THE FUNGUS *LECANICILLIUM LECANII* COLONISES THE PLANT *GOSSYPIUM HIRSUTUM* AND THE APHID *APHIS GOSSYPII*

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Abstract

The purpose of the research reported here was to determine whether the fungus *L. lecanii*, which was isolated as an endophyte from cotton (*Gossypium hirsutum*), may readily colonise cotton, an aphid pest of cotton (*Aphis gossypii*), and transfer from plant to aphid, and from aphid to plant.

L. lecanii from agar culture and growing on or in alternative hosts was used to inoculate the aphid *A. gossypii* and cotton *G. hirsutum.* Colonisation was assessed by isolating the fungus from the surface or from within each host. *L. lecanii* colonised each host from spores, and transferred from aphid to cotton and cotton to aphid. The fungus sporulated on the surface of both hosts. Internal colonisation of each host increased over time under certain conditions.

In conclusion, the entomopathogen *L. lecanii* readily colonised two potential hosts and transferred between the hosts under experimental conditions. *L. lecanii* is widely used in glasshouses to control aphids. The potential for the entomopathogen to survive in field conditions is indicated by these results. If the outcome is supported under field conditions, the fungus may be used to reduce the impact of a pest of cotton in Australia.

Key words: *Verticillium lecanii,* endophyte, epiphyte, entomopathogen, tripartite interaction, biocontrol.

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Introduction

A wide variety of fungi, referred to as endophytes, are asymptomatic colonists of plants. The relationship between endophyte and plant is understood in a few cases. For instance, seed-transmitted *Neotyphodium* spp. colonise grasses, modifying interactions between plant and insects. Laterally transmitted endophytes appear less specific in association, and they must grow in the presence of hostile plant metabolites (Schultz *et al.* 1999).

The fungus *Lecanicillium lecanii* (Zimmerman) Zare & Gams was isolated from within asymptomatic leaves of cotton *Gossypium hirsutum* L. taken from an irrigated field near Narrabri, Australia (McGee 2002). Some isolates of this fungus are mycoparasites (Kim *et al*. 2007). However, the absence of other fungi at isolation would suggest that our

isolate was not associated with another fungus. *L. lecanii* is also an entomopathogen used to control aphids and whiteflies in glasshouses (Inglis *et al.* 2001) where high humidity prevails (Gillespie & Claydon 1989). The lifecycle of *L. lecanii* is poorly documented.

Endophytic colonisation of maize (Bing & Lewis 1992) and epiphytic occupation of leaf surfaces (Meyling & Eilenberg 2006) by the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin indicates that some entomopathogens may persist on plants in the absence of insect hosts. The plant provides the fungus with a nutritious, stable and long lived (relative to an insect) environment, with the added advantage that contact with an insect host is possible. Thus *L. lecanii* may have two hosts: insect and plant. Alternatively, the isolate of *L. lecanii* from cotton may be an endophyte lacking the capacity to colonise an insect host.

The potential for transfer by *L. lecanii* between different hosts is unknown. If transfer takes place, then the tripartite interaction may have potential to regulate damaging populations of insect pests in the field. Interactions between three different organisms are simpler to study in controlled conditions than the field. The purpose of the research reported here was to determine whether *L. lecanii* may readily colonise cotton *G. hirsutum*, an aphid pest of cotton *Aphis gossypii* Glover, and transfer from plant to aphid, and from aphid to plant.

Materials and methods

A colony of *A. gossypii* was established on cotton seedlings (cv. Sicala V2) growing in sterilised potting medium (McGee *et al*. 1997) and maintained in a growth cabinet with a 13/11 h day/night cycle (28/22 C). Photosynthetically active radiation was approximately 350 µmol m^{-2} s^{-1} at the soil surface.

L. lecanii from cotton (McGee 2002) was cultured on potato dextrose agar (PDA) at 23°C. A spore/mycelium suspension (approx. 1 × 10^7 spores mL^{-1}) was harvested from cultures in sterile de-ionised water and used to inoculate aphids and leaves of cotton (Kanagaratnam *et al*. 1982).

