Part 1. A technical review on the potential use of entomopathogenic fungi for improved management of carpophilus beetles

1. Abstract

Entomopathogenic fungi may be a useful "biopesticide" tool to manage insect pests of almonds and other orchard fruit and nut crops. This review will investigate the potential of utilizing entomopathogenic fungi to control carpophilus beetles, with a particular focus on autodissemination technique. Autodissemination is a pest control strategy that uses a lure to attract the target insect species and inoculate adults with microorganisms, which then act as the dispersal agent for the disease through their natural behaviour and ecology. Carpophilus ecology fits particularly well with the potential to utilise EPFs and autodissemination for control, as (i) this strategy works best in pest populations that have a known strong attractant to lure insects, and where aggregation behaviour occurs and can be manipulated to facilitate the dispersal of a known hyper virulent agent, and (ii) adults and larvae of this pest remain hidden within mummy nuts and developing fruits for most of their life cycle. The role of entomopathogenic fungi in the control of carpophilus beetles is not well studied, therefore much of the literature considered in this review is taken from related coleopteran pests occupying similar habitats. The factors that determine the suitability of an autodissemination strategy for carpophilus beetles are evaluated, including virulence screening and selection, laboratory experiments testing horizontal transmission, autoinoculation traps, and in-field auto-dissemination trials. Suggestions are made regarding the initial steps that should be taken to develop proof of concept for EPF application to carpophilus in almonds.

2. Introduction to entomopathogenic fungi as bioinsecticides

Entomopathogenic fungi (EPFs) have considerable potential for use as biocontrol agents, since they naturally occur in soils throughout the world, and act as parasites of various arthropod species. EPFs are the most abundant type of microorganism that infect insects, with approximately 60% of insect diseases being caused by EPFs, often resulting in an epizootic (Faria & Wraight 2007). The EPFs currently used for the control of insect pests include *Beauveria* spp., *Metarhizium* spp., *Isaria* spp., and *Lecanicillium* spp. However, there is untapped diversity both within these genera and in other groups (Mascarin & Jaronski 2016). Many species and strains have been discovered and optimised and formulated by various companies internationally and marketed for use on a wide range of insect orders. The full list of commercially available and tested entomopathogenic (EPF) products and strains are listed comprehensively in Faria and Wraight (2007) and Beauveria specifically in Mascarin and Jaronski (2016).

The pathogenesis of an EPF begins with conidia (asexual spore) attachment to the epicuticle of a target insect. Following germination, penetration of the cuticle layers occurs with an array of enzymes produced by a modified hyphal body called an appressorium. Once the cuticle is breached, colonisation of the insect commences through the growth of blastospores and other hyphal bodies that produce immune suppressants, metabolites and toxins that ultimately kill the insect. The final stage of growth is the emergence of conidiophores from the cuticle and the dispersal of conidia. Most agricultural products utilise these dispersible conidia (asexual spores) in suspensions, powders, cultures or ready to use baits while blastospore and hyphal based products are less prevalent (Faria &

Wraight 2007, Mascarin & Jaronski 2016). The aqueous solutions of conidia are frequently emulsified with Tween 80 or similar product to aid in the homogenous dispersal of hydrophobic conidia, or an oil suspension is used. Different emulsifiers can have a stimulatory or inhibitory effect on germination and vegetative growth rate, dependent on strain and concentration, however this is not always transferable to insecticidal potential (Mwamburi et al. 2015).

The two main entomopathogenic fungi control strategies that are employed in orchard-analogous settings are (i) autodissemination to an overwintering population (Dowd & Vega 2003) and (ii) whole tree and understory sprays (Vera et al. 2011). Each program has specific requirements regarding formulation for application and additives that optimise the pathogen performance. For almonds, entomopathogenic fungi are better suited to post harvest (April-June) or early spring application program when pests are actively moving to or from overwinter niches at times when negating crop damage is critical. Implementation at this time may result in a greater percentage of population control, below damage threshold levels, that can be achieved with lower trap density, reducing cost while conditions will be suitable for fungal growth. Applications of biopesticides around hull-split may have limited potential due to the slower lethal time however synergy with sub-lethal doses of chemical insecticides could negate this issue (Akhanaev et al. 2016). Approaching December, fungicide application may be required to control hull rot and other diseases, and would be incompatible with an entomopathogenic fungi program. Additionally harsher climatic conditions and greater UV exposure may reduce efficacy (Mascarin & Jaronski 2016). Due to advantageous conditions for an autodissemination control strategy, this will be explored with specific reference to Carpophilus beetles. Relevant literature will be used to guide and inform the experimental procedure required to verify this strategy.

