incubated at 20 °C and 12:12 L:D regime.

In the subgroup of 24 larvae, groups of four larvae were allocated in six Blackman boxes, each box was randomly assigned with a number from 0 to 5. From box 0 until box 5, the four larvae from each box were frozen individually every 24 hours and maintained at -20 °C for further DNA extraction. As the 20 larvae subgroup of each treatment was maintained under the same conditions as the 24 larvae subgroup, they were used as a positive control to confirm that infection had taken place. This was necessary because many larvae from the 24 larvae subgroup were frozen before mycosis was confirmed. In the 20 larvae subgroup, groups of larvae were also allocated in five Blackman boxes and the mortality recorded every day until day five. Mortality and cause of mortality was confirmed as in 5.2.5.

6.2.4.2 DNA extraction from inoculated larvae

The DNA extraction process for each larva was the same as described in 6.2.2.2.

DNA samples extracted from each experimental larva were quantified using a Cary 50 Series Spectrophotometer (Varian, Inc. Palo Alto, CA. USA). Due to variation in the DNA concentration in the different samples, all the samples were diluted when necessary to achieve a final concentration of 20 ng/µl of DNA. The dilutions were performed using the same elution buffer used in 6.2.2.2.

6.2.4.3 Quantification of Zoophthora radicans *DNA in laboratory infected* Plutella xylostella *larvae inoculated either with* Zoophthora radicans *alone or dual-inoculated with* Pandora blunckii

In treatments two and three, larvae were used at each time interval to investigate the interactions between *Z. radicans* and *P. blunckii* in comparison with treatment six (control) and treatment one (when *Z. radicans* was inoculated alone). Treatments four and five were not used for DNA quantification because only *P. blunckii* conidia were inoculated and were used just as a positive control to confirm the biological activity of *P. blunckii* on *P. xylostella* larvae.

Using the optimal primer concentrations and the range of dilutions selected from the preliminary standard curve results (6.2.3.4), a series of real-time PCR reactions were performed to quantify *Z. radicans* DNA from single and dual-inoculated larvae. SYBR® Green I JumpStart™ Taq ReadyMix™ Kit (Sigma-Aldrich Ltd., Dorset, UK) was used, and the components per reaction were as follows: 12.5 µl of SYBR® Green I JumpStart™ Taq ReadyMix™, 0.05µM of the forward primer, 0.90µM of the reverse primer, 0.25 µl of the reference Dye and 1 µl (20 ng) of DNA. The reactions were made up to a final volume of 25 µl using sterile distilled water. All the reactions were performed in duplicate.

The reactions were carried out in a 7500 Real-Time PCR System (AB Applied Biossystems, Warrington, UK) using a Thermo-Fast 96 x 0.2 ml detection plate (ABGene) and Ultra Clear Cap Strips (ABGene). The thermal cycling conditions were: one cycle at 95 °C for 2 minutes and 40 cycles of 95 °C for 15 seconds, 60 °C for one minute, 72 °C for one minute and 74 °C for 20 seconds. Fluorescence was detected at the annealing step of every cycle. After amplification, a melting curve was acquired by running for 15 seconds at 95 °C, followed by one minute at 60 °C and increasing the temperature slowly for 20 minutes up to 95 °C. Fluorescence was recorded at the annealing step in each cycle of the PCR reaction. During the melting curve, fluorescence was recorded continuously during the 20 minutes. The C_t value for each sample was

automatically calculated and analysed by the 7500 real-time PCR System sequence detection software (SDS ver. 2.1). The software also transformed the C_t values automatically into ng of *Z. radicans* DNA present in each sample based on the standard curve analysis. A different Thermo-Fast 96 x 0.2 ml detection plate (ABGene) was used for each treatment and combination of fungi and each detection plate was run twice on seperate occasions. In all the real-time PCR reactions performed for each combination, the selected dilutions for the standard curve were included.

To assess whether there was a difference in the amount of *Z. radicans* DNA in infected *P. xylostella* larvae amongst treatment one to three, the data were transformed using a log10 transformation. As there were zero values in the data set and the logarithm of zero is negative infinity (Sokal and Rohlf, 1995), half of the smallest value was added to the whole data set before the log10 transformation. A different half value was used for each combination of isolates. The DNA extracted from larvae in treatment six (control) were not included in the analysis, they were only used during the real-time PCR reactions to confirm that no amplification was obtained from *P. xylostella* DNA. The data were then analysed by ANOVA using Genstat ver. 8.1.

6.3 RESULTS

6.3.1 Development of species-specific diagnostics primers for *Zoophthora radicans* and *Pandora blunckii*

Based on the sequence information for both species (Chapter 4), species-specific sets of primers were developed (Table 6.3).

Table 6.3. Species-specific sets of primers developed for *Zoophthora radicans* and *Pandora blunckii*. Letters in bold can represent two different bases at the same time: **R**= A or G, **W**= A or T and **Y**= C or T.

Zoophthora radicans	
F: 5'GTA TGC TCT CGG GT**R** TAT TGT TGG 3'	R: 5'TAG ACT AAT C**Y**A **W**AA CAA TAA TGC TC 3'
Pandora blunckii	
F: 5'TGG TTT ATT TTA AGT TTT AGC TGA GG 3'	R: 5'CAT TTG CAT TAA ACT TTA CCC TCG 3'

The primers designed for *Z. radicans* amplified a 570 bp sized product, and were tested against all the *Z. radicans* isolates (Fig. 6.2). The primers for *P. blunckii* amplified a 370 bp sized product, and were tested again all isolates of *P. blunckii* (Fig. 6.3). With the *P. blunckii* species-specific primers, secondary bands were observed in isolates NW344, NW352 and ARSEF6293 (Fig. 6.3). However, even with the secondary bands, it was still possible to distinguish between the two species.

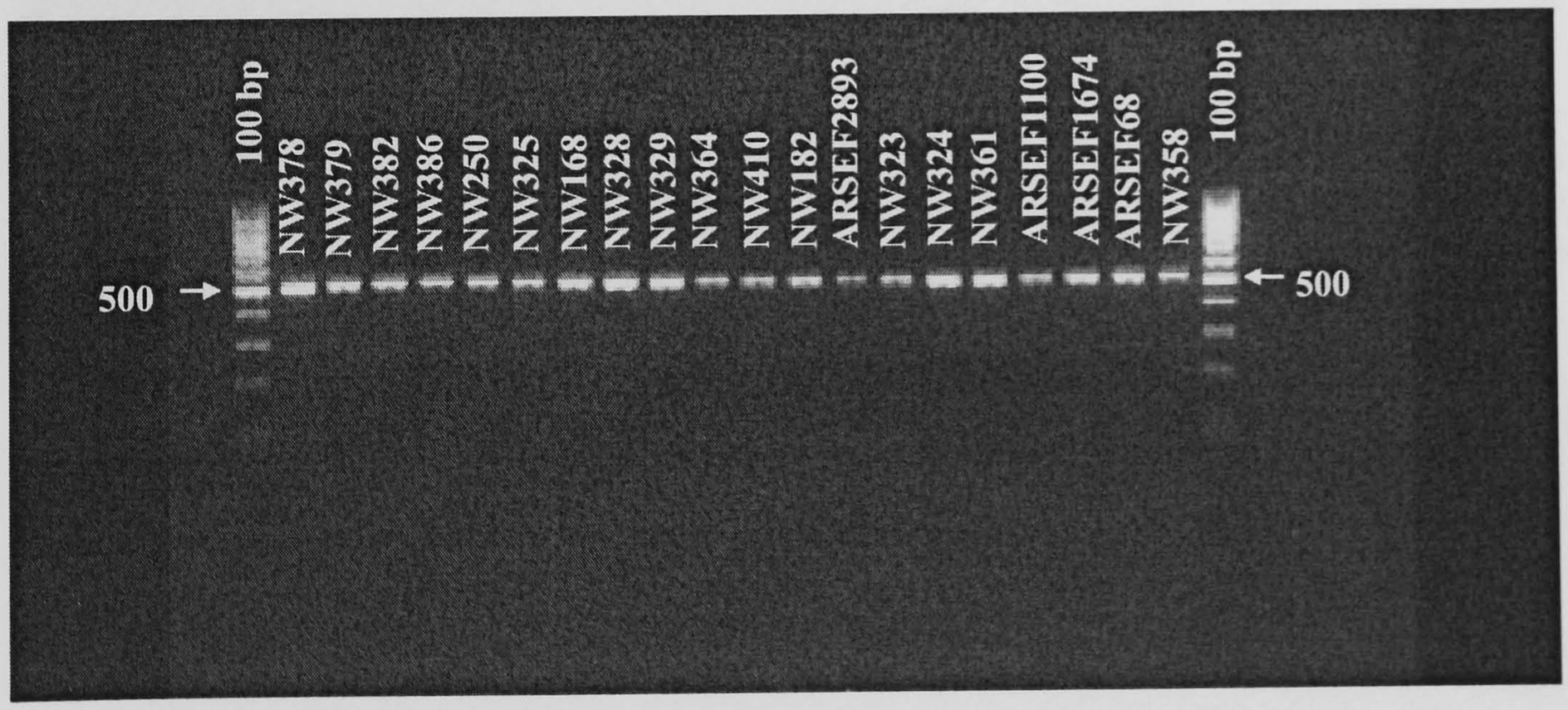


Figure 6.2. Amplification of some *Zoophthora radicans* isolates using species-specific primers. The size product is 570 bp. Size marker = 100bp. Information about the isolates are listed on Table 4.2 (Chapter 4).

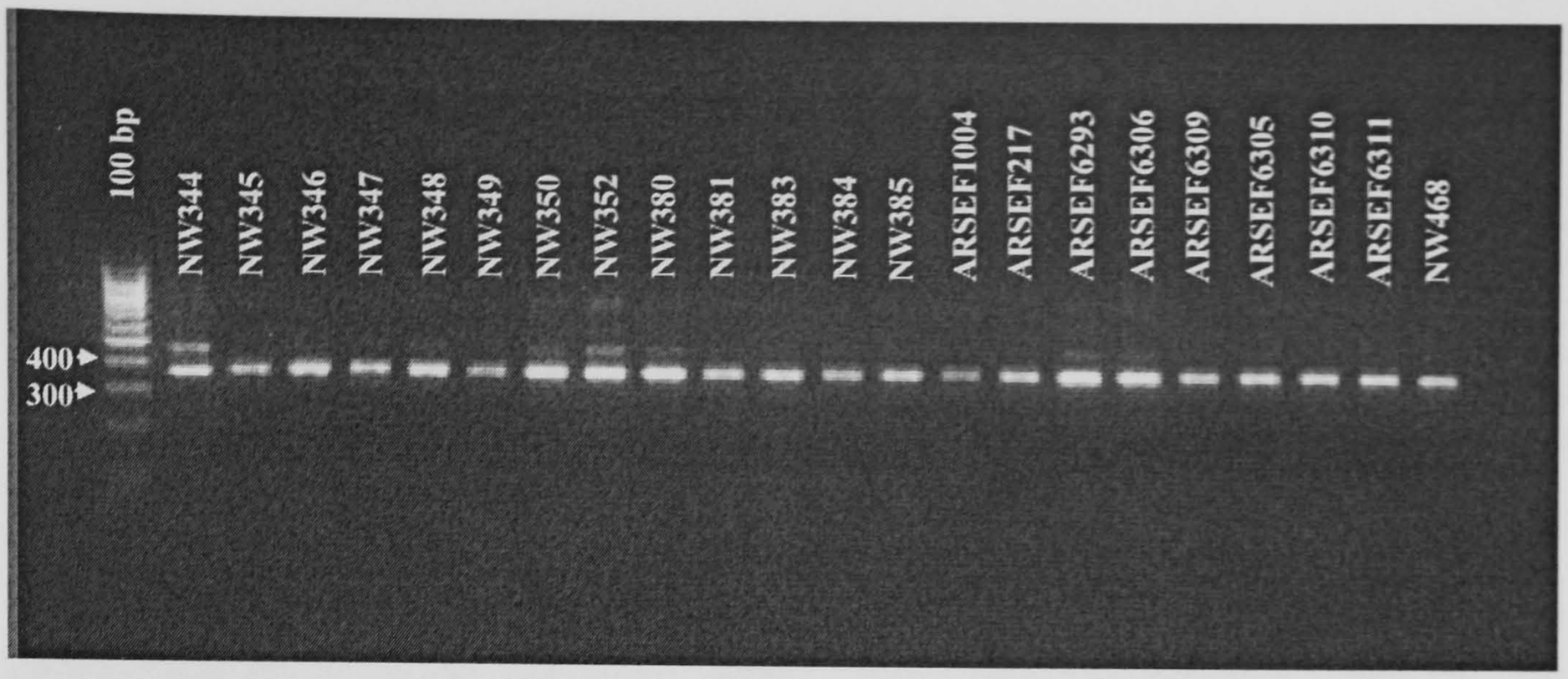


Figure 6.3. Amplification of all the *Pandora blunckii* isolates using species-specific primers. The size product is 370 bp. Size marker = 100bp. Information about the isolates are listed on Table 4.1 (Chapter 4)

The primers for each species were tested using DNA from other species as well (Table 6.4). With the *Z. radicans* primers no amplification was found for any of the other species DNA (Fig. 6.4). However, when the *P. blunckii* primer set was tested, faint bands were detected for isolates NW386 (Z. *radicans*) and ARSEF207 (*Z. occidentalis*), but no amplification in the other species (Fig. 6.5).

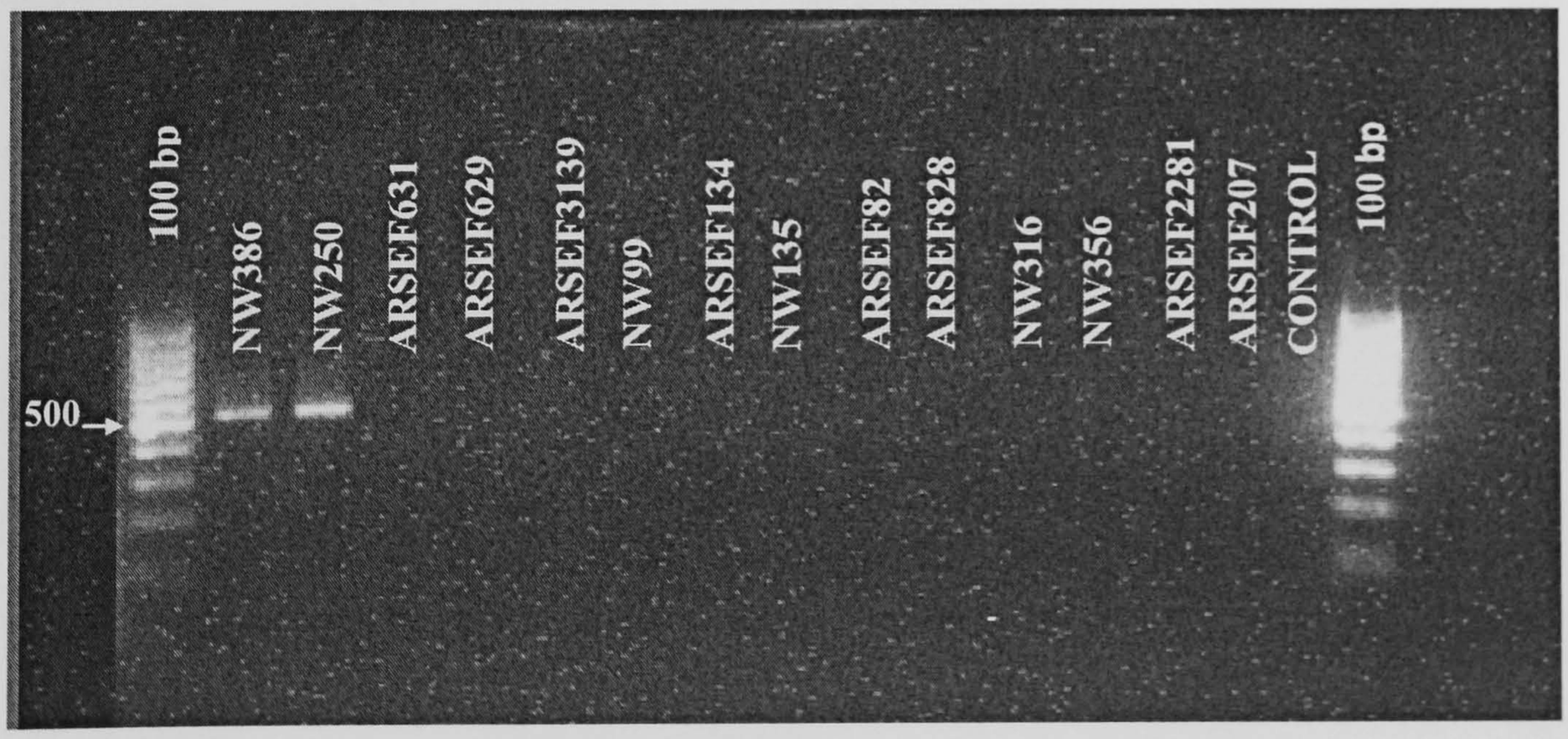


Figure 6.4. Specificity determination of the *Zoophthora radicans* species-specific primers. The primers were tested using DNA extracted from *in vitro* cultures of other entomopathogenic fungal species (Table 6.4). NW386 and NW250 isolates were used as positive controls, and sterile distilled water were used as a negative control. Size marker = 100bp.