The potential for *L. lecanii* to colonise *A. gossypii*

A surface sterilised leaf of cotton (cv. Sicala V2), from a plant maintained in an aphid-free growth cabinet, was placed on a disk of moistened sterile filter paper in a Petri dish (15 cm diameter). Twenty medium-sized adult apterous aphids were placed on the leaf. Pilot studies indicated that aphids readily fed on excised leaves for up to 14 d. Aphids were sprayed either with a spore suspension of *L. lecanii* or sterile de-ionised water as the control. Petri dishes were sealed with Parafilm™. After 7 d, mortality among aphids was calculated as the number of dead aphids in each of five replicate Petri dishes expressed as a percentage of the total number of live and dead aphids in the dish. A sub-sample of live aphids from inoculated and uninoculated dishes was either surface sterilised by dipping briefly in ethanol (70% w/v), or taken directly from a dish, and pressed gently into PDA. The aphids were incubated at 23°C for 7 d and emergence of *L. lecanii* noted. The experiment was repeated. Pooled *t*-tests were used to detect differences in mortality.

The potential for *L. lecanii* to colonise cotton

Treatments

Cotton leaves may be colonised by airborne spores of the fungus. Alternatively, the fungus may be vectored by aphids. Growth and subsequent colonisation of cotton by the fungus may be enhanced by the presence of aphid exoskeletons and honey dew, which are both common on aphid infested plants. Endophytic colonisation of cotton may be enhanced by the disruption of the epidermis that occurs when aphids feed (Pollard 1973). Therefore we designed treatments to mimic conditions at the leaf surface that may occur during aphid infestation. Sucrose (2% w/v) was added to the inoculum to provide additional energy to fungal spores for colonisation of leaves as would be present on leaves saturated with aphid honey dew. Chitin (0.025 g mL^{-1}) was added to inoculum to simulate shed aphid exoskeletons. Fine emery paper was gently pressed once against the adaxial leaf surface to cause minor disruption of the epidermis and allow direct penetration of fungal spores into the leaf. This “leaf

damage" treatment was not a direct simulation of aphid damage, as this is not possible. It was intended to allow easier access of fungal spores into the leaf during inoculation, as is expected to occur on leaves damaged by aphid feeding.

Inoculum of *L. lecanii* with the surfactant Pulse™ at a rate of 0.02% w/v was applied to the adaxial leaf surface with a camel hair brush. Seven treatment combinations were chosen: 1) spores in sucrose; 2) spores mixed with chitin; 3) spores in water applied to a damaged leaf; 4) spores mixed with chitin applied to a damaged leaf; 5) spores in sucrose applied to a damaged leaf; 6) spores in sucrose and chitin with no leaf damage; and 7) spores in sucrose and chitin with leaf damage. We controlled these treatments by applying spores in water with Pulse™ to undamaged leaves. Leaves were wrapped with clear plastic food wrap following inoculation to retain high relative humidity at the leaf surface. The plastic wrap was removed after 2 d.

Each treatment was randomly applied to a half-leaf (as delineated by the mid-vein) of the four youngest fully expanded leaves on a plant at the six true leaf stage. Each half leaf was considered to be one replicate. Five groups of five plants were inoculated, so there were five replicates (half leaves) per treatment in each group of five plants. The five groups would then be randomly selected at different points in time for re-isolation of the fungus.

Isolation

Five plants were randomly chosen and leaves removed for isolation of *L. lecanii* at seven, 14, 28, or 35 d after inoculation. Each half-leaf was pressed against PDA to determine whether the fungus was present on the leaf surface. Leaves were then surface-sterilised and incubated on PDA to determine whether the fungus was present inside the leaf. A sub-sample of surface sterilised leaf fragments was pressed against PDA to determine the effectiveness of surface sterilization (Dingle & McGee 2003). Plates were kept at 23°C for 7 d and then examined microscopically for the emergence of *L. lecanii*. The frequency of isolation of *L. lecanii* from the surface and within leaves was compared using likelihood Chi-square analysis to determine dependency between isolation and inoculation treatment. A sub-sample of inoculated leaves was also examined microscopically to determine where spores were located, whether or not spores had germinated and grown into the leaf, and where the fungus penetrated the leaf surface.