3. Virulence screen and strain selection

3.1 Bioassays

To test the susceptibility of a target pest to EPFS, a laboratory bioassay is generally conducted. Most bioassays on Coleoptera employ the use of emulsified conidial suspensions in water at concentrations of $1x10^6$ to $1x10^8$ conidia/ml that is applied as a whole insect immersion or contamination of substrate. This is a simple and precise way to administer a set dosage to provide an assessment of the strain virulence on the target pest. Looking into fungal bioassays conducted on other beetle orders provides insight into the potential of each fungus and guides experimental design. Whole insect immersion was used to test adult emerald ash borer (*Agrilus planipennis*) in suspensions of three strains of *Beauveria bassiana* and two strains of *Metarhizium anisopliae* at 10^6 and 10^7 conidia/ml (Liu & Bauer 2006). The Mycotech Corp. developed *B. bassiana* strain GHA had the lowest mean survival time (MST) of 4.6 and 4.2 days at both concentrations while the remaining isolates had an MST of 5.6 or less indicating that all strains were quite effective against the target, but that one strain (GHA) was the most virulent. In selecting an agent for sweet potato weevil, Ondiaka et al. (2008) conducted a more comprehensive conidial suspension spray assay. Eight strains of *M. anisopliae* and four of *B. bassiana* were selected for mortality rate bioassays and median lethal time (LT_{50}) assessment. The inclusion of this data provided a clearer picture of the virulence of the large range of strains as several isolates had similar mortality rates but different LT_{50}. In addition to this information, spore germination counts for each strain were conducted to confirm the viability of the preparations. The methods from these studies are a suitable guide for bioassay design with carpophilus beetles, using a wide range of strains, assessing mortality rate and LT_{50}, checking spore viability and testing a range of concentrations give a

detailed assessment of virulence. The immersion inoculation method is simple procedure to administer a consistent conidial dose, however given the anticipated trap inoculation method a conidial powder impregnated fibre may be appropriate.

There are few bioassay studies testing EPF virulence on carpophilus or nitidulid beetles. Muerrle et al. (2006), looked at small hive beetle (*Aethina tumida*) and demonstrated a 74% mortality rate with a *B. bassiana* conidial suspension impregnated on a cotton bud medium compared to much lower rates with other *M. anisopliae* and *H. illustrus* species. This study is useful as a rough guide for showing that nitidulid beetles are susceptible to a particular species, however poor bioassay design limits the strength of evidence as there was no indication of suspension concentration, spore viability, and toxic conidial load. Spore concentration and germination rate was considered bioassays conducted by Husberg and Hokkanen (2001) of *M. anisopliae* on *Meligethes aeneus* (nitidulidae). The suspension spray on substrate assay registered an average mortality rate of 85% was confirmed as a result of mycosis with incubation of surface sterilised dead beetles. In unpublished data from Dowd and Vega 90% of the test *Carpophilus lugubris* insects died three days after walking through a sporulating culture plate of commercial *B. bassiana* strain AF-4 and led them to conduct further studies on autodissemination potential that will be discussed later. This method of inoculation is expected to transfer very high rates of conidia that would not be replicated in field traps, a known dose bioassay technique is recommended.

Beetle species	Inoculation method	Agent(s)	Result	Reference
Meligethes aeneus (Coleoptera: Nitidulidae)	Conidia suspension on substrate	*M. anisopliae*	85% mortality rate after 5 days	Husberg & Hokkanen 2001
Aethina tumida (Coleoptera: Nitidulidae)	Conidia suspension on substrate	*B. bassiana*	74.00 ± 8.94% mortality after 24 days	Muerrle et al. 2006
		M. anisopliae	12.00 ± 8.37% mortality after 24 days	
		H. illustris	2.00 ± 4.47% mortality after 24 days	
Carpophilus lugubris (Coleoptera: Nitidulidae)	Beetle walking through sporulating cultures	*B. bassiana* strain AF-4	90% mortality after 3 days	Fernando Vega unpublished data

3.2. Summary of EPFs tested on nitidulid beetles

The papers discussed specify that *B. bassiana* and *M. anisopliae* are pathogenic to nitidulid beetles and testing with these species is a good place to start. However, as there is considerable variation of virulence within these species, testing as many isolates as available is recommended. In Australia there are two APVMA registered *Beauveria bassiana* products for use on thrips, aphids and similar soft bodied insects, and five Metarhizium products, two granule formulations for beetle larvae in soil and three different suspension formulations (oil immersion, oil suspension, ULV) for Australian Plague Locust. Internationally there are 37 *B. bassiana*, 22 *M. anisopliae*, 7 *B. brongniartii* and 1 *I. fumosorosea* commercially listed agents for use against coleoptera with one *M. anisopliae* strain listed for Nitidulidae (Faria & Wraight 2007). With the limited availability of commercially available products in Australia isolation of fungi from the target pest range may be required.

3.3. Temperature

In considering the effectiveness of a fungal biocontrol program, of equal importance to strain virulence/pathogenesis is the optimal temperature range of the strain. Average temperature will vary widely depending on the program and season of application (Mildura winter average temperature