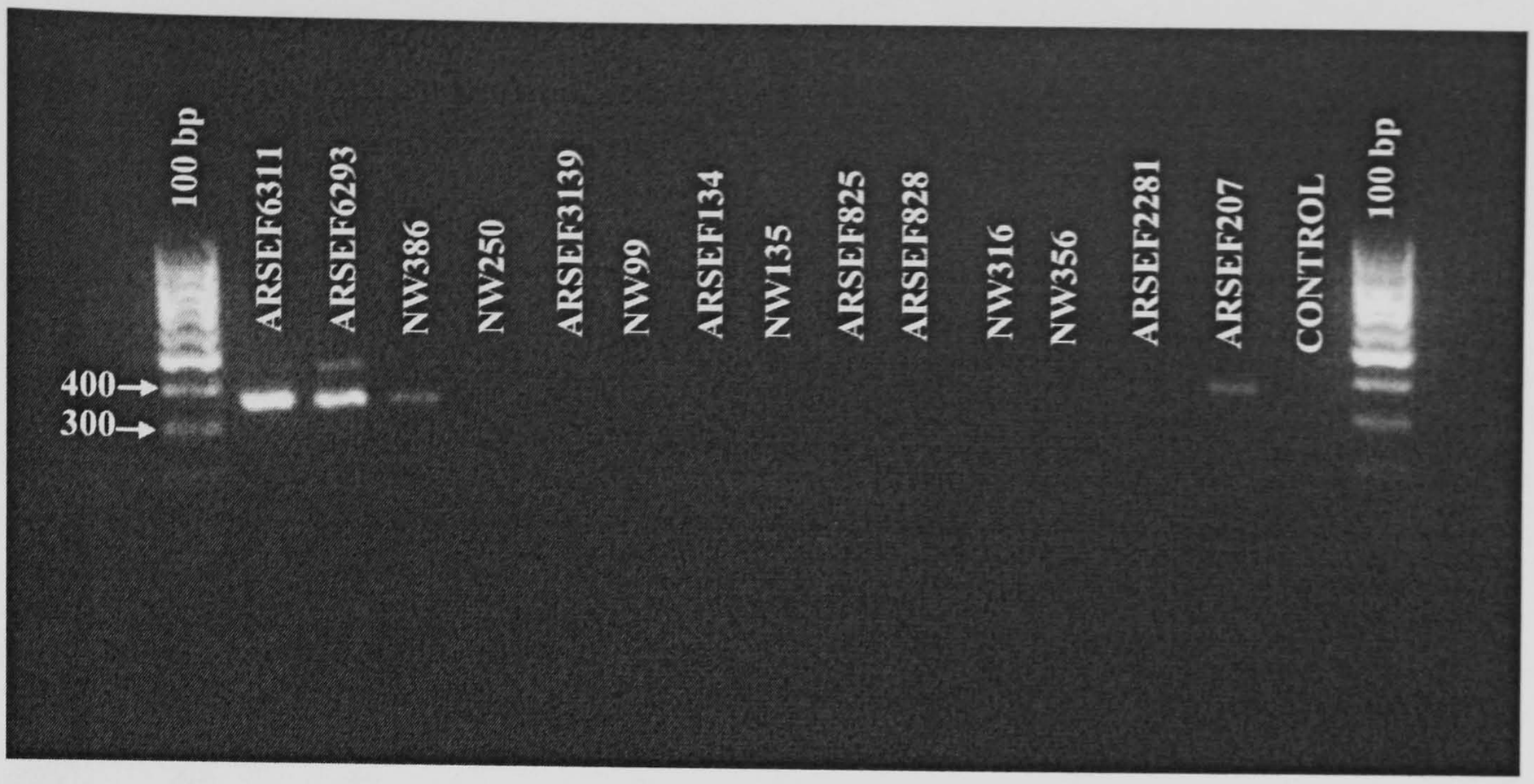


Figure 6.5. Specificity determination of the *Pandora blunckii* species-specific primers. The primers were tested using DNA extracted from *in vitro* cultures of other entomopathogenic fungal species (Table 6.4). ARSEF6311 and ARSEF6293 isolates were used as positive controls, and sterile distilled water were used as a negative control. Size marker = 100bp.

Table 6.4. Isolates of different species tested to confirm specificity of the species-specific primers developed for *Zoophthora radicans* and *Pandora blunckii*. NW isolates came from the Rothamsted Research collection, ARSEF isolates came from the USDA-ARSEF culture collection, curated by Dr. Richard Humber

Isolate code	Species	Country
ARSEF3139	*Conidiobolus obscurus*	USA
NW99	*C. obscurus*	France
ARSEF134	*Pandora delphacis*	Japan
NW135	*P. delphacis*	UK
ARSEF825	*P. kondoiensis*	Australia
ARSEF828	*P. kondoiensis*	Australia
NW316	*P. neoaphidis*	UK
NW356	*P. neoaphidis*	UK
ARSEF2281	*Zoophthora phalloides*	Serbia
ARSEF207	*Z. occidentalis*	USA

6.3.2 Specific detection of *Pandora blunckii* and *Zoophthora radicans* in laboratory infected *Plutella xylostella* larvae using conventional PCR

6.3.2.1. Infection of larvae

No overall percentage mortality could be obtained because the larvae were frozen for DNA extraction before death. The dose for each fungus used to infect the larvae was estimated. Larvae inoculated only with *Z. radicans* (NW378) conidia had an average dose of 1.7 conidia/mm^2. Larvae inoculated only with *P. blunckii* (ARSEF 6293) had an average dose of 170.6 conidia/mm^2. Larvae inoculated with both pathogens received an average dose of 2.5 conidia/mm^2 and 88 conidia/mm^2 of *Z. radicans* and *P. blunckii* respectively.

6.3.2.2 Specific detection of Pandora blunckii *and* Zoophthora radicans *in laboratory infected* Plutella xylostella *larvae using conventional PCR*

The species-specific primers for both species amplified either only *Z. radicans* (Fig. 6.6) or only *P. blunckii* (Fig. 6.7) from DNA extracted from single and dual-inoculated larvae. No product was found from DNA extracted from uninfected larvae (Figs. 6.6 and 6.7).

When the *P. blunckii* species-specific primers were tested, a double band was amplified, the lower band corresponded to the correct size amplified from *P. blunckii* DNA extracted from *in vitro* cultures. Even with the double band, it was still possible to differentiate between both species.

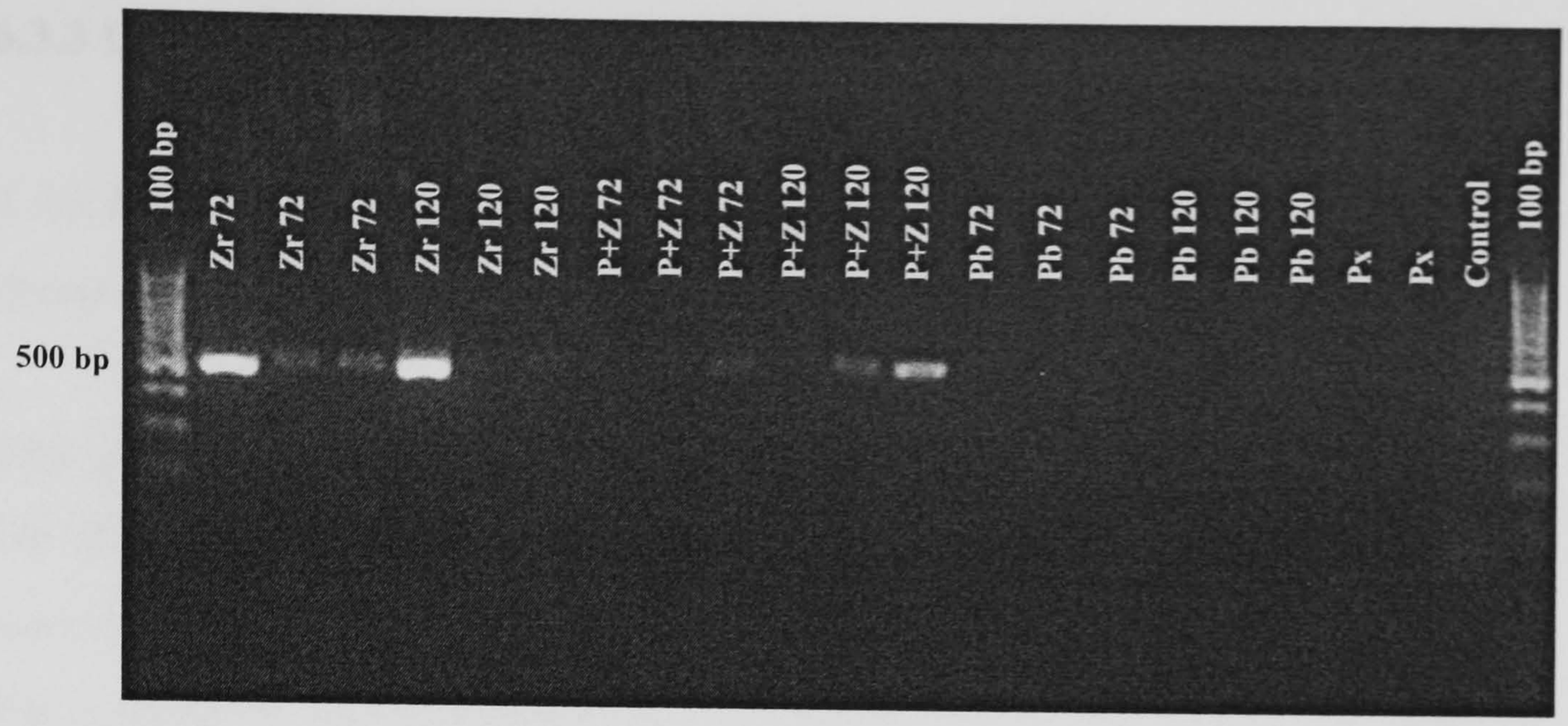


Figure 6.6. Amplification of *Zoophthora radicans* (NW378) using genomic DNA extracted from infected larvae and species-specific primers. Zr = Genomic DNA extracted from *Plutella xylostella* larvae inoculated only with *Zoophtora radicans,* P+Z = Genomic DNA extracted from *Plutella xylostella* larvae dual-inoculated with *Pandora blunckii* and *Zoophthora radicans*, Px = Genomic DNA extracted from *Plutella xylostella* larvae without fungal inoculation. The size product is 490 bp. Size marker = 100bp.

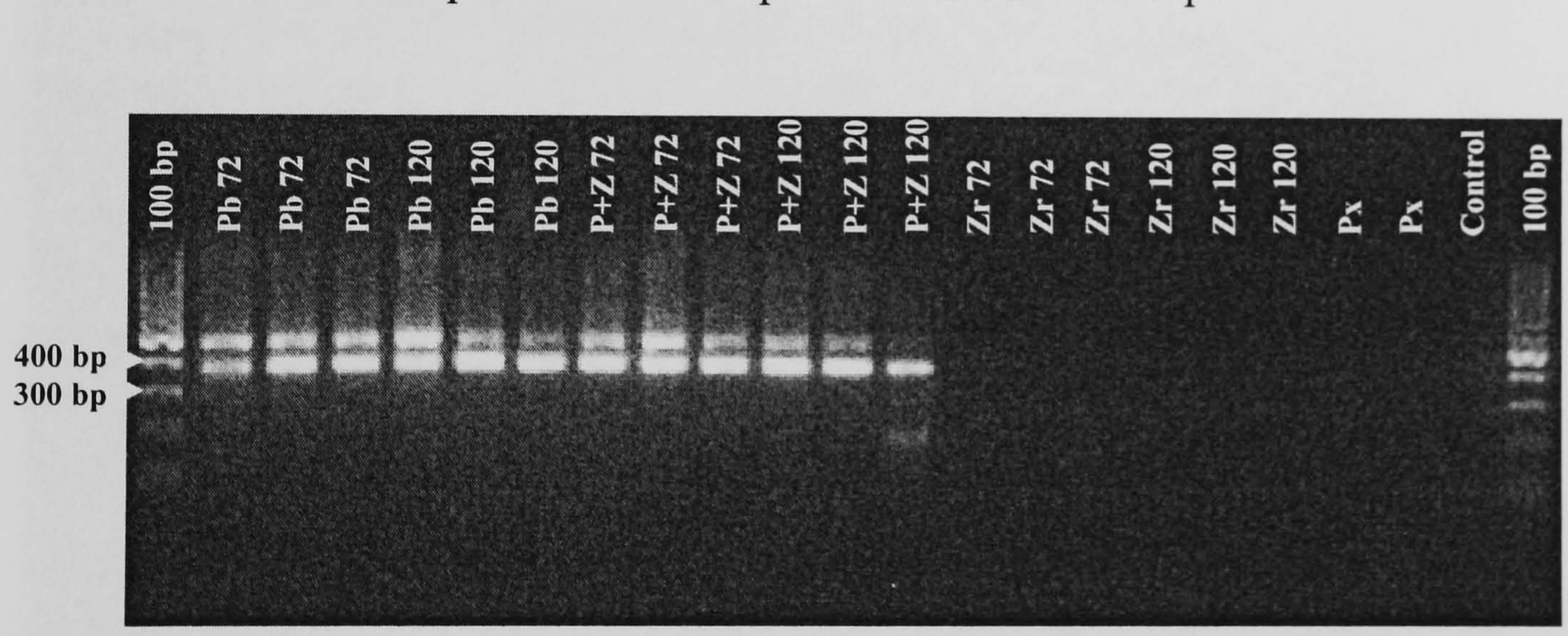


Figure 6.7. Amplification of *Pandora blunckii* (ARSEF6293) using genomic DNA extracted from infected larvae and species-specific primers. Pb = Genomic DNA extracted from *Plutella xylostella* larvae inoculated only with *P. blunckii,* P+Z = Genomic DNA extracted from *Plutella xylostella* larvae dual-inoculated with *Pandora blunckii* and *Zoophthora radicans*, Px = Genomic DNA extracted from *P. xylostella* larvae without fungal inoculation. The size product is 370 bp. Size marker = 100bp.

6.3.3 Development of quantitative real-time PCR assays

6.3.3.1 Primer concentration optimisation for the Zoophthora radicans *species-specific primers*

The gradients with the lowest C_t values were G3 (Fig. 6.8), G6 (Fig. 6.9) and G9 (Fig. 6.10). The primer concentrations for each of these gradients are shown in Table 6.1. There was little difference between these three gradients, but gradient 3 was selected as the optimal primer concentration for further experiments. In the control, some amplification was observed at the end of the PCR reaction (Fig. 6.11). No product was expected in this gradient because no DNA was used. After the melting curve analysis, the product found on the control had a different melting temperature compared to the melting temperature of the amplified product on the other gradients. The melting temperature of the expected PCR product was 74.4 °C, and approximately 72 °C on the unexpected PCR product (Fig. 6.12), suggesting primer dimer production.

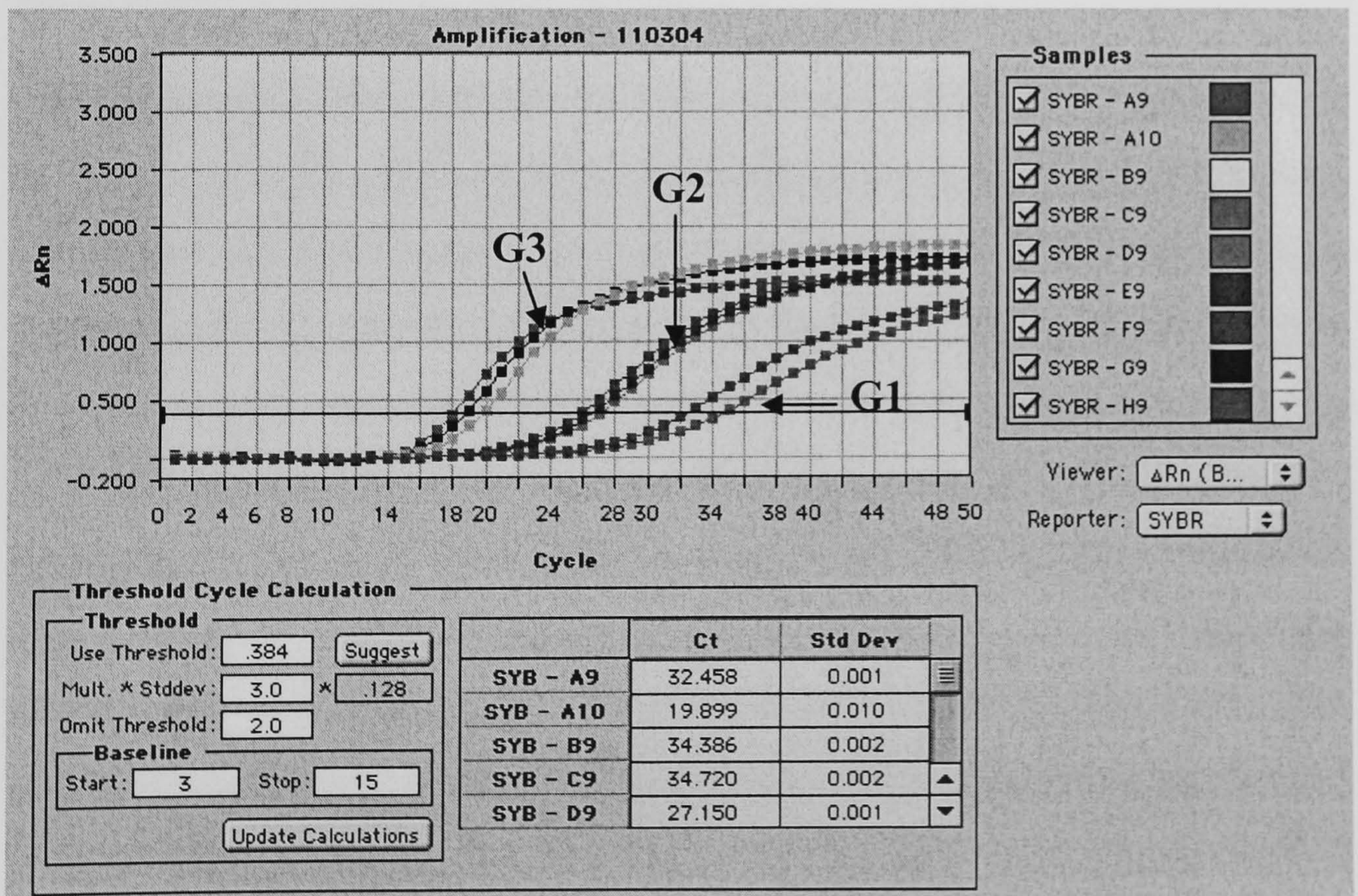


Figure 6.8. Amplification plot showing the C_t values for gradients (G) 1 to 3. Primer concentrations are shown on Table 6.1. The darker horizontal line represents the detection threshold value.