Transfer of *L. lecanii* from inoculated aphids to uninoculated cotton

Five leaves, heavily infested with *A. gossypii*, were each placed on disks of moistened filter paper in Petri dishes. Aphids were sprayed with a suspension of *L. lecanii* and dishes were sealed with Parafilm™. After 24 h, 15 aphids from each dish were carefully transferred with a camel hair brush onto each of five fresh uninoculated cotton leaves under the same experimental conditions. The brush was briefly soaked in 70% ETOH to kill any spores on the brush. After 6 d, aphids were removed from leaves, leaves were cut into fragments, pressed against PDA to determine colonisation of the leaf surface, and then surface-sterilized in 70% ETOH and 5% hypochlorite for 5 minutes and placed on PDA to determine internal colonisation by *L. lecanii*. A sub-sample of aphids was also pressed into PDA, or surface sterilised by briefly soaking in 70% ETOH and then pressed into PDA, incubated for 7 d, and examined for emergence of *L. lecanii*.

Transfer of *L. lecanii* from inoculated cotton to uninoculated aphids

Leaves of similar size and age on cotton plants were either 1) inoculated with *L. lecanii,* or 2) painted with sterile distilled water, or 3) left untreated. Experimental leaves were excised from the plant 7 d after inoculation, rinsed in sterile distilled water, and placed adaxial side down on a moistened 5 cm disk of Whatman no. 1 filter paper in a Petri dish (15 cm diameter). Ten uninoculated aphids were placed on each leaf. Dishes were sealed with Parafilm. After 7 d, a sub-sample of aphids from each treatment was placed on PDA, or surface sterilised and then placed on PDA. A sub-sample of leaf fragments from each treatment was also pressed against PDA, and then surface-sterilized and placed on PDA. Aphids, leaf presses and leaf fragments were incubated for one week and then examined for emergence of *L. lecanii*.

Results

The potential for *L. lecanii* to colonise *A. gossypii*

The mortality of inoculated aphids was 50 and 56% in each experiment. The mortality of uninoculated aphids was 15 and 10% in each experiment respectively, and these differences were significant in each experiment ($P < 0.01$). *L. lecanii* was isolated from two of 15 and 6 of 25 inoculated aphids previously surface sterilized with ethanol, and from 14 of 20 and 15 of 15 un-sterilized aphids, respectively. The fungus was not isolated from any uninoculated aphids. The fungus was also observed growing from the exoskeleton of living aphids and was apparent on shed exoskeletons and cadavers. Some colonised, dead aphids were attached by hyphae to the inner surface of the Petri dish.

The potential for *L. lecanii* to colonise cotton

Endophytic colonisation

Isolation of *L. lecanii* from surface sterilised cotton leaves was dependent upon the method used to apply inoculum (Likelihood $\chi^2 = 68.62$, $p < 0.001$, df = 7) and the length of time from inoculation to isolation (Likelihood $\chi^2 = 30.09$, $p < 0.001$, df = 4). *L. lecanii* was isolated more frequently from damaged surface sterilised leaves ($y = 103.875x - 0.75$, $r^2 = 0.316$, $n = 16$, $P = 0.233$) than undamaged surface sterilised leaves ($y = 53.813x - 1.06$, $r^2 = 0.574$, $n = 16$, $P = 0.02$) over time. The fungus was isolated from 100% of damaged leaves after 35 d but much less frequently from undamaged leaves.

Epiphytic colonisation

Inoculation treatment did not influence isolation of *L. lecanii* from the surface of leaves (Likelihood $\chi^2 = 11.82$, $p = 0.107$, df = 7). Frequency of isolation of the fungus from the leaf surface decreased as time from inoculation increased (Likelihood $\chi^2 = 45.16$, $p < 0.001$, df = 4). The decrease was linear over time ($y = 114x - 2.068$, $r^2 = 0.63$, $n = 32$, $P < 0.001$).

Microscopy

Spores of *L. lecanii* were aggregated adjacent to veins of inoculated leaves. Spores germinated on the leaf epidermis and appeared to penetrate directly from appressoria on the surface of epidermal cells and between epidermal cells.