13°C, summer average 25°C), a fungus that will thrive in the required setting is needed. Two comprehensive studies by Fargues et al. (1997) and Ouedraogo et al. (1997) investigated the temperature effect on *in vitro* vegetative growth of numerous *B. bassiana*, *M. anisopliae* and *M. flavoviride* isolates from a diverse range of climatic regions. The optimum growth rate was usually observed at 25 to 30°C but several strains of *B. bassiana* and *M. anisopliae* had growth rates of approximately 60% at 15°C. The information from these studies shows the potential of effective fungal growth of both *Beauveria* and *Metarhizium* at lower temperatures, however *in vitro* vegetative growth and pathogen establishment leading to insect mortality is not necessarily linked. To explore the interactive effect of low temperatures on EPF efficacy on coleopteran species, Doberski (1981) conducted a $1x10^5$ conidia/ml fungal dip bioassay on elm bark beetle larvae at temperatures from 2 to 20 degrees with six EPF isolates. The Beauveria and Paecilomyces (now known as Isaria) isolates reduced survival time at 6°C but was twice as effective at 10°C. A more recent study by Klingen et al. (2015) tested six EPF isolates collected from Norway on black vine weevil. A 50% mortality rate was recorded by one *B. pseudobassiana* and one *M. brunneum* strain at 12°C with an associated LT_{50} of four weeks. These results are supportive of action winter application indicating the fungal pathogens are active below the 13°C average of Mildura. The longer median lethal time associated with lower temperature is not a major hinderance to the program as the target beetles and larvae will have long development times in these periods.

The rate of sporulation of cadavers is dependent on temperature and humidity. Cadaver sporulation is important for direct horizontal transmission of the agent and habitat contamination, which will be discussed further in proceeding sections. Arthurs and Thomas (2001) found optimal conidia production at 25°C in with cadavers in contact with damp substrate, while relatively little *M. anisopliae* conidia production occurred on acridid cadavers at 15°C, reduced to zero at 10°C. Fargues and Luz (1998) observed significantly reduced *B. bassiana* sporulation in hemipteran cadavers when relative humidity was reduced however, Carpophilus beetles have been observed to prefer mummy nuts located around irrigation systems where the nuts are moist, possibly negating this factor. Temperature is going to be a key factor in selecting a strain that will have a high mortality rate, LT_{50} and sufficient cadaver sporulation in order to have a significant impact on Carpophilus populations.

4. Autodissemination

An EPF-based control system utilising attractant lures to trap and inoculate wild pest populations with an agent, and then automatically distribute the agent to the remaining population, is particularly appealing. It is potentially a low cost, low environmental impact method of pest control due to the targeted nature of the program, which requires minimal amounts of inoculum and has limited off-target damage. This strategy works best in pest populations that (i) have a known strong attractant to lure insects, and (ii) where aggregation behaviour occurs and can be manipulated to facilitate the dispersal of a known hyper virulent agent that can cause an epizootic in target populations. The factors that determine the success of an autodissemination program are the rate of attraction and inoculation in the trap and the dispersal through horizontal transmission from infected beetle to untreated beetle. Horizontal transmission is mediated by the aggregation and proximity of beetles, the ratio of treated to untreated beetles, the rate of transfer through sexual contact, duration of contact, the time after inoculation to contact, and cadaver sporulation among other factors. Experimentally testing these factors will establish the likelihood of success of this program in Carpophilus beetles and will be discussed in the proceeding sections.

4.1 Horizontal transmission – laboratory trials

Insect ratio on transmission rate of aggregated adults

Horizontal transmission of fungal spores from inoculated adults to untreated conspecifics either through mating or aggregation is the critical aspect determining the success or failure of an entomopathogen autodissemination program. The ratio of fungus infected beetles and untreated beetles has been shown to affect the mortality rate of untreated specimens. This is an important factor to consider as autodissemination strategies rely on a minority of inoculated adults passing the pathogen to the rest of the population: therefore, a high transfer rate is critical. Modest declines in mortality rate were seen in spruce bark beetle, with ratios increasing from 1:1 to 1:20 *B. bassiana* treated to untreated beetles of 90% and 75% respectively after 7 days (Kreutz et al 2004). Supporting this, Kocacevik et al. (2016) found 100% mortality rates in all bioassays of ratios from 100% to 25% on the same species after 15 days using *Beauveria pseudobassiana,* indicating that a lethal conidia dose can be transferred to a large number of beetles from a single treated conspecific. A study by Getahun et al. (2016) of *Metarhizium* spp. on sorghum chafer tested the effect of exposure time of the untreated insect to an infected adult on the mortality rate and found 17 – 34.4% mortality rate from a 2-hour exposure, and 47-59% mortality from 24 h after 15 days, demonstrating greater transmission with longer interaction. Horizontal dissemination of a fungal biocontrol agent among carpophilus beetles aggregating in overwintering niches is supported by the findings presented as it is expected the adults will have a long duration of close contact. Experimental trials assessing this factor in carpophilus beetles is advised, with emphasis placed on the impact of multiple mummy nut refuges on insect interaction.

Sexual transmission

Sexual transmission is a significant component of horizontal transmission, initiating an extended period of contact for potential dispersal of an agent. Kreutz et al. (2004) found a single sexual contact between a *B.bassiana* treated spruce bark beetle male and an untreated female was observed to transfer a lethal dose of $1.2x10^4$ conidia, with a mean survival time of 3.7 days and a mycosis rate of 96.5%. Similar transmission rates were observed with a male or female vector with red palm weevil (Dembilio et al. 2010). Ugine et al. (2014) examined the effect of time between inoculation point and secondary contact by increasing the postexposure time in sexual transmission of *M. brunneum* with Asian longhorn beetle. The indirectly exposed females had a 100% mortality rate when exposed to males inoculated from 0 h to 48 h previous, while the median days to death (LT_{50}) increase by 25% after 24 h and 90% after 48 h indicating that while conidia transfer decreased over time after 48 hours a lethal dose was transferred. The studies presented here give a good indication that transmission through sexual contact can be high even under a range of constraints expected in field settings. These experiments would be appropriate to replicate with *Carpophilus* spp. and expanded to include the impact of flight on attachment of conidia and transmission rate.