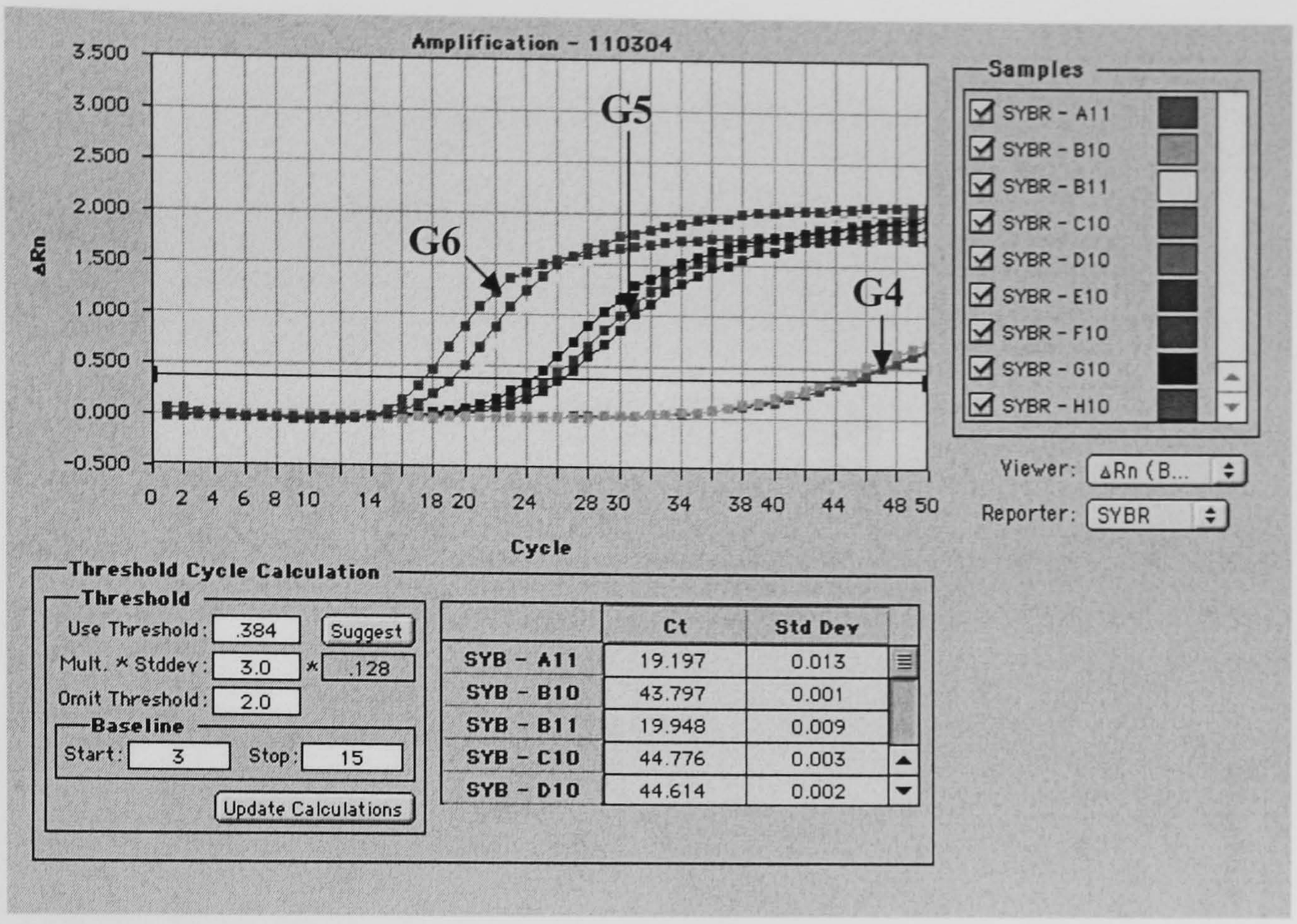


Figure 6.9. Amplification plot showing the C_t values for gradients (G) 4 to 6. Primer concentrations are shown on Table 6.1. The darker horizontal line represents the detection threshold value.

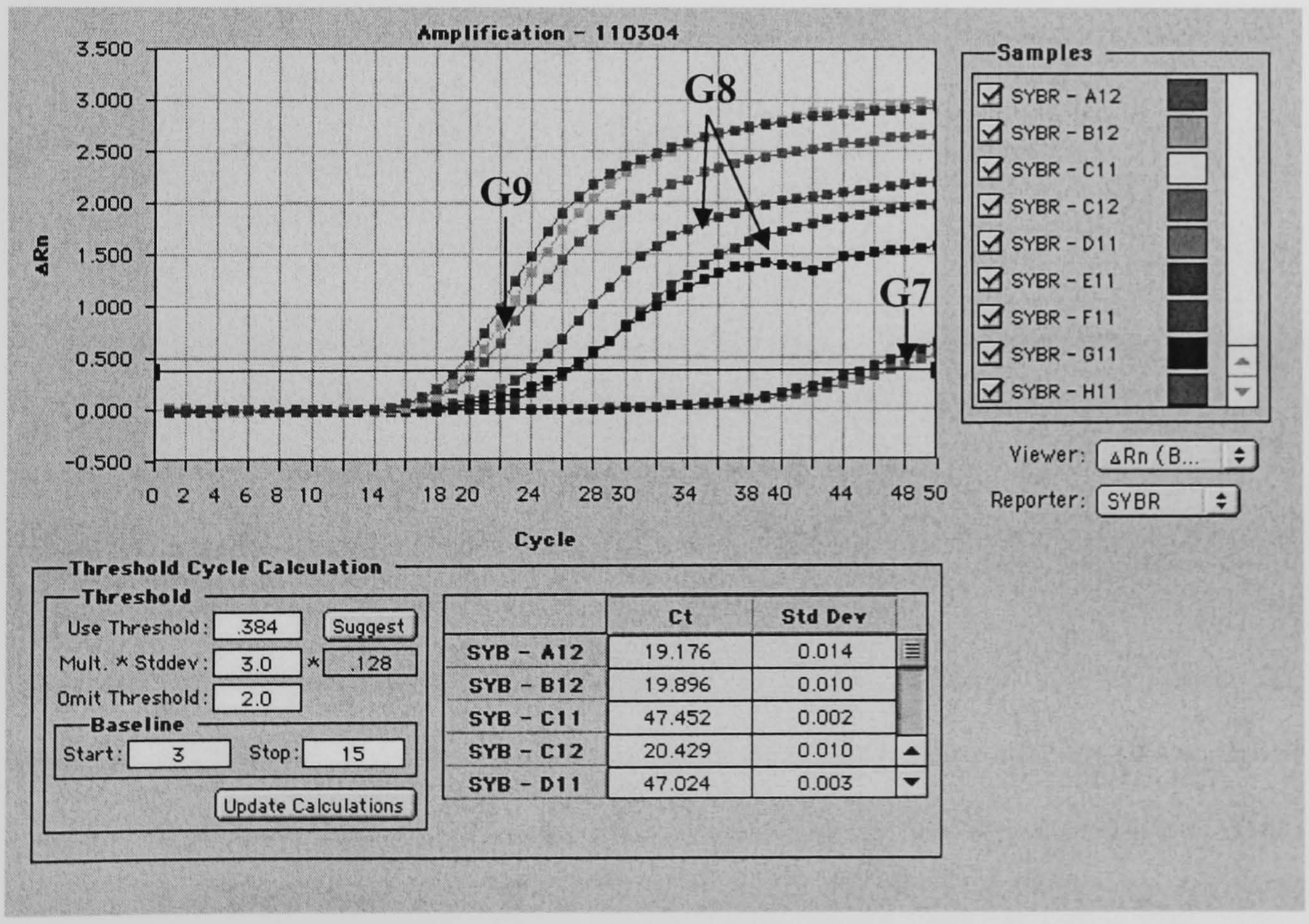


Figure 6.10. Amplification plot showing the C_t values for gradients (G) 7 to 9. Primer concentrations are shown on Table 6.1. The darker horizontal line represents the detection threshold value.

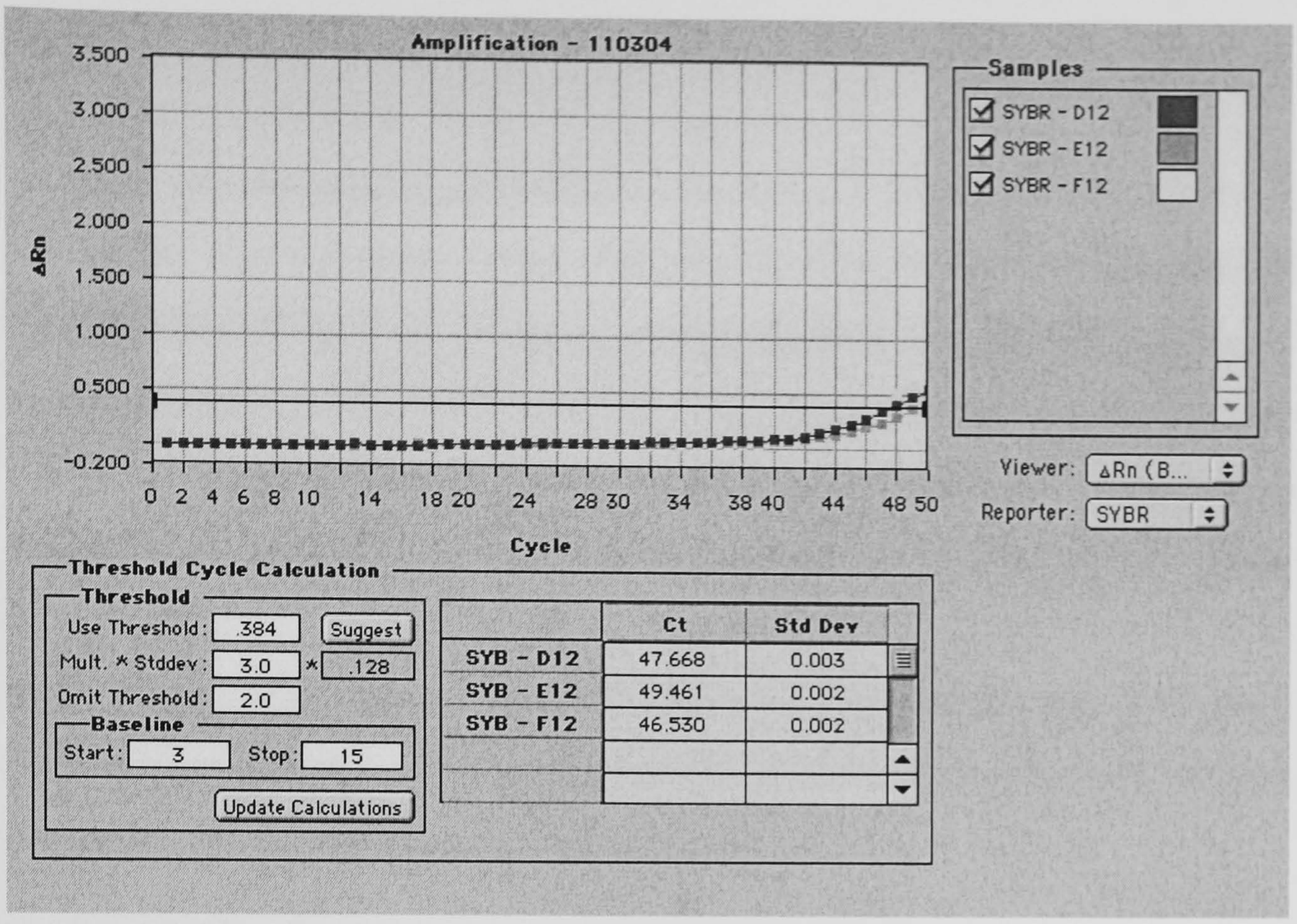


Figure 6.11. Amplification plot shows the C_t value for gradient (G) 10 (no DNA control). Primer concentrations are shown on Table 6.1. The darker horizontal line represents the detection threshold value.

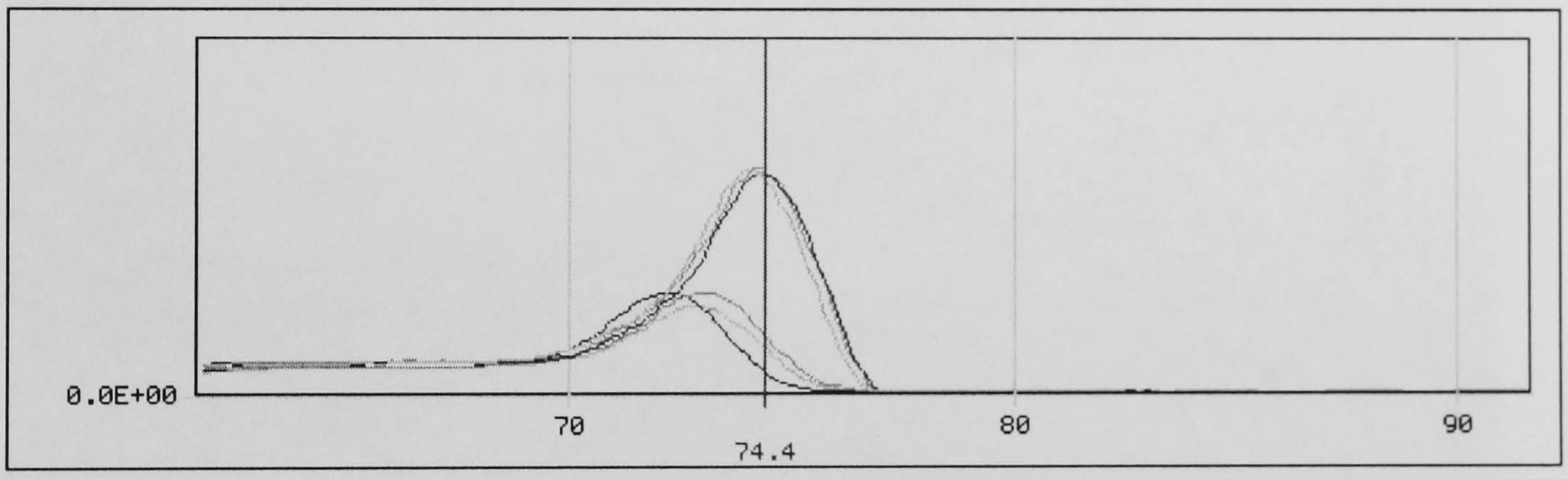


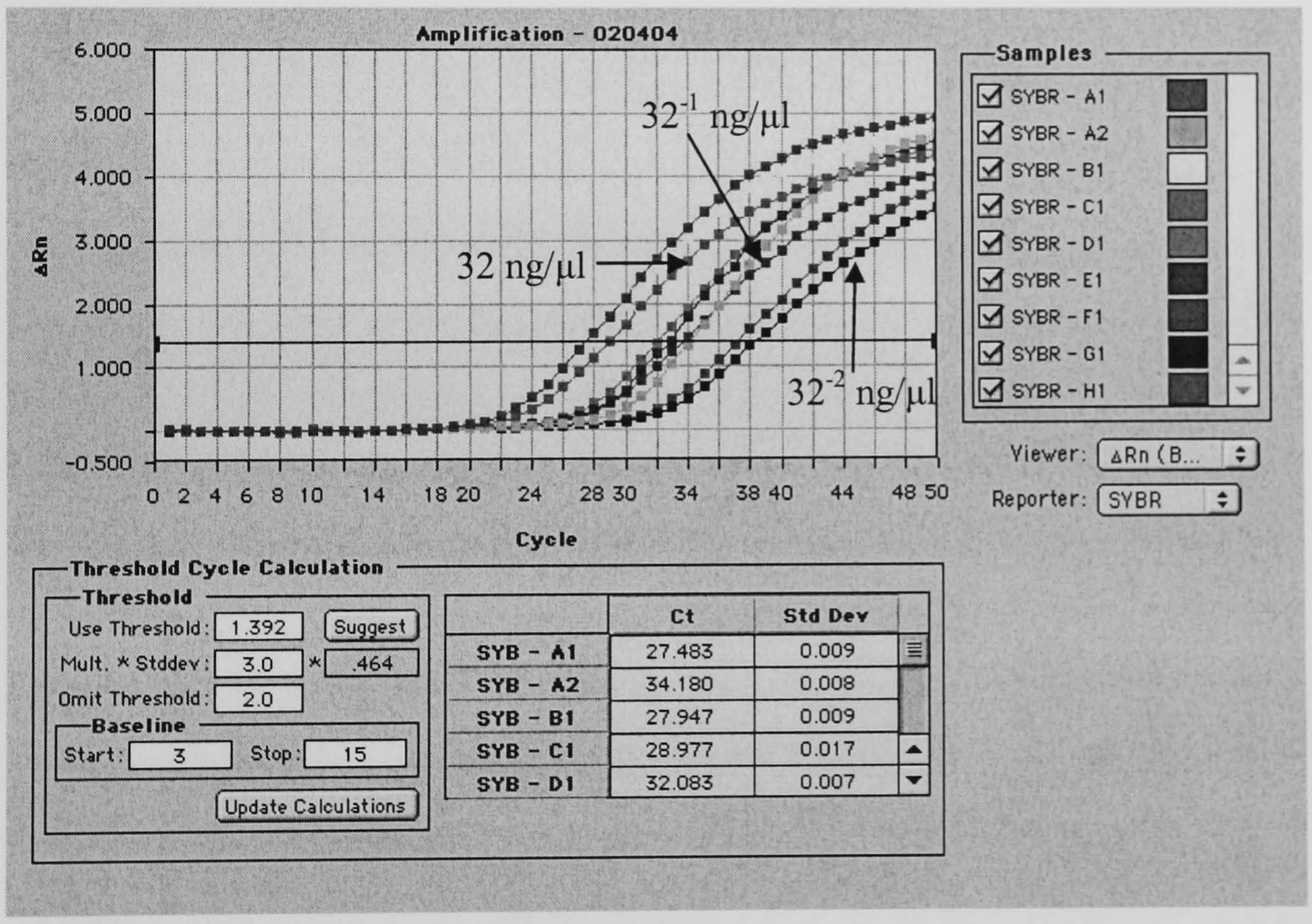
Figure 6.12. Melting temperatures obtained for the expected PCR product (74.4 ˚C), and for the unexpected product in the control (72 ˚C). The curves showing the melting temperature for the expected products were those obtained for G3 as an example.