The potential for *L. lecanii* to transfer from inoculated aphids to uninoculated cotton

L. lecanii was isolated from the surface of 100% of leaf fragments, and from within an average of 35% of leaf fragments after 7 d. The fungus was also isolated from 82% of non surface-sterilised aphids, but not from surface sterilised aphids.

Transfer of *L. lecanii* from inoculated cotton to uninoculated aphids

L. lecanii was isolated from 75% of aphids on inoculated leaves and from no aphids from uninoculated leaves. The fungus was not isolated from surface-sterilised aphids. The fungus was present at the surface of all inoculated leaves, and was detected on one uninoculated leaf, indicating minor cross-contamination potentially during the isolation process.

Discussion

An apparently endophytic isolate of *L. lecanii* colonised leaves of cotton and aphids under the experimental conditions. The fungus transferred from leaf to insect and insect to leaf. Though the degree of colonisation differed between experiments, the fungus killed aphids, and did not cause obvious disease in leaves of cotton. While the fungus did not always kill aphids within 7 d, in two experiments direct inoculation with spores resulted in substantial mortality, indicating that density of inoculum and time are directly related to aphid mortality. While the fungus did not persist for long on the leaf surface, it was isolated from within leaves up to 35 d after inoculation suggesting an endophytic life strategy. Longer incubation and leaf damage increased the likelihood of the inoculated fungus being recovered from within the leaf suggesting that insects are important in the establishment of this isolate of *L. lecanii* as an

endophyte in cotton. Microscopic examination confirmed the presence of the fungus on the surface of leaves and indicated that spores of the fungus germinated and grew on the leaf surface, and penetrated the leaf following inoculation. While leaf damage increased colonisation, germ tubes of the fungus directly penetrated surfaces of leaves, indicating possible fungal colonisation of intact leaves. *L. lecanii* has the potential to colonise both the leaves of cotton and one aphid pest of cotton, confirming the status of the isolate of *L. lecanii* used in this study as both endophyte and entomopathogen.

The high humidity under our experimental conditions was intended to enhance colonisation of plant and insect, and to investigate the possibility of transfer between hosts. Colonisation of insects by *L. lecanii* is increased under conditions of high humidity (Gillespie & Claydon 1989). Utilization of *L. lecanii* as a biocontrol agent of *A. gossypii* in the field will require periods of high humidity to facilitate colonisation of insect and plant. Extended periods of high humidity do occur in cotton fields in Australia, specifically during the middle of the growing season, when the canopy of the crop is closed and the crop is irrigated weekly (pers. comm. Dr L.J. Wilson CSIRO Entomology, Narrabri, Australia). In addition, *A. gossypii* is most commonly found in the lower canopy (Hardee *et al*. 1994), where humidity is high, thus indicating potential for direct contact between the fungus and aphid within the closed canopy.

Conidia of *Lecanicillium* spp. are unlikely to survive exposure to environmentally realistic levels of ultraviolet (UV) radiation (Braga *et al*. 2002). Sadras & Wilson (1997) demonstrated that the canopy of a mid-late season cotton crop can intercept > 80% of photosynthetically active radiation. Consequently, it is unlikely that conidia in the lower canopy are exposed to deleterious levels of UV radiation. However, this remains to be tested.

These findings indicate potential for an interaction between *L. lecanii*, *A. gossypii* and cotton in the field that may explain the isolation of endophytic *L. lecanii* from field grown cotton. Colonisation of the plant provides the fungus with a stable and nutritious insect-attracting environment. If the aphid reached pest densities, leaf damage may increase the likelihood of fungal colonisation of plant tissues. In addition, the presence of endophytic colonies might increase colonisation of aphids. Colonised aphids may disperse the fungus between plant tissues, and plants, becoming foci of inoculum *post mortem*. Lastly, the fungus may also parasitise fungi associated with plants and aphids. These hypotheses remain to be explored under field conditions.

The presence of the fungus within the plant may induce plant responses (Schultz *et al*. 1999) resulting in changes in aphid behavior (Underwood 1999). Thus good reason exists to explore the potential for this fungus to reduce feeding and/or reproduction of aphids in the field.

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