Cadaver transmission

Sporulating cadavers are an additional factor that contribute to horizontal transmission through insect/cadaver interaction and contamination of habitat substrate. Sweet potato weevil adults in a container with a sporulating cadaver had a mortality rate of 63% after 12 days (Dotaona et al. 2017). The result is supportive in the context of *Carpophilus* spp. as it is expected that aggregation points in mummy nuts and other small niches would be conducive to this mode of transmission, in addition to providing a valuable contamination point for larvae and other pests occupying this niche. Removal and destruction of mummy nuts is a large part of current pest control methods as carpophilus beetles and

carob moth are thought to utilise them for overwinter shelter. If these refuges can be contaminated with a large amount of conidia through cadaver sporulation this labour-intensive aspect to pest management may be mitigated. As mentioned previously cadaver sporulation can be highly impacted by temperature and humidity, testing of these variables in the context of almond orchards will be required as it will be a key factor for the extended transmission of the agent.

4.3. Autoinoculation trap

To study attraction and inoculation Vega et al. (1995) produced a device that exhibited high rates of attraction and contamination of *Carpophilus lugubris* with a marker dye from a simple trap design. Pheromone packages (male and female attracting) have been developed by Bartelt and Hossain (2010) that elicit a very high attraction of carpophilus species associated with stone fruit, and these have been effectively integrated into attract-and-kill traps together with "co-attractant" blends based on fermenting peaches. A new powerful attractant specifically for carpophilus in almonds is currently under development in Hort Innovation project AL16009. Various modifications have been made to existing commercial pheromone traps with ease to deliver a lethal dose and auto-release effect (Klein & Lacey 1999). Mota et al. (2017) tested an autoinoculation device designed for coffee berry borer that had *B. bassiana* conidia impregnated in fabric on trap exit tubes. $2.9x10^5$ conidia were acquired per insect after a 5 second contact, which had an associated mortality rate of 88.5% when maintained in the lab. Similarly, the trap developed by Lyons et al. (2012) delivered a *B. bassiana* dose of $5.79x10^5$ conidia per emerald ash borer beetle, resulting in an MST of 13.7 days after the trap and inoculum had 29 days in the field. Delivery of a substantial conidial load is essential in achieving a high mortality rate, median lethal time (LT_{50}) and effective horizontal transmission, if this can be achieved in an autoinoculation trap with a long operative field time the economic case for this method of pest control is strengthened. The trap designs discussed here indicate that these requirements can be met.

4.5 Autodissemination field trails

The body of evidence supporting the horizontal transmission of EPFs among a number of different beetle species in lab-based bioassays demonstrates the potential use of these agents in an autodissemination pest control scheme, and in-field studies have been conducted to develop the theory in various beetle species. In-field large cage experiments containing four spruce trunks with a 1:1 ratio of *B. bassiana* (in the form of Boverol® conidia dust) treated spruce bark beetle adults and untreated conspecifics showed significant reductions in bore damage, numbers of larvae and pupae and a 99% mortality rate of adults compared to 52% in the control treatment (Kreutz et al. 2004). Yasuda (1999), testing *B. bassiana* autodissemination for control of sweet potato weevil, found the system to be effective in the attraction, inoculation and killing of males with a peak mortality rate of 96.2% after 3 weeks of trap placement that remained as high as 60% four weeks after the removal of the trap. Horizontal transmission to female weevils was indicated through a roughly 30% increase in female mortality of the trial period and a 30% incidence of fungal contamination of live trapped beetles compared to 5% in the control at the three-week mark. While results from this trial were promising, subsequent studies on EPFs and sweet potato weevil conducted by Ondiaka et al. (2008) and Reddy et al. (2014) focused on the efficacy of broad scale sprays and insecticide synergy. Autodissemination was discussed as a potential control strategy but was not explored in these studies. Mota et al. (2017) had a lot of success with the autoinoculation device discussed earlier and development of a trap with higher attraction rates is underway, however commercial application is yet to be seen. Although these studies show promising results for autodissemination programs on coleopteran pests, none have yet progressed beyond pilot studies thus far.