6.3.3.2 Standard curve

From the 10 serial dilutions of *Z. radicans* DNA used for the standard curve, clear C_t values could be obtained only from 32 ng/µl to 32^{-3} ng/µl (Figs. 6.13 and 6.14). Dilutions corresponding to 32^{-3} and 32^{-4} ng/µl were not easily

separated (Fig. 6.14). No signal was detected for the remaining dilutions (Fig. 6.15).

A signal was detected in all samples containing *Z. radicans* DNA (Table 6.5). No signal was detected in the samples containing only *P. blunckii* DNA (ARSEF6293) (Fig. 6.16, Table 6.5). Amplification was detected in one of the three replicates of the NTC samples, (Fig. 6.17), but melting curve analysis confirmed that the product was a primer-dimer because it had a lower melting temperature compared to the *Z. radicans* samples (Fig. 6.18). Although some amplification was detected in all replicates where only *P. xylostella* DNA was used, in only one replicate was this above the detection threshold (Fig. 6.19). Melting curve analysis confirmed that the products found in the *P. xylostella* samples were primer-dimers (Fig. 6.20).



	Ct	Std Dev
SYB - A1	27.483	0.009
SYB - A2	34.180	0.008
SYB - B1	27.947	0.009
SYB - C1	28.977	0.017
SYB - D1	32.083	0.007

Figure 6.13. Amplification plots obtained with 32, 32^{-1} and 32^{-2} ng/µl of *Zoophthora radicans* DNA. The darker horizontal line represents the detection threshold.

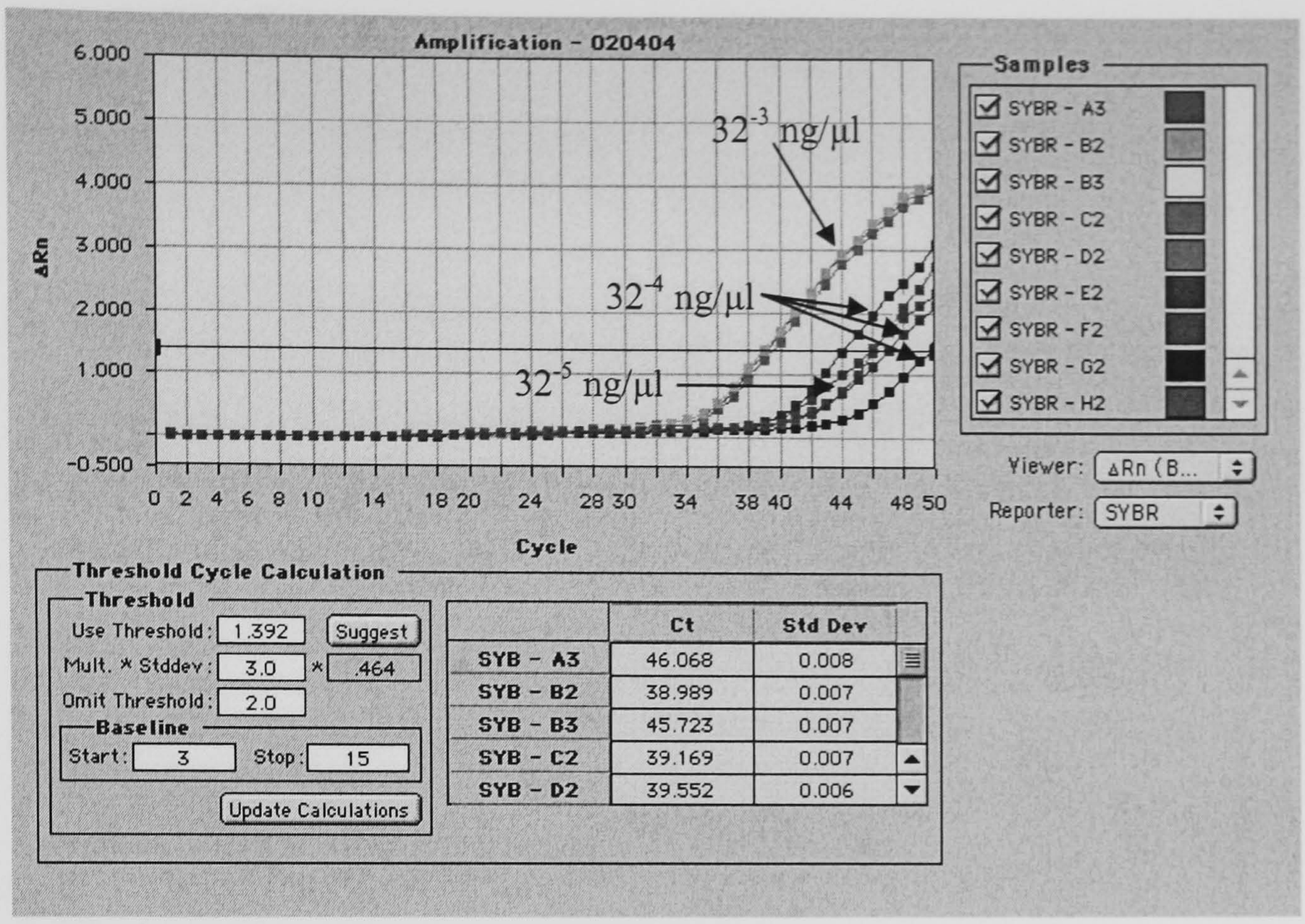


Figure 6.14. Amplification plots obtained with 32^{-3}, 32^{-4} and 32^{-5} ng/µl of *Zoophthora radicans* DNA. The darker horizontal line represents the detection threshold.

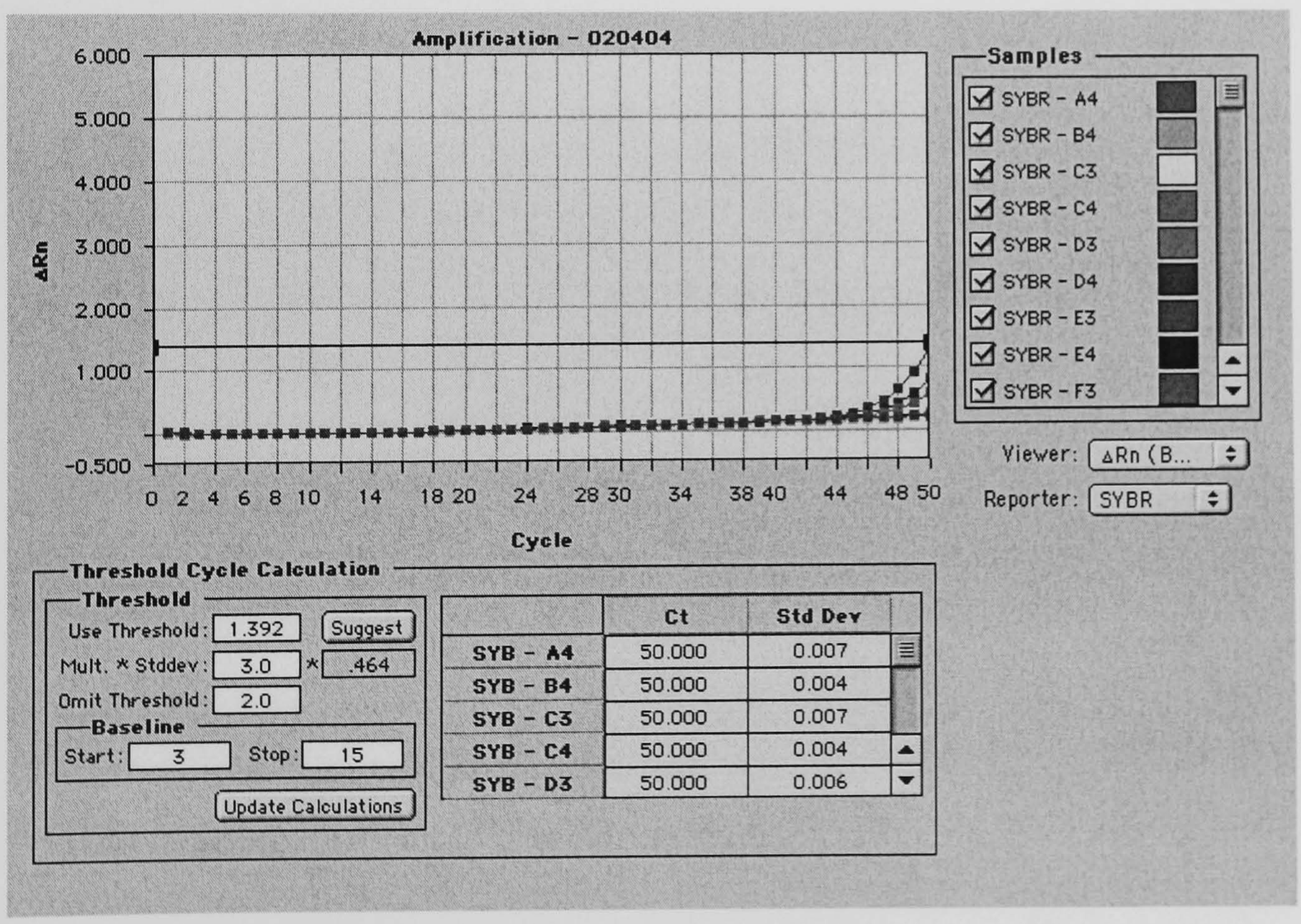


Figure 6.15. Amplification plots obtained with 32^{-6}, 32^{-7}, 32^{-8} and 32^{-9} ng/µl of *Zoophthora radicans* DNA. The darker horizontal line represents the detection threshold.

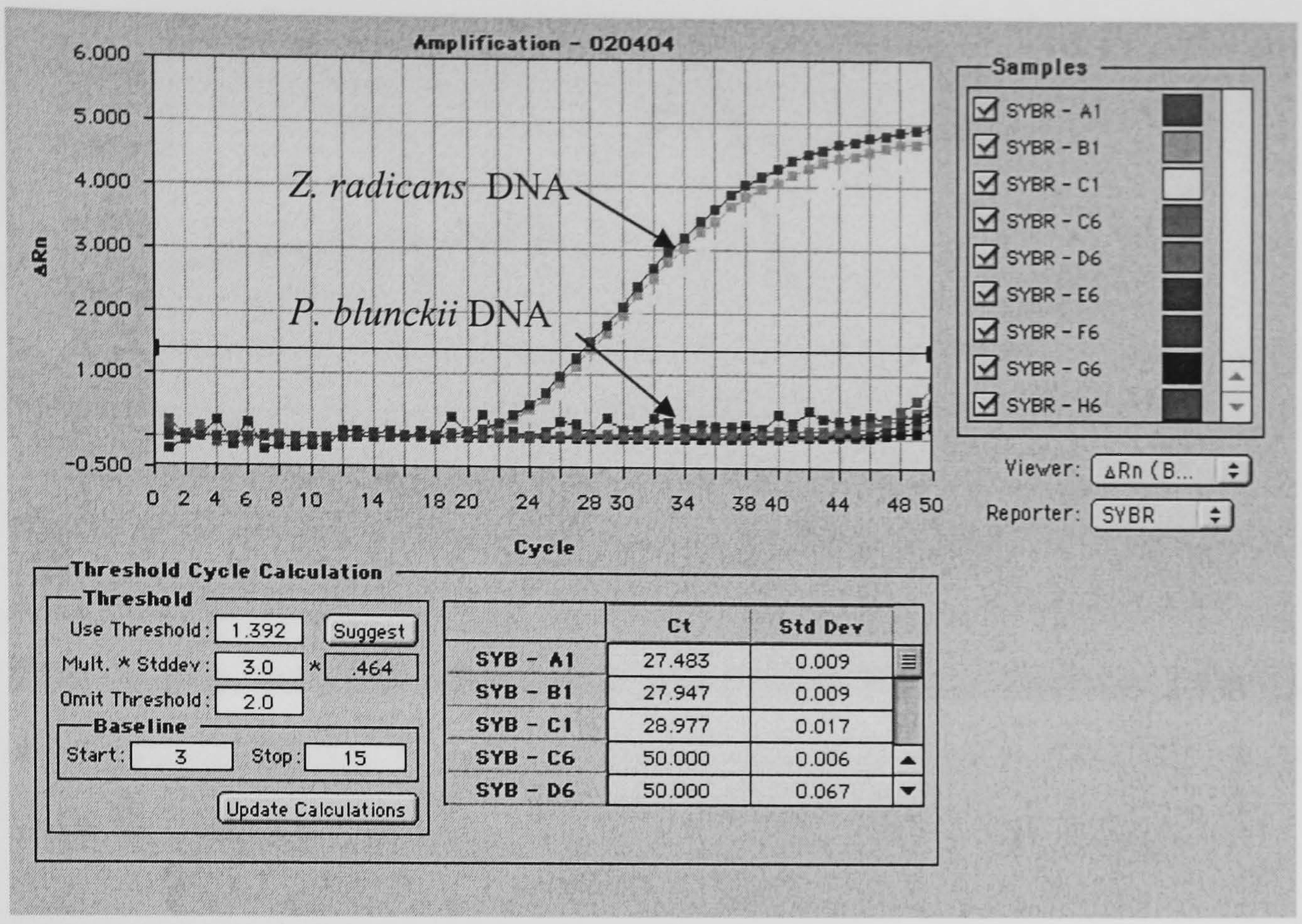


Figure 6.16. Amplification plots obtained with the 32 ng/µl of *Zoophthora radicans* DNA compared to the amplification plots obtained with *Pandora blunckii* DNA. The darker horizontal line represents the detection threshold.

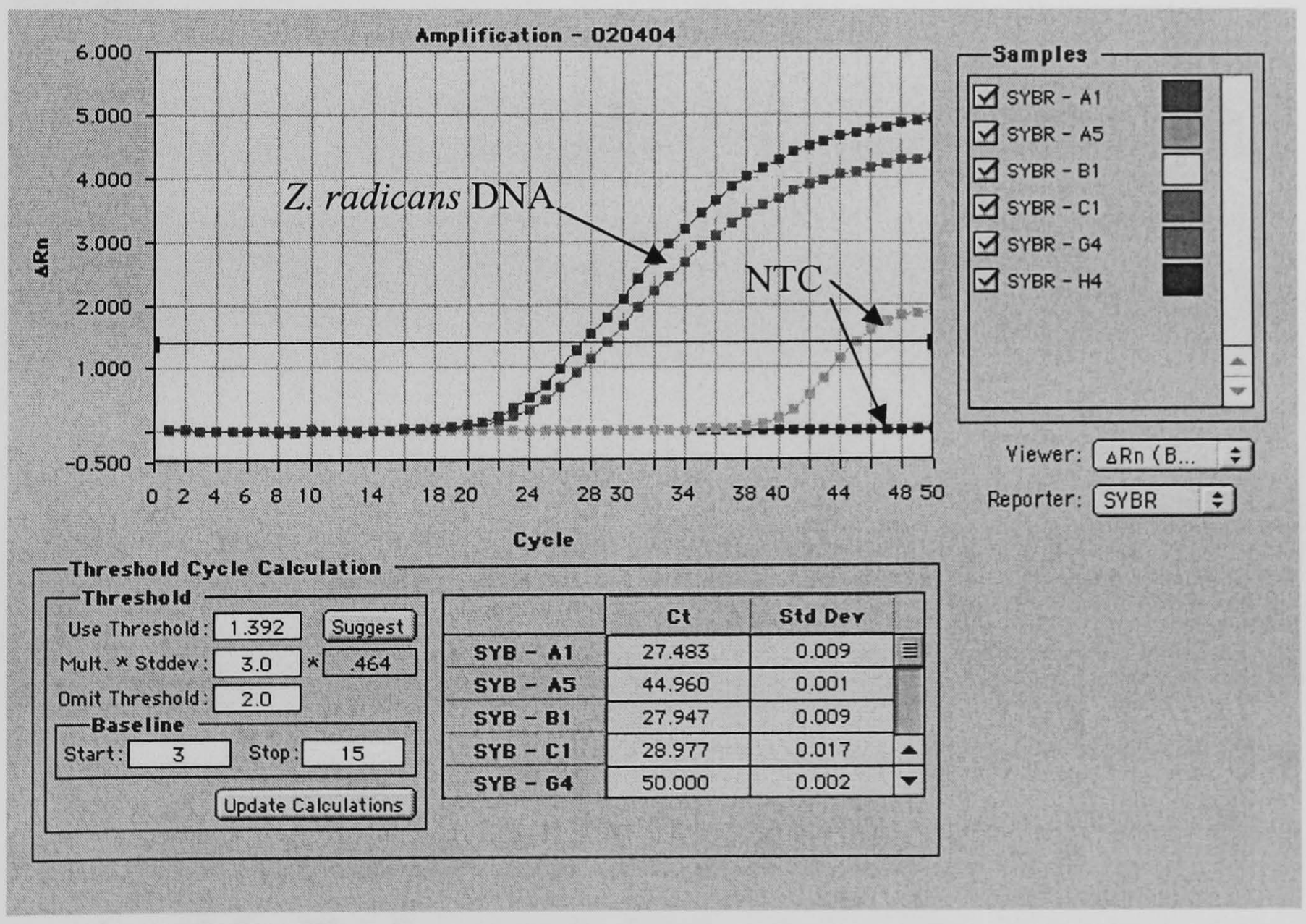


Figure 6.17. Amplification plots obtained with 32 ng/µl of *Zoophthora radicans* DNA compared to the amplifications obtained with the no temple control (NTC). The darker horizontal line represents the detection threshold.

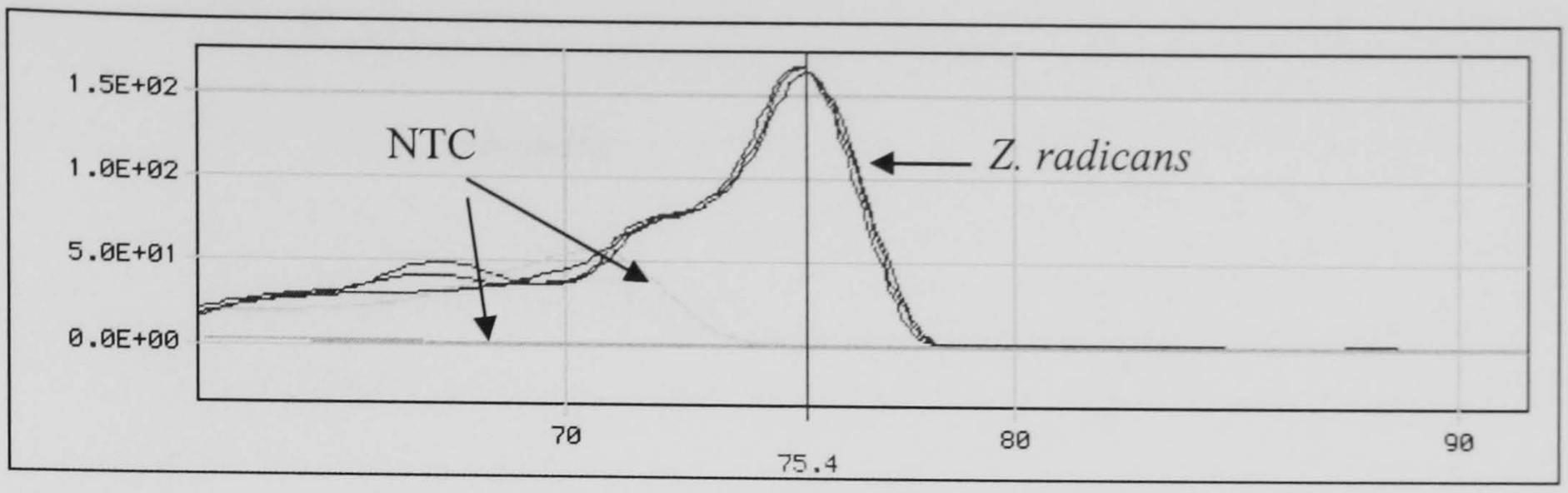


Figure 6.18. Melting curve analysis for the PCR product obtained from the amplification of the 32 ng/µl of *Zoophthora radicans* DNA sample and the PCR product obtained in one replicate of the NTC amplification. The graph shows the differences in melting temperatures confirming that the NTC product is a primer-dimer.

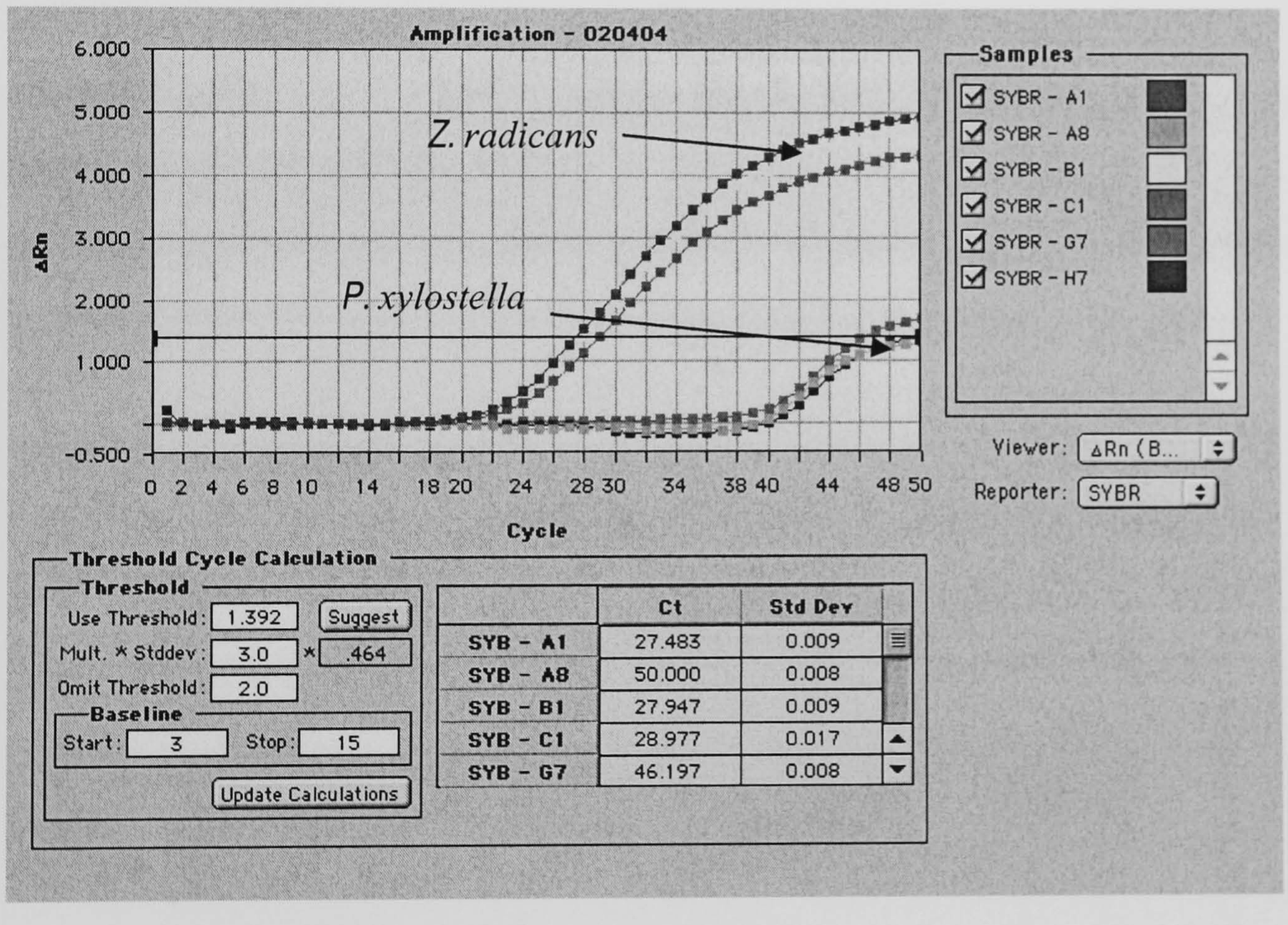


Figure 6.19. Amplification plots obtained with 32 ng/µl of *Z. radicans* DNA sample and the *Plutella xylostella* DNA samples. The darker horizontal line represents the detection threshold.

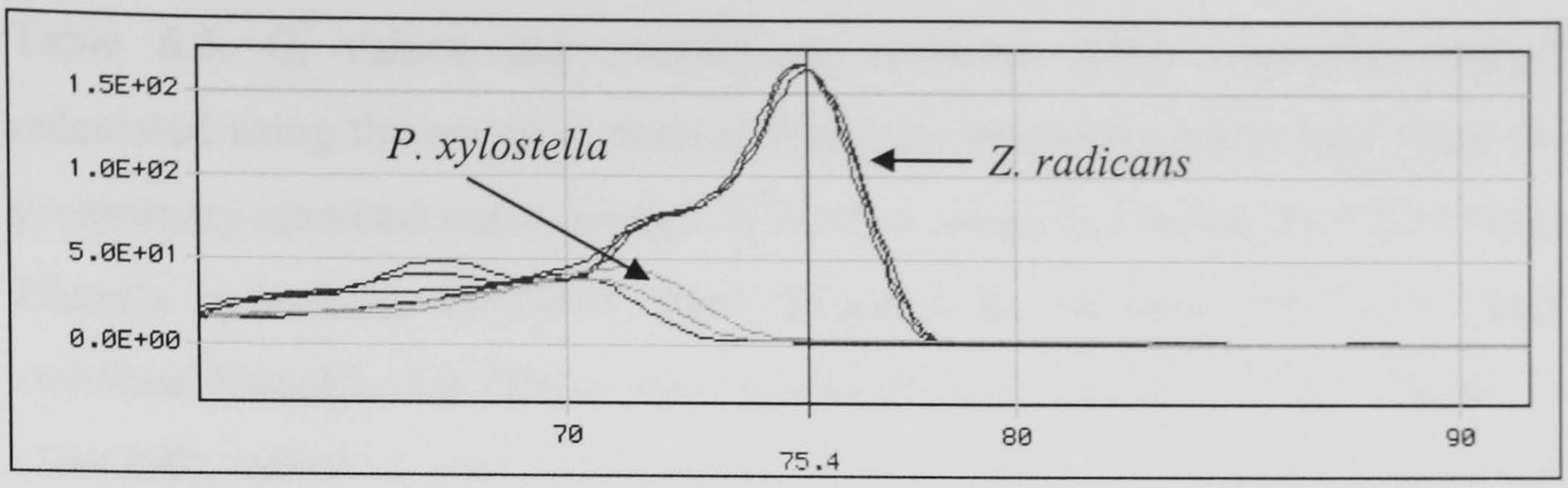


Figure 6.20. Melting curve analysis for the PCR product obtained from the amplification of the 32 ng/µl of *Zoophthora radicans* DNA sample and the PCR product obtained with the *Plutella xylostella* samples amplification. The graph shows the differences in melting temperatures confirming that the PCR product obtained with the *Plutella xylostella* samples were primer-dimers.

Using only the results obtained from dilutions 32 ng/µl to 32^{-4} ng/µl, a regression analysis was performed. The linear correlation coefficient of the standard curve was r^2=0.9274. Using the equation generated by the linear regression (Y=-4.3843(log10X) + 34.584), the unknown quantities where *Z. radicans* DNA was present where estimated (Table 6.5). The same range of dilutions was used for the further real-time PCR assays.

Table 6.5. C_t values and *Zoophthora radicans* DNA quantities (ng/µl) calculated using the equation derived from the regression performed using the preliminary standard curve results. NTC=No template control, Px=DNA from *Plutella xylostella*, Zr=DNA from *Zoophthora radicans*, Pb=DNA from *Pandora blunckii*. All DNAs were extracted from infected larvae, except Zr (NW250), which is pure DNA extracted from an *in vitro* culture which was used as a positive control. Number in brackets represents different larvae, which were run in triplicate. Highlighted rows are samples where no C_t values should have been detected, these values were confirmed through the melting curve analysis that was primer-dimers and not fungal DNA related (Fig. 6.18 and 6.19).

Sample	C_t value	DNA quantity (ng/µl)
NTC	44.96	0.004299
NTC	No C_t	0
NTC	No C_t	0
Zr (NW250)	49.72	3.53E-04
Zr (NW250)	49.71	3.55E-04
Zr (NW250)	37.69	0.195687
Px+Zr (1)	47.18	0.001340
Px+Zr (1)	46.73	0.001697
Px+Zr (1)	47.63	0.001058
Px+Zr (2)	47.2	0.001326
Px+Zr (2)	47.39	0.001200
Px+Zr (2)	48.22	7.76E-04
Px+Pb (1)	No C_t	0
Px+Pb (1)	No C_t	0
Px+Pb (1)	No C_t	0
Px+Pb (2)	No C_t	0
Px+Pb (2)	No C_t	0
Px+Pb (2)	No C_t	0
Px+Pb+Zr (1)	44.1	0.006753
Px+Pb+Zr (1)	45.94	0.002570
Px+Pb+Zr (1)	45.32	0.003558
Px+Pb+Zr (2)	49.47	4.02E-04
Px+Pb+Zr (2)	48.91	0.000540
Px+Pb+Zr (2)	49.94	3.14E-04
Px	No C_t	0
Px	46.2	0.002242
Px	No C_t	0

6.3.4 Use of quantitative real-time PCR assays to investigate the interactions between *Zoophthora radicans* and *Pandora blunckii* during the time course of an infection

6.3.4.1 Total mortality and mortality caused by each species in single and dual- inoculated larvae

In the combination NW250 and ARSEF6293 isolates from *Z. radicans* and *P. blunckii* respectively, the dose of *Z. radicans* conidia applied to treatments one to three were 78, 80 and 82 conidia/mm^2 respectively. One hundred per cent mortality was obtained in all the treatments except the control (Fig. 6.21). In the first treatment, 95% of the larvae were infected by *Z. radicans* as expected, and 5 % were unknown mortality as no indication of any infection was found such as resting spores or external sporulation. In treatments two and three the actual number of insects infected only by *Z. radicans* was drastically reduced suggesting a negative interaction with *P. blunckii*. The reduction in *Z. radicans* infected larvae showed a relationship with dose because in treatment two, where the dose relationship was 80 and 41 conidia/mm^2 for NW250 and ASREF6293 respectively, only 35% of larvae were infected by *Z. radicans*. In treatment three where the dose relationship was 80 and 108 conidia/mm^2 for NW250 and ARSEF6293 respectively, only 5% of the larvae were infected by *Z. radicans*. In treatments two and three, an increased number of larvae infected only with *P. blunckii* was observed and it was, again, according to the dose. A large number of dual-inoculated larvae were present in treatment two.

The treatments where the largest dose of *P. blunckii* was applied (treatments three and five) also showed the largest percentage of unknown mortality (30% in both cases).

In the NW386 and ARSEF6293 isolate combination, similar doses amongst the three batches of 44 larvae inoculated with *Z. radicans* were not be achieved. The doses were 86, 41 and 79 conidia/mm^2 for treatments one to three respectively. Treatment number two obtained the smallest dose of the three treatments, and was not included in the real-time PCR quantification

experiment. Between 90 and 100% mortality was obtained in all the treatments except the control (Fig. 6.22). In the first treatment, 100% of the larvae were infected by *Z. radicans* as expected. In treatments two and three the actual number of insects infected only by *Z. radicans* was reduced suggesting a negative interaction with *P. blunckii*. The reduction in the number of *Z. radicans* infected larvae showed a relationship with dose because in treatment two, where the dose relationship was 41 and 24 conidia/mm^2 for NW386 and ASREF6293 respectively, 65% of larvae were infected by *Z. radicans*. In treatment three where the dose relationship was 79 and 120 conidia/mm^2 for NW386 and ARSEF6293 respectively, only 5% of the larvae were infected by *Z. radicans*. In treatments two and three, the increase in the percentage of *P. blunckii* infected larvae seems to have a relationship with dose, because the larger the dose of *P. blunckii,* the larger the number of larvae infected with *P. blunckii*.