Focussing specifically on carpophilus beetle, the ability of sap beetle (*Carpophilus lugubris*) to carry and spread *B. bassiana* was tested by Dowd and Vega (2003) in a multiyear field study. The study did not record a reduction in population size or associated damage of *C. lugubris,* however a high frequency of beetles were re-trapped with the test strain indicating that it was present amongst the overwintering population. Recording the presence in the field of the agent with re-trapping and DNA verification is an important method to quantify dissemination and should be employed along with damage and population surveys. *Carpophilus freemani* have also been investigated for use as a vector of *B. bassiana* to European corn borer (*Ostrinia nubilalis*) and have demonstrated the capacity to carry spores on their cuticle and in faeces that led to the mortality of *O. nubilalis* larvae occupying the same corn tunnel. However, there was no quantitative measure of the rate of transmitted spores or pathogenicity on *C. freemani* (Bruck & Lewis 2002). This study is supportive of *Carpophilus* spp. vectoring fungal spores and indicates the potential for lethal doses of conidia to be transferred to the cryptic habitat these beetles occupy. Field studies will be useful to indicate when autodissemination traps will be most effective as substantial inter-niche movement is required to vector the agent in epizootic proportions. Beetles are likely to be inactive during the winter limiting the transmission potential at this time, however beetles moving into these niches after harvest and emergence after winter are possible implementation opportunities.

5. Conclusion

The current pest control for carpophilus beetles in almonds and other fruit and nut orchards are labour intensive, high cost methods, which have limited coverage. This highlights the need for developing novel and comprehensive pest control strategies that can function effectively as part of an IPDM program. We present evidence from a range of studies suggesting that *Carpophilus* species are an ideal candidate for an EPF-autodissemination control program aimed at reducing pest populations. The aggregating behaviour of the insect, together with the mummy nut habitat within the orchard, support high fungal virulence, effective horizontal transmission, and high coverage in a cryptic habitat (inside nuts); and not only for this species but for the carob moth, which shares the same niche. Entomopathogen autodissemination is very host specific, with limited impact on beneficial predators and parasites that support orchard health and suppress other pests from emerging. The fungal pathogens discussed are naturally abundant and pose a low risk to the wider ecosystem or humans through both occupational exposure and consumption. Many factors can limit the effectiveness of an autodissemination program once it goes to the field, and emphasis should be placed on rigorous laboratory-based bioassays before field trials commence. Biopesticide programs can be particularly variable and it is therefore crucial to ensure growers are not let down with an unsatisfactory performance resulting from poorly planned field trials in which they are involved. Efforts to alleviate this variability include advances in genetic modification, formulation, and synergistic use with other microbial agents and sub-lethal pesticide that increase virulence and hardiness of fungal pathogens, while optimisation of fermentation is helping to reduce production costs and product stability. Development of a successful autodissemination program with carpophilus beetles in almond orchards would be readily transferable to other affected fruit and nut orchards, and would encourage ecologically beneficial pest control strategies within the wider agriculture system.

6. Recommended experimental trials for a carpophilus beetle autodissemination program

1. Virulence screen and strain selection experiments
    a. Mortality rate at varying concentrations
    b. Median lethal time
    c. Temperature range of agent
2. Laboratory based horizontal transmission experiments
    a. Mortality rate with increasing ratio of treated to untreated beetles with multiple mummy nut refuges (with assessment of fungal contamination of the mummy nuts at the end of trial
    b. Cadaver spore production in mummy nuts over seasonal conditions
    c. Mortality rate from sexual transfer
    d. Impact of time between inoculation and transmission on mortality (plus effect of flight)
3. Autoinoculation trap development and testing
    a. Rate of attraction
    b. Conidial dose per inoculation
    c. Field longevity of spores
4. Field trials
    a. Rate of attraction in the field
    b. Survey for the presence of the agent in the field of mummy nuts for adults with specific agent (DNA verified)
    c. Assessment of population impact through re-trapping

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Part 2. A literature search for use of neem products to control insects in almond orchards

1. Background & Methods

Certain natural products may offer an alternative choice to synthetic pesticides, due to their reduced negative impacts on human health, beneficial insects and the environment. Neem oil derived from the seeds of the neem tree *Azadirachta indica* has a long history of use in India as a pesticide, cosmetic, and natural remedy. The Almond industry has shown interest in exploring the use of neem oil to control both carob moth and Carpophilus beetle. Section two conducts a literature search on the use of neem (azadirachtin) oils in the context of controlling almond pests. The literature search utilised the Victorian Government Library Services and *Google* search engine were used. Both the common name "neem" and the active ingredient "Azadirachtin" were used in combination with pest names. As carpophilus and carob moth are the major issues to the almond industry, "lepidoptera" and "coleoptera" / "beetles" were also used in the literature search.

2. Result

2.1 Overview of neem and azadirachtin

Azadirachta indica is native to seasonally dry, tropical woodlands of north-east India and probably some other parts of Asia. It has been planted in Africa, Australia, Latin America, and Asia (Koul 2005) mainly to establish plantations for production of neem extract to control a range of pests. Neem can tolerate severe drought and saline soils (Radwanski et al 1981). Fruits are the most important source of the ingredients that could potentially be used to control insects.

Neem oil is a mixture of compounds, however Azadirachtin is the most active component and some purified formulations are used as pesticide products. The quantity of azadirachtin present in the seed kernels may vary considerably depending on environmental and genetic factors. Azadirachtin has deterrent, anti-ovipositional, antifeedant, growth-disrupting(growth-regulating), fecundity and fitness-reducing properties on insects (Schmutterer 1990, Mordue (Luntz) and Balckwell 1993). Several other active compounds were also isolated from neem seed kernels, such as salannin, salannol, salannolacetate, 3-deacetylsalannin, azadiradion, 14-epoxyazaradion, gedunin, nimbinen, and deacetylnimbinen (Jones et al 1989).