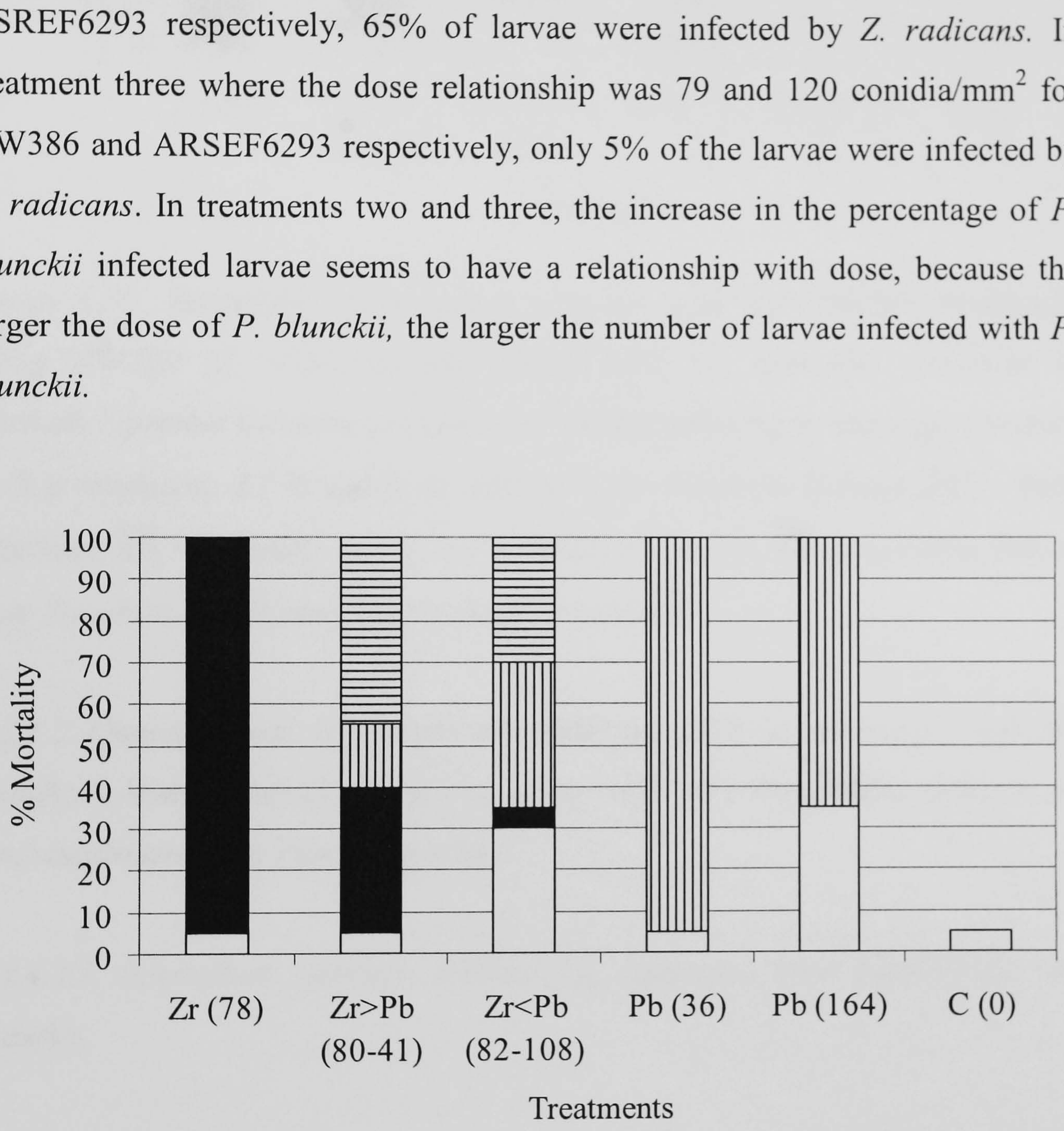


Figure 6.21. Percentage of infection obtained with the different treatments using NW250 (*Z. radicans*) and ARSEF6293 (*P. blunckii*). Numbers in brackets represent the dose (conidia/mm^2) of the pathogen or pathogens used in that treatment. Zr=*Zoophthora radicans*, Pb=*Pandora blunckii*, ▤ = dual-infection, ▥ = mortality due to only *Pandora blunckii*, ■ = mortality due to only *Zoophthora radicans*, □ = unknown mortality.

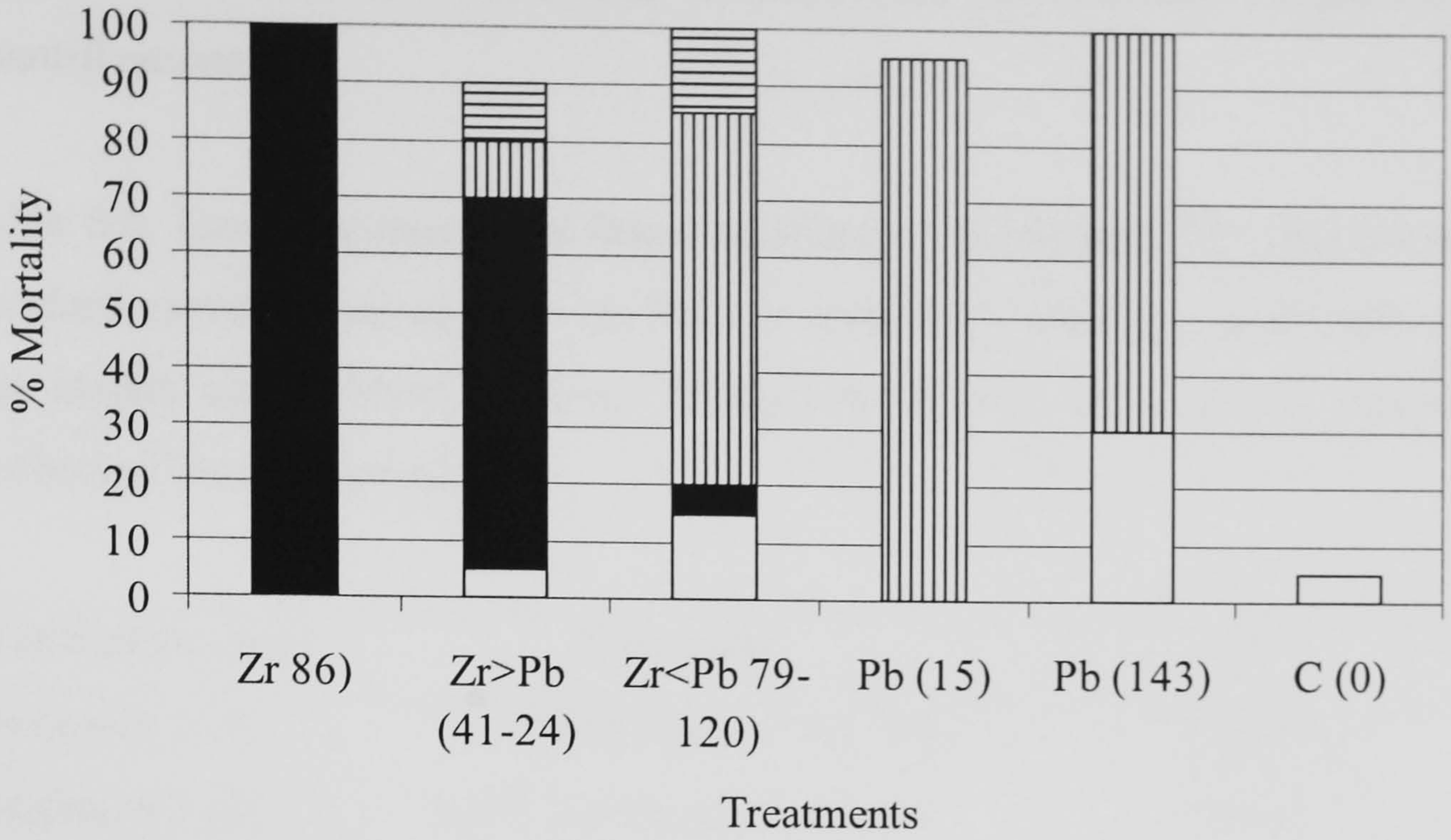


Figure 6.22. Percentage of infection obtained with the different treatments using NW386 (*Z. radicans*) and ARSEF6293 (*P. blunckii*). Numbers in brackets represent the dose (conidia/mm^2) of the pathogen or pathogens present in that treatment. Zr=*Zoophthora radicans*, Pb=*Pandora blunckii*, ▤ = dual-infection, ▥ = mortality due to only *Pandora blunckii*, ■ = mortality due to only *Zoophthora radicans*, □ = unknown mortality.

6.3.4.2 Quantification of Zoophthora radicans *DNA in laboratory infected* Plutella xylostella *larvae inoculated either with only* Zoophthora radicans *or dual-inoculated with* Pandora blunckii

6.3.4.2.1 Interaction between NW250 (Z. radicans) *and ARSEF6293* (P. blunckii

The similarity in dose amongst treatments one to three allowed for a valid statistical comparison (6.3.4.1). As infection in single and dual-inoculated treatments was successfully achieved in all the treatments except in the control (Fig.6.21), this confirmed that the DNA extracted from the 24 larvae subgroup from all the treatments contained DNA from both pathogens.

The linear correlation coefficients of the standard curves for each real-time PCR reaction performed for this combination are summarised in Table 6.6. The

high values of the R^2 coefficients demonstrated the accuracy of the PCR quantifications.

Table 6.6. Linear equation and linear correlation coefficient (R^2) from all the standard curves obtained from all the real-time PCR reactions performed for this isolate combination. Number in brackets showed the replicate number performed for each treatment.

Treatment (rep)	Equation	R^2
Treatment 1 (1)	Y=-6.8900log(x) + 35.762	0.9867
Treatment 1 (2)	Y=-7.5075log(x) + 34.434	0.9828
Treatment 2 (1)	Y=-5.9075log(x) + 36.168	0.9498
Treatment 2 (2)	Y=-5.3700log(x) + 35.776	0.9878
Treatment 3 (1)	Y=-6.4575log(x) + 36.219	0.9960
Treatment 3 (2)	Y=-6.6400log(x) + 34.614	0.9858

Significant differences were found in the quantities of *Z. radicans* DNA in infected *P. xylostella* larvae amongst the three treatments on days 0 ($F_{2,8}$=12.83, P=0.003), 2 ($F_{2,9}$=6.61, P=0.017) and 4 ($F_{2,9}$=30.76, P=<0.001). No significant differences were found on days 1 ($F_{2,8}$=0.68, P=0.532), 3 ($F_{2,9}$=0.49, P=0.627) and 5 ($F_{2,6}$=1.80, P=0.244). Different trends in the quantities of *Z. radicans* DNA in single (treatment one) and dual-inoculated with *P. blunckii* (treatments two and three) *P. xylostella* larvae during the infection course were observed (Fig. 6.23). In treatment one, the quantities of *Z. radicans* DNA showed a gradual increase through time, with a slight decrease on day 5. In treatment two, an increase was observed until day four, but on day five a drastic decrease of fungal DNA was observed. In treatment three an increase in DNA was observed until day three and after that the quantities decreased drastically.

On day three, treatments two and three showed a greater quantity of *Z. radicans* DNA than in treatment one on the same day. On day four, only treatment two had more DNA than treatment one (Fig. 6.23).

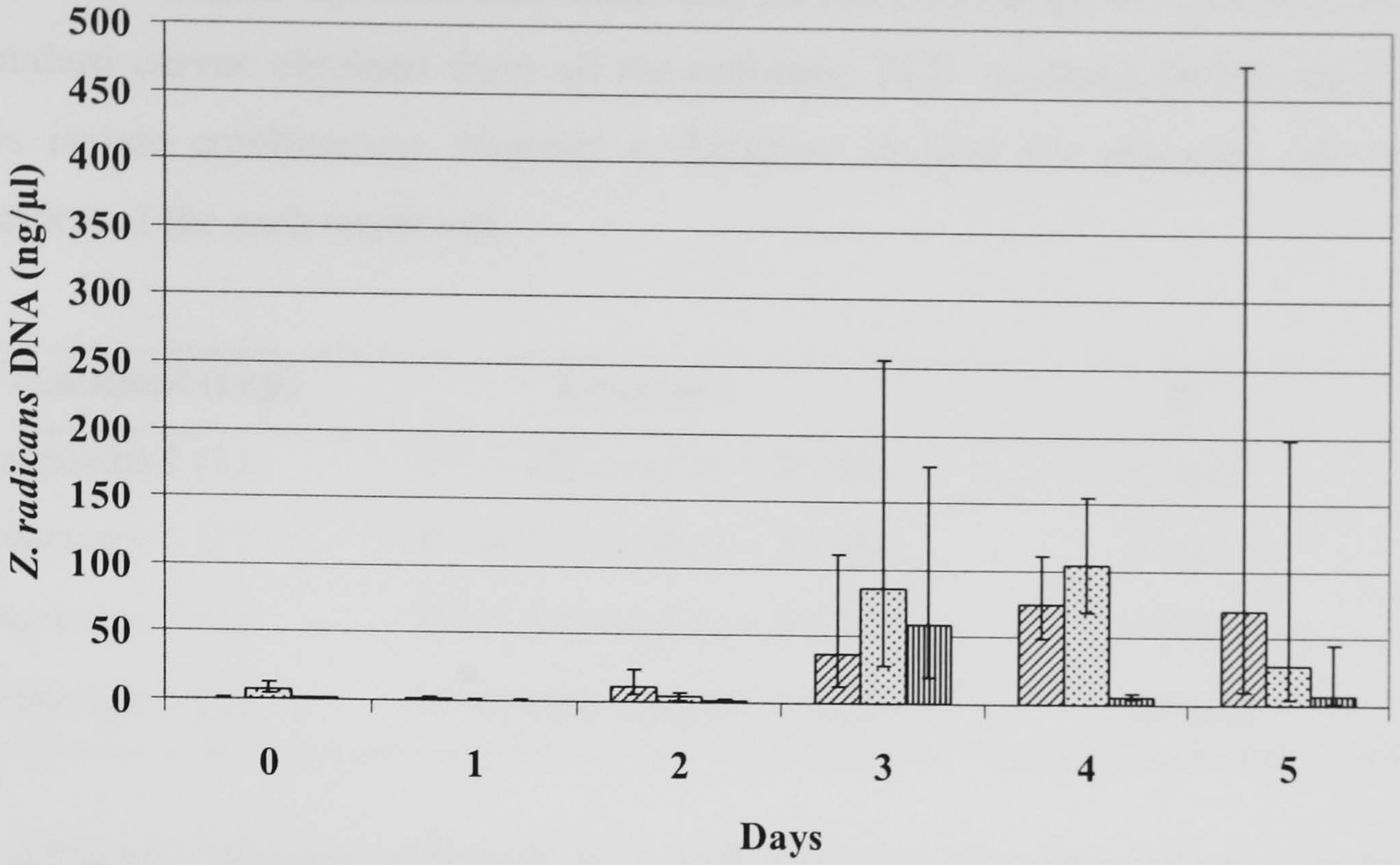


Figure 6.23. Changes in quantities of *Zoophthora radicans* DNA (NW250) in single (T1 ▨) and dual-inoculated with *Pandora blunckii* (T2 ▩ and T3 ▥) larvae (*P. xylostella*) during the time course of infection. Error bars represent Confidence Intervals (CI) 95% back transformed from log10.

6.3.4.2.2 Interaction between NW386 (Z. radicans) *and ARSEF6293* (P. blunckii)

As similar doses of *Z. radicans* was obtained only for treatments one and three, statistical analyses were only carried out for these two treatments (6.3.4.1). Infection in single and dual-inoculated treatments was observed but not in the control (Fig. 6.22), which confirmed that the DNA extracted from the 24 larvae subgroup from all the treatments contained DNA from both species of fungus.

The linear correlation coefficients of the standard curves for each real-time PCR reaction performed for this combination are summarised in Table 6.7. The high values of the R^2 coefficients demonstrated the accuracy of the PCR quantifications.

Table 6.7. Linear equation and linear correlation coefficient (R^2) from all the standard curves obtained from all the real-time PCR reactions performed for this isolate combination. Number in brackets showed the replicate number performed for each treatment.

Treatment (rep)	Equation	R^2
Treatment 1 (1)	Y=-7.4625log(x) + 34.843	0.9601
Treatment 1 (2)	Y=-5.9130log(x) + 20.501	0.9889
Treatment 3 (1)	Y=-7.4675log(x) + 34.759	0.9652
Treatment 3 (2)	Y=-6.6485log(x) + 31.865	0.9623

A difference between quantities of *Z. radicans* DNA was detected only on day two ($F_{1,6}$=5.98, P=0.050), the probability is not highly significant, which suggests that if this experiment is repeated under the same conditions, the mean quantities of *Z. radicans* DNA might not be different. No significant differences were found between quantities of *Z. radicans* DNA on the other sample days, day zero ($F_{1,6}$=0.28, P=0.615), day one ($F_{1,5}$=0.29, P=0.611), day three ($F_{1,4}$=0.60, P=0.482), day four ($F_{1,6}$=1.63, P=0.249) and day five ($F_{1,4}$=1.08, P=0.357).

The trends in the quantities of *Z. radicans* DNA in both treatments were similar. In treatment one, there was an increase in the quantity of DNA until day four, when a slight decrease was recorded. In treatment three, there was an increase in the quantity of *Z. radicans* DNA until day three, but after that, the quantity of DNA decreased (Fig. 6.24). On day two, where a difference in the quantities of *Z. radicans* DNA amongst treatments was found, the infected larvae had a larger quantity of *Z. radicans* DNA in treatment three than in treatment one (Fig. 6.25).

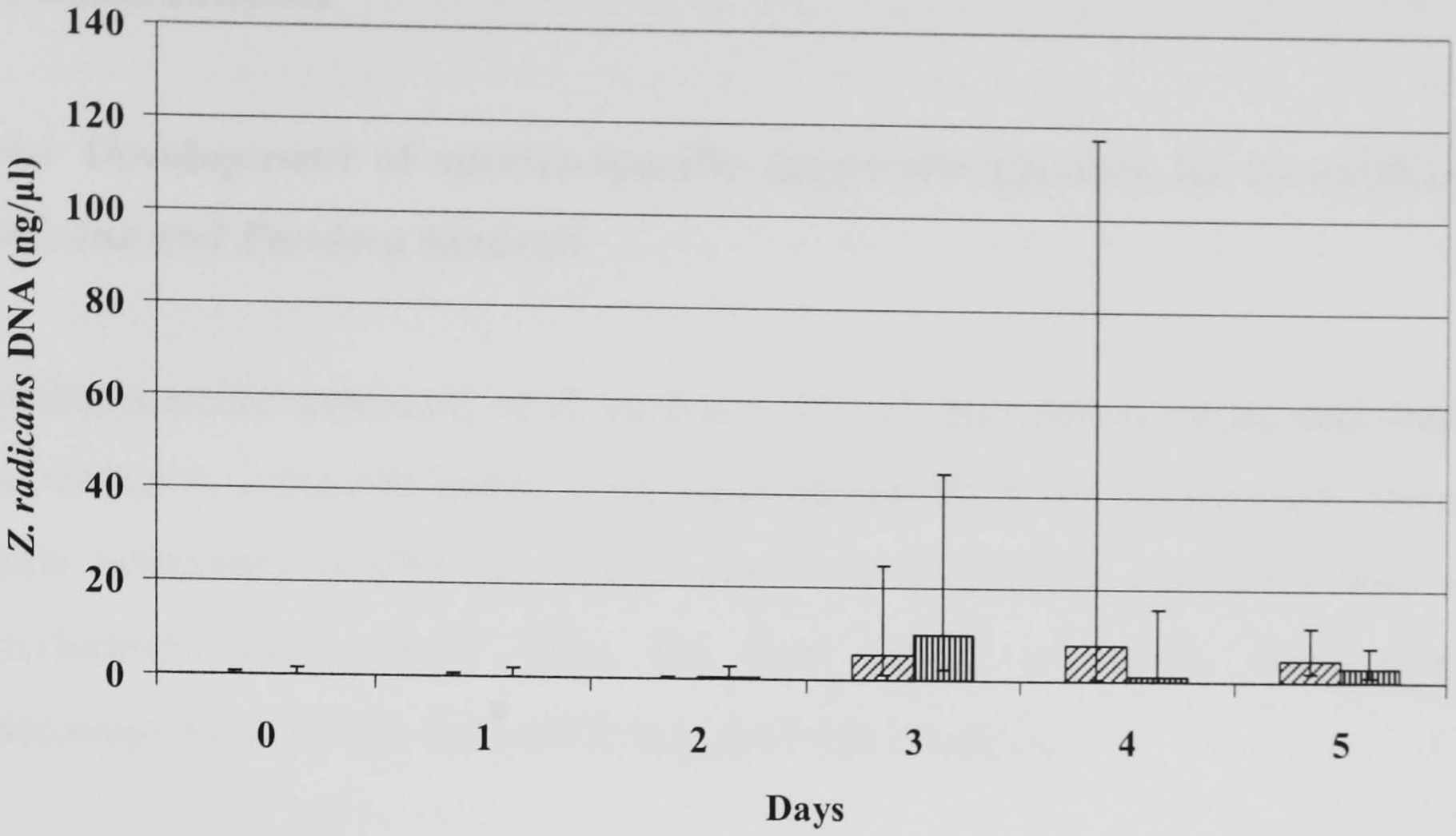


Figure 6.24. Changes in quantities of *Zoophthora radicans* DNA (NW386) in single (T1 ▨) and dual-inoculated with *Pandora blunckii* (T3 ▥) larvae (*P. xylostella*) during a time course infection. Error bars represent Confidence Intervals (CI) 95% back transformed from log10.

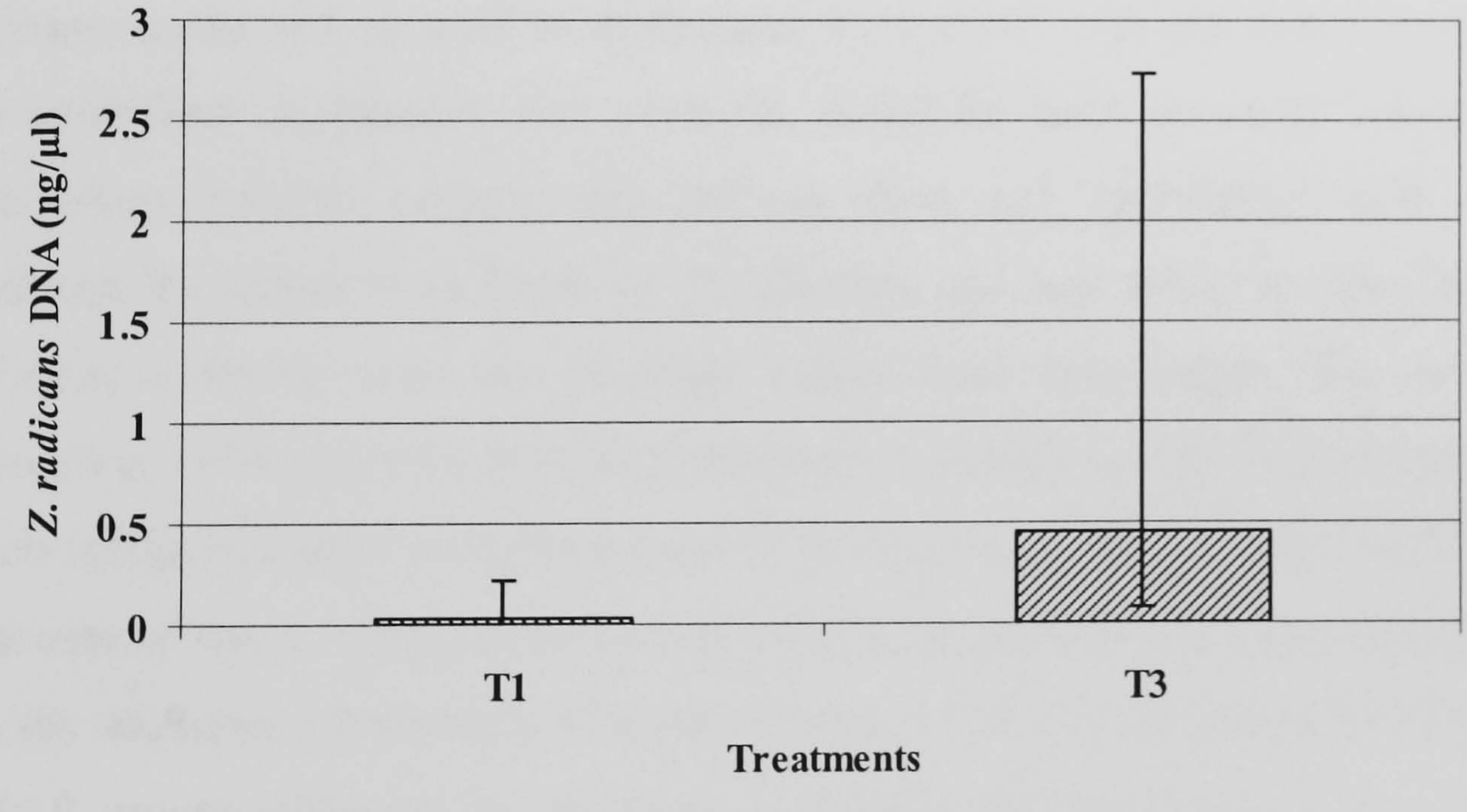


Figure 6.25. Differences in quantities of *Zoophthora radicans* DNA between larvae infected only with the *Zoophthora radicans* isolate NW386 (T1), and larvae dual-inoculated with *Pandora blunckii* conidia (T3). In treatment three, more conidia of *Pandora blunckii* than *Zoophthora radicans* were inoculated. The quantities were measured two days after inoculation. The error bars represent the Confidence Intervals (CI) 95% back transformed from log10.

6.4 DISCUSSION

6.4.1 Development of species-specific diagnostic primers for *Zoophthora radicans* and *Pandora blunckii*

Species-specific detection of *Z. radicans* and *P. blunckii* in single and dual-inoculated *P. xylostella* larvae using conventional PCR was successfully tested under laboratory conditions. It was important to confirm that there was no interference from DNA from the host insect or DNA from other microorganisms within the host during the PCR reaction.

The specificity of both species-specific primers was also tested against other related fungal pathogens. The *Z. radicans* species-specific primers amplified only isolates from this species and could therefore be used to detect this fungus in the field in cadavers or as conidia/resting spores in the environment (e.g. soil/foliage). However, the *P. blunckii* species-specific primers seemed to be less specific because some faint bands were produced when DNA from other related fungal species was used. In spite of this reduced specificity, these primers could still be used to distinguish between *P. blunckii* and *Z. radicans* in controlled conditions. For example, could be used to verify whether a laboratory infected cadaver that did not show any sporulation, was really infected by either *Z. radicans* or *P. blunckii* and just failed to sporulate or whether mortality was due to other causes than this fungus. The reduced specificity of the species-specific primers of *P. blunckii* might be because these were designed based on information from only one isolate (ARSEF6293). In the case of the *Z. radicans* species-specific primers, these were designed based on the sequence information of seven isolates, which also belonged to different RFLP groups (Chapter 4). As there is genetic variability amongst different isolates of the same fungal species (e.g. Maurer *et al.*, 1997; Fargues *et al.*, 2002; Tymon *et al.*, 2004; Tymon and Pell, 2005), in order to design better species-specific primers, the sequence information from more than one isolate must be considered.

The species-specific primers of *Z. radicans* and *P. blunckii* were specific enough to distinguish between both species under controlled conditions, as confirmed in 6.3.2.2. However, currently only the *Z. radicans* species-specific primers have the potential to be used in the field for detecting and monitoring this species. These primers were designed based on the ITS region which is of high copy number within the genome (Voet and Voet, 1995; Bridge, 2002) and, therefore, it is possible to detect even low levels of the fungus. The *P. blunckii* species-specific primers need to be redesigned based on sequence information from more isolates.

6.4.2 Use of quantitative real-time PCR assay to investigate the interactions between *Zoophthora radicans* and *Pandora blunckii* during the time course of an infection

Only the *Z. radicans* species-specific primers could be optimised for real-time PCR. The *P. blunckii* species-specific primers produced primer-dimers making the quantification very difficult (data not shown). This might be because it was not 100% species-specific to *P. blunckii* as already discussed. As SYBR® Green I assays were selected, and this dye is specific to any double stranded DNA (Morrison *et al.*, 1998), the production of primer-dimers might lead to false fluorescence detection unrelated to the presence of *P. blunckii* DNA. The use of strand-specific chemistry methods such as TaqMan®, would have helped to optimise both sets of primers, as this method has a double specificity level, firstly, the primers themselves and secondly, the dual-labelled probe (Heid *et al.*, 1996). However, the fact that the experiments were carried out with isolates of different sequence (*Z. radicans* isolates, Chapter 4) makes it very difficult to design a dual-labelled probe that would work with all the isolates (Lee *et al.*, 2004), and so SYBR® Green I was more suitable for this experiment and for *Z. radicans* particularly. A major advantage of this variability between the *Z. radicans* isolates is the potential to design isolate-specific primers for conventional PCR as well as TaqMan® assay, which would be ideal to investigate intra-specific interactions. This was not carried out during this research, but would be an interesting future study.

Two combinations of isolates were used, the *Z. radicans* isolates NW250 and NW386 against the *P. blunckii* isolate ARSEF6293. The *Z. radicans* isolates had different biological attributes; NW250 was more virulent than NW386; and only NW386 produced resting spores. In the *in vitro* interactions experiment, ARSEF6293 affected the *in vitro* growth of NW250 but not NW386. Isolate ARSEF6293 had a similar virulence to NW250 but was more virulent than NW386. Also, isolate ARSEF6293 normally produced cadavers with either ephemeral external sporulation or no sporulation at all (Chapter 5).

When growth of both *Z. radicans* isolates on single inoculated larvae was compared, although this cannot be done statistically, the results suggested that NW250 produced more fungal biomass than NW386. The greatest DNA production for NW250 was on day four (73.9 ngDNA/µl). For NW386 the greatest production was also on day four but only reached 7.9 ngDNA/µl. One possible reason for this may be because NW386 produced resting spores. It may be more difficult to extract DNA from resting spores due to the thick double wall (Pell *et al.*, 2001). However, it is more likely that the result is because NW250 always had greater *in vitro* growth than NW386 (Chapter 2), and that these differences in growth rate were maintained when growing within the host. This would allow NW250 to attain greater fungal biomass than NW386. A positive relationship between growth rate and virulence has been proposed (e.g. Messenger *et al.*, 1999; Lipsitch and Moxon, 1997; Mackinnon and Read, 1999), which suggest that isolate NW250, having a greater growth rate than NW386, would be more virulent than NW386, which also agrees with the results obtained in the dose-response bioassays (Chapter 5).

6.4.2.1 Interactions between NW250 and ARSEF6293

According to the quantities of *Z. radicans* DNA estimated in treatment one on the different days, the results suggest that *Z. radicans* biomass increased in the larvae as the infection progressed (Fig. 6.23). Comparing the trends in the different treatments, an overall negative effect on this growth can be observed in the presence of *P. blunckii* in treatments two and three, and the severity of the negative effect was related to the dose of ARSEF6293. However, in

treatment two, on days three and four, stimulation in the growth of *Z. radicans* was observed as a response to the presence of ARSEF6293. The stimulation of growth of a fungal species in the presence of competitors has been found *in vitro* between wood decaying fungi (Wald *et al.*, 2004). They found that the fungi *Creolophus cirrhatus* (Pers.) and *Hericium coralloides* (Scop.) increased the extension rates of their colonies in the presence of other fungi (e.g. *Vuilleminia comedens* (Nees), *Ganoderma applanatum* (Persoon) Patouillard, *Phlebia radiate* Fr., *Trametes versicolor* (L.: Fries) Pilát and *Hypholoma fasciculare* (Huds.) Quél.) in experiments carried out on agar plates. One of the possible reasons given by the authors was that this may be an attempt to escape a competitor. It is difficult to know the actual reason for this behaviour, but it seems to be in response to the presence of a competitor to avoid exclusion. This stimulation in the growth of NW250 as a response to the presence of ARSEF6293 was not observed in the *in vitro* interactions experiment (Chapter 3), which suggests that the stimulation observed in growth (which was apparent for both *Z. radicans* isolates) was related to additional factors within the larva as well as the presence of ARSEF6293.

A drastic reduction in quantities of *Z. radicans* DNA was observed on day five when the quantity of DNA in treatment two was less than treatment one. However, NW250 was not completely excluded; the superiority in dose of NW250 compared to ARSEF6293 may have helped NW250 to survive inside the larvae. If these results are translated to mortality (Fig. 6.21), the percentage of larvae infected only by NW250 was reduced to 35 % from 95 % achieved in treatment one. Only 15 % of the larvae were infected by ARSEF6293 and 45% of the larvae were dual-infected. In this particular experiment, the percentages of dual-infected larvae were slightly greater than the percentages obtained in the *in vivo* interactions experiments carried out in Petri dishes (Chapter 5). The results obtained in the *in vivo* interactions experiments were combining the data from three different replicates carried out on different occasions. Although the overall results in Chapter 5 showed a very low proportion of dual-infected larvae, there were some occasions where the proportion of dual-infected larvae were greater, similar to the percentage obtained in this particular experiment that was described in this chapter. It is likely that repeating this experiment on

more occasions, the percentage of dual-infected larvae will change as observed in the *in vivo* interactions experiments of the previous chapter.

In treatment three, when ARSEF6293 was inoculated at a higher dose than NW250, the effect follows a similar pattern. A large increase of the quantity of NW250 DNA was observed on day three. However, on days four and five the NW250 DNA level was reduced almost to zero (Fig. 6.23). These results expressed in mortality showed that the mortality caused only by NW250 was only 5%, the mortality due to only ARSEF6293 increased to 35% and the dual-infection was reduced to 30%. Again, NW250 still persisted in the larval population either out-competing ARSEF6293 in a small percentage of larvae (5%), or dual-infecting larvae. In treatment three, there was a large increase in the number of cadavers with unknown causes of mortality (from 5% in treatment one to 30% in treatment three). As the percentage of cadavers with unknown mortality was greater in the treatments than in the controls, it is assumed that these cadavers were fungus related but failed to sporulate externally (non-sporulating cadavers).

6.4.2.2 Interactions between NW386 and ARSEF6293

Unfortunately in the NW386 and ARSEF6293 combination, only treatments one and three could be compared statistically. This was because treatment two received a smaller dose of NW386 compared to treatments one and three, and so, if any differences in quantity of DNA were detected compared to the other treatments, it would have been difficult to attribute this to the presence of ARSEF6293, or to the smaller dose received. Statistically, ARSEF6293 did not overall have an effect on NW386 when dual-inoculated onto *P. xylostella* larvae. However, the trend in the quantities of NW386 was similar to that obtained with NW250. Stimulation in the growth of NW386 in response to the presence of ARSEF6293 was detected only until day 3 and decreased on the subsequent days. Unfortunately, the variability in the data meant this trend was not statistically significant. This agrees with the mortality results obtained, where on treatment three, the mortality due to NW386 was reduced to 5 % compared to treatment one where 100 % mortality was obtained with isolate

NW386. The mortality due to ARSEF6293 was 65 %. The percentage of dual infected larvae was 15 %. The dual infected larvae and the 5% infected larvae with NW386 alone may represent the quantity of NW386 DNA that remained at the end of the infection process. Comparing only the results obtained in treatment three, the mortality due to *Z. radicans* only obtained with NW250 and NW386 was 5 %, but the percentage of dual-infected larvae was greater with NW250 (30%) than NW386 (15%) (Figs. 6.21.and 6.22 respectively), which could be a consequence of the greater virulence of NW250 than NW386 (Chapter 5). This suggested that NW250 was a better competitor than NW386 because it had more opportunity to survive via dual infected larvae. However, it is more likely that NW386 had more real opportunity to survive long term by producing resting spores, which was not possible for NW250. For NW250 all the dual-infected cadavers had ephemeral sporulation producing few conidia or even producing non-sporulating cadavers. Dispersal of NW250 depends entirely on the immediate production of conidia and so would be affected more than NW386 which produced resting spores.