Mathur (2013) considered the use of neem extract to be more acceptable as a part of Integrated Pest Management due to its relative safety to biocontrol agents, specificity and different mode of action compared to the broad-spectrum pesticides such as carbamates, cyclodiene organochlorines etc. They can be used in a variety of crops and ornamentals for insect control. Mordue (Luntz) and Blackwell (1993) reported that azadirachtin can be effectively used as an antifeedant and insect growth regulator to control insects from several different orders. Azadirachtin showed antifeedant effects against some coleopterans, such as colorado potato beetle (*Leptinotarsa decemlineata*) (Zehnder and Warthen 1988). As an insect growth regulator (IGR), azadirachtin caused weight reduction in many lepidoptera species (Isman 1993), increased the duration of immature stages of *Spodoptera littoralis* (Adel and Sehnal 2000), and inhibited/delayed moulting of American cockroach (Quadri and Narsaiah 1978). Azadirachtin can also be used as a nematicide (Lynn et al 2010). The

efficacy of Neem oils is mainly depens on the amount and the formulation. Neem oils can be formulated as granules, wettable powders, emulsifiable concentrates and dust.

Neem and many naturally occurring pesticides are often slow acting as crop protectants. Neem can play a significant role in resistance management due to its different mode of action. Lowery and Smirle (2000) demonstrated that neem products can be used in reducing levels of detoxification enzymes by blockage of protein synthesis and therefore may be more effective in resistant strains of insects. Trisyono and Whalon (2000) also reported that 0.25% Neemix combined with *Bacillus thuringiensis* (Bt) can be used as a resistance breaking compound when used against the Bt resistant strain of Colorado potato beetle.

2.2 Influence on insect behaviour and physiology

Heyde et al (1984) reported that the application of 3% neem oil resulted in fewer brown plant hoppers (*Nilaparvata lugens*) landing. Here, neem acted as an olfactory repellent, and no insect contact with treated plants was necessary. Oviposition repellence has also been reported: many lepidopterans including cabbage webworm *Crocidolomia binotalis*; the cotton bollworm *Helicoverpa armigera*; and the fall armyworm *Spodoptera frugiperda*; stopped laying eggs on plants treated with neem products. Some beetles (*Callosobruchus* spp.) have also shown oviposition avoidance in the presence of neem (Schmutterer 1990).

When rice plants were treated with 1 to 50% emulsion of neem oil, food intake by some homopteran insects (for example *N. lugens*, *Sogatella frucifera* and *Nephotettis virescens)* was reduced significantly compared to control plants. Saxena et al (1984) found similar results with *N. lugens,* when these insects were feeding on rice plants grown in soils mixed with neem cake (Heyde et al. 1984). The Japanese beetle*, Popillia japonica*, stopped feeding on soybean leaves when treated with 1 % aqueous emulsion from the neutral portion of an ethanolic neem seed kernel extract; Azadirachtin was also effective. However, spraying the leaves of roses and grapes with neem seed extract did not generate any repellent effect against beetle (Ladd 1981). Schmutterer (1990) reported that many lepidopteran larvae including *Spodoptera littoralis*, *S. frugiperda*, *S. exempta*, *Heliothis virescens*, *Helicoverpa zea*, *H. armigera* reduced their feeding when plants were sprayed with Azadirachtin. Simmonds et al (1984) reported that oligophagous species were more sensitive to Azadirachtin compared to polyphagous ones. Adel and Sehnal (2000) concluded that the antifeedant effect and toxicity due to Azadirachtin and its related products were obvious in the early instars. They found that adding 10 ppm Azadirachtin to the diet of S. *littoralis* was repellent to 2nd instars but was acceptable to their higher instars (4th and 6th), and some of which completed their life cycle but the adults were sterile.

Mehaoua et al (2013) reported that neem based products can stop or slow down the development of eggs and larvae, blocking metamorphosis of larvae and nymphs by inhibition of chitin synthesis. Heyde et al (1984) concluded that foliar application of neem oil and enriched formulated neem seed kernel extract could negatively affect the moulting process, increase the duration of the nymphal period, and increase dose dependent mortality in *N lugens* and *N virescens*. The Mexican bean beetle*, Epilachna varivenstis*, is one of the most investigated insects in relation to growth regulating effects of neem derivatives. Methanolic neem seed kernel extract in high concentrations or Azadirachtin, in most cases caused one to four dark brown to black spots on the dorsal side of the thorax of treated fourth instar larvae of *E varivenstis* (Schmutterer 1981, 1987). Most of these affected larvae failed to moult but lived for up to a month. Histological studies found that these black spots consisted of melanized, degenerated cells of the imaginal wing disks. The epidermis of these larvae was also partly damaged (Schluter 1981, 1987).