The confidence intervals (CI 95%) demonstrated a large variability in the quantifications of DNA from isolates NW250 and NW386, either single or dual-inoculated with ARSEF6293. It is possible that the larvae used for the DNA quantification had indeed different amounts of fungal DNA, which was a consequence of the large variations in the dose received by each larva within each treatment. Shah *et al.* (2003) demonstrated differences between the doses estimated from the cover-slips and the actual dose of *Pandora neoaphidis* received by aphid species. Also, the number of replicates of the experiment was probably small leading to this large variability in the quantities of *Z. radicans* DNA, or most likely due to a combination of these factors.

Overall, both *Z. radicans* isolates were not completely out-competed by ARSEF6293. For example, if the environment where the interactions took place had been homogenous such as liquid medium, it would be likely that a virulent isolate (e.g. ARSEF6293) would out-compete the less virulent isolate (e.g. NW386). However, because larvae are not homogeneous environments, this may allow the *Z. radicans* isolates to survive. It is possible that both

pathogens were spatially separated, allowing both species to dominate in different places within the larvae (Massey *et al.*, 2004). Kerr *et al.* (2002) also found that different bacterial strains with different levels of virulence may co-exist in a Petri dish if it is considered as a heterogeneous system where the bacteria strains are not completely isolated but separated enough to allow competition only on the boundaries of each colony. When the same strains were dual-inoculated on liquid medium, the virulent strain out-competed the other strains.

Nowak and May (1994) have defined within-host competition as a gradient between two extremes: super-infections, where the most virulent pathogen eliminates those less virulent, and co-infections, where the pathogens differ little in virulence and the resources are shared via scramble competition. Scramble competition is defined as competition where the resources are shared much more equally between the competing individuals, and hence the negative effects are also spread amongst the competitors (Begon *et al.*, 1996), and the negative effects can be pathogen fitness reduction (Hughes *et al.*, 2004).

The results of this experiment showed that *P. blunckii* and *Z. radicans* had a co-infection pattern, because neither out-competed the other completely. Although there were differences in virulence amongst the three isolates, these may not have been sufficiently different biologically to allow any of them to dominate. Considering only the isolates that produced cadavers with abundant sporulation, ARSEF6311 and NW250, dual-infected larvae by these two isolates showed very little external sporulation, or nothing at all (personal observation), which suggests scramble competition. This will affect future transmission of both pathogens.

The production of resting spores by NW386 gives this isolate an ecological advantage over NW250. Although both isolates were almost excluded from the larvae, the quantities of NW250 DNA that remained inside the larva may sporulate and eventually produce a few conidia, in small patches (personal obaservation), but it is also possible that the cadaver will not produce any NW250 conidia. However, the small quantities of NW386 DNA that remained

inside the dual-infected larva will certainly turn into resting spores, and once the resting spores are free in the environment they will persist for some time and then germinate and transmit with the potential to escape competition.

In conclusion, *P. blunckii* isolate ARSEF6293 had a negative effect on the *in vivo* development of *Z. radicans* isolates NW386 and NW250 within the host. Although both *Z. radicans* isolates showed an overproduction of fungal biomass after days three and four of infection as a defence against ARSEF6293, eventually *P. blunckii* almost excluded both *Z. radicans* isolates, particularly when ARSEF6293 had a larger inoculation dose than the *Z. radicans* isolates. It is possible that both species were spatially separated allowing them to have multiple interactions within the larva, where the dominant isolate would be influenced by order of arrival at the micro habitat, allowing co-existence. Although co-existence was possible, it had a negative effect; mortality of the host was achieved, but this meant a reduction in fitness of both pathogens, decreasing reproduction or production of infective units, therefore, affecting persistence and dispersion. If both *Z. radicans* isolates, NW250 and NW386, compete against ARSEF6293 when at a numerical disadvantage, both will be out-competed in the short term. However, isolate NW386 had more chances to transmit and survive in the long term because it produced resting spores. Isolates like NW250, that did not produce resting spores, are more likely to be competitively excluded and their persistence and transmission strongly affected. The real-time PCR quantification represents an important ecological tool allowing understanding of the interactions between these two species, not only at the end of the interaction, but also during the infection process within the host.

CHAPTER 7. GENERAL DISCUSSION

Pandora blunckii and *Z. radicans* have been isolated from infected larvae in the same *P. xylostella* population, and from different populations in the same area (Riethmacher and Kranz, 1994; Velasco *et al.*, 2000). This demonstrates that both species can co-exist. However, nothing was known of the possible mechanisms used by these pathogens to co-exist, or what might happen if one were augmented as part of a microbial control strategy.

As initially a large number of isolates from each species were available, fewer isolates had to be selected to test particular hypotheses in interaction studies. These were selected based on their *in vitro* growth characteristics at different temperatures (Chapter 2). Considering that, overall, *P. blunckii* had the greatest growth at 20 °C, and *Z. radicans* at 25 °C (Chapter 2), the initial hypothesis was that the outcomes of an interaction may depend on the temperature at which the interaction takes place. For that reason, the initial *in vitro* interactions were done at these two temperatures (Chapter 3). The results showed that temperature did not have a great effect on the outcomes of interactions.

Regardless of temperature, a number of interesting interactions were demonstrated. Firstly an isolate from a different species (*Pandora* sp.) had a great negative effect on the *in vitro* growth of both *Z. radicans* and *P. blunckii*. This isolate was not used in the subsequent *in vivo* experiments because it was found not to be pathogenic against *P. xylostella*, but it demonstrated that a non-pathogenic fungus may still have an effect on the interaction between two pathogenic fungi, and this represents an important aspect of research in the future. Secondly, one *P. blunckii* isolate (ARSEF6293) also had a great negative effect on the *in vitro* growth of the majority of the *Z. radicans* isolates. This isolate and another, ARSEF6311, that did not have such a strong negative effect on other isolates, were selected.

There was not a particular *Z. radicans* isolate that had a positive or negative effect on the *P. blunckii* isolates. However, isolate NW386 was selected

because it was not affected by any *P. blunckii* isolate, and most importantly, because it was isolated in Mexico, and was therefore, potentially co-evolved with ARSEF6293. With both isolates from Mexico, future research between them may be carried out in this country in the field, therefore, information on their interactions are important. Isolate NW250 was selected because it was affected by the presence of ARSEF6293 in *in vitro* studies and also because there was a lot of research carried out using this isolate previously and so it also served as a reference (e.g. Pell *et al.*, 1993a, b; Yeo *et al.*, 2001; Furlong and Pell, 1997; Furlong and Pell, 2000; Furlong and Pell, 2001). In addition, NW250 was from *P. xylostella* populations from Malaysia and, therefore, not co-evolved with ARSEF6293.

Using these four isolates, *in vivo* interactions in *P. xylostella* were evaluated. Prior to this experiment, the virulence of each isolate against *P. xylostella* was estimated. It has been suggested that virulence can have an important influence on the outcomes of interactions between pathogens (e.g. Nowak and May, 1994; De Roode *et al.*, 2005). The *P. blunckii* isolates showed very similar LC_{50} values, and overall, they were more virulent than the *Z. radicans* isolates, particularly NW386. Isolate NW250 showed a similar virulence to the *P. blunckii* isolates. These bioassays were also carried out at 20 and 25 °C. The trends in virulence for the different isolates were similar at both temperatures, but at 25 °C all the isolates were more virulent than at 20 °C. Although this suggested that by influencing virulence, temperature may have an effect on the outcomes of interactions, at 25 °C, all the isolates particularly NW250 and to a lesser degree ARSEF6311, produced a lot more cadavers that were unable to sporulate than at 20 °C. This could not be assessed for isolate NW386, because this isolate always produced resting spores, and it seemed that temperature did not have a great influence on this ability. As the presence of conidia was the main parameter to evaluate the outcomes of an interaction, *in vivo* interactions experiments were carried out only at 20 °C.

The hypothesis for this experiment was that the most virulent isolates would out-compete and exclude the less virulent. This was based on the suggestions of other authors, where highly virulent pathogens also had greater growth rates

and could, therefore, invade the host faster than less virulent isolates. This meant that virulent isolates had more opportunities to produce reproductive stages or conidia and eventually a greater chance for transmission (e.g. Nowak and May, 1994; De Roode *et al.*, 2005). The difficulty of delivering predetermined doses for *P. blunckii* and *Z. radicans* made it difficult to use the actual dose as a variable. As a result, the doses were only classified as high, low or no dose. According to the results, the initial dose of each isolate at inoculation had a greater effect on the outcomes than virulence. For example, an isolate with a "high" inoculation dose compared to its competitor would be more likely to out-compete the other isolate regardless of their relative virulence. To measure the actual role of virulence in the outcomes of interactions, it would be necessary to develop more accurate methods to deliver and quantify predetermined doses.

Nevertheless, the results also gave valuable information about another mechanism that isolates may utilize to survive direct competition with another isolate that had a dose advantage at inoculation and was more virulent. Isolate NW386 always produced resting spores and NW250 never did. It has been suggested that resting spores are produced as a consequence of adverse environmental conditions, but the actual reason still remains unclear (Glare *et al.*, 1989; Hajek and St. Leger, 1994). Isolate NW386, regardless of whether it is competing with a *P. blunckii* isolate or if it was at a numerical disadvantage, produced more dual-infected cadavers than NW250. This suggests that NW386, even if it is outnumbered, can produce resting spores that will be released to the environment and eventually germinate when the competitor may no longer be present. This attribute gave NW386 an ecological advantage over NW250 in competition with ARSEF6293, because although the latter may still have been present inside a dual-infected cadaver, that cadaver only produced *P. blunckii* conidia. Isolate NW250 would remain as mycelium that did not sporulate externally, therefore ecologically it was excluded because it did not produce any conidia that would allow this isolate to transmit and persist.

Zoophthora radicans and *P. blunckii* can be present in the same geographical area, and possibly infecting the same *P. xylostella* population. It is possible that

the Mexican *Z. radicans* isolates, such as NW386, produce resting spores as mechanisms to survive direct confrontation with *P. blunckii* which may be more virulent. This was supported by the fact that all the Mexican isolates coming from the same region as NW386, produced resting spores (data not shown). When isolate NW250 from Malaysia was collected, no larvae infected with *P. blunckii* were found (Pell, J.K., personal communication), which suggests that this isolate did not have a potential competitor, or at least no *P. blunckii* isolates, and therefore, was not under selection pressure to produce resting spores. However, this could not be confirmed completely. It would be important to assess the ability to produce resting spores by other Malaysian isolates from the same region, to asses whether this is a characteristic possessed by many Malaysian *Z. radicans* isolates, or just NW250. The Rothamsted fungal collection possesses these isolates, and so this study is possible in the future.

The *Z. radicans* isolates had a large genetic variability (Chapter 4), but in this study, no clear relationship was found between the RFLP groupings and host or geographical origin (Chapter 4). The genetic variability found amongst *Z. radicans* isolates suggests a large capacity for adaptation to different environments (Cooke and Rayner, 1984). This adaptability includes different responses to temperature (Chapter 2) and, potentially, a wide insect host range (e.g. Galaini-Wraight *et al.*, 1991; Mcguire *et al.*, 1987; Vandenberg and Soper, 1987). This was specifically demonstrated for isolate NW250 by Furlong and Pell (1996) by infecting the parasitoid *D. semiclausum.* However, this parasitoid was 70 and 133 fold less susceptible than the larvae and adult of *P. xylostella* respectively. This suggests an alternative mechanism by which the generalist *Z. radicans* may avoid direct competition with the specialist *P. blunckii*, by infecting alternative hosts.

It is important to remember that these inter-specific interactions were evaluated using a reduced number of isolates. As demonstrated previously, all these isolates had significant variability in their *in vitro* growth at different temperatures (Chapter 2), and also large genetic variability, particularly amongst the *Z. radicans* isolates (Chapter 4). It is very likely that the outcomes

could be different using different isolates, as the most important feature of natural populations is genetically based variation within them (Cooke and Rayner, 1984). This variability may contribute to the development of other mechanisms for co-existence that have not been found during this research.

An important factor that was not evaluated experimentally during this project. but could have an impact in the outcomes of an interaction, is any possible change in the behaviour of the insect host either during the infection (Furlong *et al.*, 1997) or before an infection may occur, as an avoidance behaviour of the host assessing any potential risk (Meyling and Pell, 2006). It is very likely that these behaviour changes can modify the possibility of being infected by a single pathogen or even dual infected. Specific experiments need to be designed and carried out in order to assess the impact of these behavioural changes in the interaction outcomes.

The DNA quantification of *Z. radicans* isolates when co-inoculated with different doses of ARSEF6293 (Chapter 6) gave interesting insights into what may be happening during the infection process. For example, when ARSEF6293 and NW250 were co-inoculated in *P. xylostella* larvae, and the former had a larger dose than NW250, three days after inoculation, NW250 had produced larger amounts of fungal mycelium than when it was inoculated alone. This was presumably as a response to the presence of the competitor ARSEF6293. However, on days four and five these amounts were greatly reduced, particularly when ARSEF6293 had an initial numerical advantage in dose over NW250. This suggests that probably NW250 was only a better competitor than ARSEF6293 during the parasitic stage of the infection (from day 1 to day 3), and that ARSEF6293 had better saprophytic development (from day 4 and 5). This has been suggested for other species of entomopathogenic fungi (e.g. Thomas *et al.*, 2003). Unfortunately, because quantitative PCR detection could not be developed for *P. blunckii*, the *in vivo* growth of ARSEF6293 could not be assessed. Had ARSEF6293 really been out-competed by NW250 during the first three days of infection, or had both pathogens grown in the same proportions, sharing the resources, and leading to faster death of the host? What happened on days 4 and 5? While the quantities

of NW250 mycelium within the larva are reduced on days 4 and 5, is the quantity of ARSEF6293 reduced as well? If both were reduced, it may be the result of scramble competition for the very limited resources. This may lead to a reduction in fitness of both pathogens and the production of non-sporulating cadavers. In contrast, if the amounts of ARSEF6293 were increasing on days 4 and 5, possibly due to better saprophytic growth than NW250, the former one used all the resources left. This would lead to an extinction of NW250 in that particular system. Eventually, the cadaver produces ARSEF6293 conidia only, and therefore ARSEF6293 wins the competition and excludes isolate NW250 but the mechanisms can only be determined if quantitative PCR is available for both competitors. This represents an interesting future research study if the molecular techniques can be developed. For this, the sequence of more *P. blunckii* isolates must be obtained, to improve the species-specific primers of *P. blunckii* and optimise their use in quantitative real-time PCR.

The effect of fluctuating temperature on these interactions was not evaluated. All the *in vivo* interaction experiments were carried out at constant temperatures, but it is known that fluctuating temperatures can influence the outcomes of dual-inoculations, particularly if the pathogens had a different range of optimal temperatures (Thomas *et al.*, 2003; Fargues and Bon, 2004). For example, it seems that the parasitic and saprophytic phases of the infection may be influenced differently by temperature (e.g. Luz *et al.*, 1999), or the interaction with other pathogens (Thomas *et al.*, 2003), so what would happen if different temperatures were used at each of those stages? It is very likely that the outcomes would change compared to the results obtained at constant temperatures.

A final question that arose from these experiments would be from a biological control point of view. Would it be advisable to inoculate in the field a combination of both species? The answer would be no, because although one of the objectives could be achieved, and that is the death of the host, eventually the increased opportunities of encounters between these two species, and therefore dual-inoculated larvae, would lead to a negative impact on the persistence of both pathogens. All the dual-infected larvae, and all the larvae

that only produced conidia of one species but were dual-inoculated, showed a great reduction in their sporulation capacity compared to a single inoculated larvae. This suggests that dual inoculation is not the best strategy to obtain long term control, because eventually the persistence of each species would be greatly reduced. However, to test this, looking over repeated generations would be necessary and represents another potential future study. If a large amount of one of the species is augmented in the environment, there is a risk of competitive exclusion of the other species and the ecological consequences of doing that are unknown.

Maybe the best strategy for these pathogens is their use in conservation biological control. As it would be impossible to remove one species from the environment and try to avoid dual infections, it would be advisable to maintain the balance for both species.

The isolates used in this study were isolated from cadavers in the field with abundant sporulation and conidia production. If under field conditions both isolates produced cadavers with abundant sporulation, it suggests that both species may co-exist in the same geographical area, probably without competition, allowing them to produce conidia and persist. This also suggests that, in the field, additional factors must exist that favour their transmission and persistence and that were not present in the laboratory. As described earlier, during the bioassays and *in vivo* interaction experiments, ARSEF6293 produced cadavers with ephemeral sporulation or non-sporulating cadavers, and NW386 always produced resting spores and never conidia.

The results of this only reflect part of the story and do not identify all the mechanisms of co-existence between these two species. More research is required to make more definite conclusions, but this can now be based on the data provided by this study.

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