Schmutterer (1990) reported in his review paper that Lepidoptera is one of the most sensitive groups of insects in relation to the growth regulating effects of neem and its derivatives. Incorporation of ground neem seed kernel in different concentrations (0.02, 0.2 and 0.5% in artificial diet) and fed 2-nd instar larvae of gypsy moth *Lymantria dispar*, resulted in almost 100% moulting inhibition. Fifth instar larvae of tobacco horn worm, *Manduca sexta*, showed high mortality when fed on diet mixed with 5 to 50 ppm of methanolic neem seed kernel extract.

2.3 Prospect for use of Azadirachtin against Carob moth

Mordue (Luntz) and Nisbet (2000) reported that for Lepidoptera, Azadirachtin, with effective doses (<1 to 50 ppm) caused 50% inhibition of feeding, depending on the species. Mordue (Luntz) and Balckwell (1993) reported that Azadirachtin application to the first instar larvae of carob moth inhibited larval development, growth and caused larval death. Mehaoua et al (2013) reported positive correlation in carob moth larval mortality depending on the doses, irrespective of exposure time to Azadirachtin. Chougourou et al. (2012) reported similar conclusions of dose related mortality. Mehaoua et al (2013) further reported that Azadirachtin, irrespective of dose, reduced the fertility of carob moth females. Manal and Frantisek (2000) reported that although some immature insects might not be killed by Azadirachtin, fertility of emerged adults was reduced. Tang et al (2001) reported that Azadirachtin is more toxic to carob moth than to aphids.

Side effects of neem on biological control agents

Due to relatively weak contact effects of neem-based pesticides, these products are in most cases not harmful or only slightly harmful to the most important natural enemies of pests. Saxena et al (1984) concluded that the wolf spider *Lycosa pseudoannulata*, an important predator of plant and leaf hoppers in rice, was not impacted by the use of neem oil to control these hoppers. Mansour et al (1987) also reported that extract of neem seed kernels was less toxic to the predatory mite *Phytoseiulus persimilis*, compared to the pest spider mites *Tetranychus cinnabarinus*. Srivastava and Parmar (1985) also concluded that predacious coccinellids were not affected by spray formulations with high neem content applied to sorghum, whereas the aphid, *Melanaphis sacchari*, was controlled successfully.

Limitations with neem products

Schmutterer (1990) reported that neem products, like many other botanical products, have limited persistence in field conditions and considered that many environmental factors such as temperature, ultraviolet light, pH on treated plant parts, rainfall and other environmental factors may exert significant negative impact on the active ingredients. Schmutterer also reported that residual effects of most of the neem-based products are in general very short and mostly around five to seven days. This relatively short residual effect of neem products suggests a requirement for multiple applications with intervals of seven to ten days. Many synthetic pesticides also have frequent application interval requirement, so this is not a disadvantage of neem.

Neem-based products might need to be applied in high doses compared to the synthetic pesticides with strong contact effects. Schmutterer (1990) further reported that several neem-based products when applied against adult insects, for example bugs and beetles, do not always lead to obvious immediate mortality, but instead result in substantial reduction in fecundity. Therefore, the user needs to understand that the impact might not be obvious until the following generation.

Conclusions

Neem products primarily work as a larvicide against lepidopteran pests, including carob moth, and any surviving larvae produce moths that have significantly reduce female fertility. This suggests that

application of sprays in conjunction with mating disruption may be worth investigation. Considering the mode of action of the neem products and their relatively low toxicities on natural enemies, we recommend evaluating Azadirachtin and related products under Australian field conditions to determine the practical application potential for use in IPM for almond pests.

Although the relatively short residual activities of neem and related products could be considered a disadvantage from the economic point of view for the Australian almond industry, their application in conjunction with mating disruption may overcome this limitation. Future research in the practical use of Azadirachtinin should also consider a better formulation to increase longevity and therefore probably their effectiveness.

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Part 3. Table of alternative pesticides.

(see next page)

Table 1. A list of the commercially available insecticides registered on almond in the USA and Australia has been compiled. The off-target toxicity and impact on many beneficial insects and predatory mites is documented. The table lists registered chemicals and their IRAC mode of action (www.irac-online.org). Trade names are not used . Ratings for pollinators are as follows: I, Do not apply or allow to drift to plants that are flowering (Red). II, Do not apply or allow to drift to plants that are flowering, except when the application is made between sunset and midnight if allowed by the label and regulations (Orange). III; No bee precaution, except when required by the label or regulations (Green). IRAC mode of action, Groups and Sub-Group allow users to identify products with the same mode of action.

From Table 1 we selected chemicals that are not registered for use in almond in Australia but are used against caterpillars by the US almond industry and have low-moderate impact on beneficials and pollinators. These chemicals, are listed in Table 2. We suggest further study on these chemicals to generate efficacy data.

Table 1: Pesticides registered for use on almonds in Australia and/or USA

Active ingredients	Mode of action classification/group number	Country where registered		Targeted Physiology	Target pests in Almonds	Relative toxicity and Duration of impact on natural enemies								
		USA	Australia			Pollinators	Predatory mites	Predatory beetles	Lacewings	Parasitic wasps	Trichogramma	Predatory bugs	Entomopathogenic nematodes	Duration
Abamectin	6	x	x	Nerve and Muscle	Mites, leafminers	i	H	M/H	H	H	H	H	L	short
Acequinocyl	20B	x		Respiration	Mites	iii	L	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Bacillus thuringiensis ssp. Kurstaki	11A	x		Midgut	Caterpillars	iii	L	L	L	L	L	L	L	Short
Bifenazate	20D	x	x	Respiration	Mites	ii	H	L	L	L	(-)	L	(-)	Short
Bifenthrin	3A			Nerve and Muscle	insects, mites	i	H	H	H	H	H	H	L	
Buprofezin	16	x		Growth and Development	Sucking insects, beetles	ii	L	M	L	L		H	L	Long
Carbaryl	1A	x		Nerve and Muscle	Insects, mites	i	L/H	H	M/H	H	H	H	L	Long
Chlorantraniliprole	28		x	Nerve and Muscle	Carob moth	iii	L	H	L	L/M	L	L	(-)	Short
Clothianidin	4A		x	Nerve and Muscle	Aphids, CM, OFM and Carpophilus ?	i	M/H	H	H	H	(-)	(-)	(-)	(-)
Chlorpyrifos	1B	x		Nerve and Muscle	Insects, mites	i	L	L/M	H	L	H	L/M	H	Moderate
Clofentezine	10A	x	x	Growth and Development	Mites	iii	L	L	L	L	L	L	(-)	Short
Cyfluthrin	3A	x		Nerve and Muscle	Insects, mites	i	H	H		H		H		Moderate
Diazinon	1B	x		Nerve and Muscle	Insects, mites	i	L	H	H	H	H	H	L	Moderate tp long
Diflubenzuron	15	x		Growth and Development	Caterpillars	ii	L	H		L		H	(-)	(-)
Emamectin benzoate	6	x		Nerve and Muscle	Caterpillars	i	(-)	(-)		(-)		(-)	(-)	(-)
Esfenvalerate	3A	x		Nerve and Muscle	Insects, mites	i	H	M		H		H	(-)	Moderate
Etoxazole	10B	x	x	Growth and Development	Mites	ii	H	(-)	H	L	(-)	H	(-)	Very long especially to predatory mites
Fenbutatin oxide	12B	x		Respiration	Mites	iii	L	L		L		L		Short
Fenpropathrin	3A	x		Nerve and Muscle	Insects, mites	i	H	H		H		H	(-)	(-)
Fenpyroximate	21A	x		Respiration	Mites and some insects	iii	H	L		L		L	(-)	Very long especially to predatory mites
Hexythiazox	10A	x		Growth and Development	Mites	ii	L	L		L		L	(-)	Short to moderate
lambda-cyhalothrin	3A	x		Nerve and Muscle	Plant bugs, beetles, caterpillars	i	H	H		H		H	(-)	Moderate
Metaflumizone	22B	x		Nerve and Muscle	Ants	iii	L	L	L	L		H	(-)	(-)
Methoprene	7A	x		Growth and Development	Ants	iii	L	L		L		L	(-)	(-)
Methoxyfenozide	18	x	x	Growth and Development	Caterpillars	ii	L	L	L	L	(-)	H	L	None
Parafinic Oil	(-)			Unknown or Non-Specific	Mites and San Jose scale	iii	(-)	(-)	(-)	H	(-)	(-)	(-)	
Phosmet	1B	x		Nerve and Muscle	Insects, mites	i	H	H		H		H		Moderate to long
Petroleum Oils	(-)		x	Unknown or Non-Specific	Insects, mites	ii	M/H	L	L	M/H	(-)	(-)	L	Short to none
Pirimicarb	1A		x	Nerve and Muscle	Aphid	iii	M/H	L	L	M/H	H	L/M	(-)	
Propagite	12C	x		Respiration	Mites	iii	M	L		L		L	(-)	Short
Pymetrozine	9B		x	Nerve and Muscle	Aphids	iii	L	L	L	M/H	(-)	L	(-)	
Pyriproxyfen	7C	x		Growth and Development	Scale, beetles	ii	L	H		L		H	(-)	Long
Spinetoram	5	x		Nerve and Muscle	Caterpillars, aphid, scale	ii	L/H	M		L/M		M	(-)	Moderate
Spinosad	5	x		Nerve and Muscle	Caterpillars, aphid, scale	ii	L/H	M		L/M		M	(-)	Short to moderate
Spirodiclofen	23	x		Growth and Development	Mites	ii	L	(-)		(-)		(-)	(-)	(-)
Sulfur		x		Unknown or Non-Specific	Mites and thrips	iii	L/H	(-)		H		L/M	(-)	Short
Sulfoxaflor	4C		x	Nerve and Muscle	Apids	iii	L	L	L	H	(-)	M/H	(-)	

Relative toxicity	H = High	L= Low	(-) = No information

Duration
Short= Hours to days
Moderate= Days to two weeks
Long= Many weeks to months

Table 2: Suggested list of alternative pesticides for further study in Australia. These pesticides are registered to use by the US almond industry to control Lepidopteran pest.

Active ingredients	**Mode of action classification/group number**
Bacillus thuringiensis ssp. Kurstaki	11A
Diflubenzuron	15
Methoxyfenozide	18
Spinetoram	5
Spinosad	5