

Characterization of Spanish strains of *Verticillium lecanii*

Luis Vicente Lopez-Llorca and Toñi Carbonell

Departamento de Ciencias Ambientales y Recursos Naturales, Universidad de Alicante, Spain

Summary

We have characterized biologically and physiologically eight *Verticillium lecanii* strains from several origins including insect pests. Of all the temperatures tested, 25°C was the best for growth and at 40°C none of the strains could grow. At 4 and 7°C, growth was reduced in comparison to warmer temperatures. The strains had better development at pH close to 7 ($F = 27,64$, $P < 0,01$) than at pH 3. Self-inhibition of germination of strain 50 was found when more than 0.78 conidia/cm² were plated on corn meal agar (CMA). Germination of conidia was close to 100% for all strains except one, three days after inoculation. Among extracellular enzymatic activities studied the fungal strains showed strongest proteolytic activities followed by lipolytic and chitinolytic activities. Some strains showed significant differences ($P < 0.05$) in conidia production. Most of the fungicides tested (especially benomyl) inhibited radial growth of strain 50 on CMA. Pathogenicity (as median lethal time, LT₅₀) of *V. lecanii* strains on larvae of *Galleria mellonella* varied from 2.66 ± 0.33 to 4.27 ± 0.25 days. We conclude that *in vitro* tests *per se* are not sufficient to select the best biocontrol strains of entomopathogenic fungi. Pathogenicity is a complex process in which the presence, timing and regulation of many factors including those covered in this paper, as well as their interactions, are probably involved.

Key words

Entomopathogenic Fungi, *Verticillium lecanii*, Strain characterization

Caracterización de cepas españolas de *Verticillium lecanii*

Resumen

Se han caracterizado biológica y fisiológicamente ocho cepas de *Verticillium lecanii* de diferentes orígenes, incluyendo plagas de insectos. De las temperaturas ensayadas, 25°C fue la óptima de crecimiento mientras que 40°C fue la peor ya que impidió el desarrollo fúngico. A 4 y 7°C, existió crecimiento aunque reducido respecto a temperaturas superiores. *V. lecanii* creció mejor a pH cercano a 7 ($F = 27,64$, $P < 0,01$) que a pH 3. Se produjo autoinhibición de la germinación en agar extracto de maíz (AEM) con más de 0,78 conidios/cm². La germinación de conidios fue cercana al 100% en todas las cepas estudiadas excepto en una. Los ensayos en medio sólido mostraron que la actividad proteolítica extracelular de las cepas fue la más temprana y abundante de las estudiadas, seguida de la lipolítica y quitinolítica. Algunas cepas mostraron diferencias significativas ($P < 0,05$) en la producción de conidios. La mayoría de fungicidas ensayados (especialmente benomilo) inhibieron el crecimiento en AEM de la cepa 50 de *V. lecanii*. La patogenicidad (tiempo letal medio, TL₅₀) de *V. lecanii* sobre larvas de *Galleria mellonella* fue de $2,66 \pm 0,33$ a $4,27 \pm 0,25$ días. Podemos concluir que los ensayos *in vitro per se* no proporcionan información suficiente para seleccionar cepas de hongos entomopatógenos. La patogenicidad fúngica es un proceso complejo en el que están probablemente implicados la presencia, regulación y efecto en el tiempo de muchos factores, incluidos los de este estudio.

Dirección para correspondencia:

Dr. Luis Vicente López-Llorca
Departamento de Ciencias Ambientales y Recursos
Naturales, Universidad de Alicante, Apdo. Correos 99,
03080 Alicante, Spain
Tel.: +34 96 5903400 ext. 3381; Fax.: +34 96 5903815;
E-mail: lvlopez@carn.ua.es

Aceptado para publicación el 9 de febrero de 1999

Adequate development of entomopathogenic fungi as biocontrol agents requires a selection schedule of species and strains adapted to specific pests, environmental conditions and crops. Laboratory bioassays may play an important role in such a screening process. Both biology and physiology of entomopathogenic fungi depend on environmental parameters such as temperature and relative humidity [1] or nutrient availability [2]. This is probably the reason why entomopathogens are most successful in controlled environments such as greenhouses [3]. Adverse environmental conditions such as low moisture, UV radiation, extreme temperatures or chemicals such as some fungicides may effect negatively the performance of mycoinsecticides [4]. For practical use of these antagonists a sound knowledge of their behaviour in respect to environmental factors is then of paramount importance. In many instances the best practice to control an insect pest is to obtain the entomopathogens from the same host or its environment [4].

Temperature is the environmental factor that affects most the development of entomopathogenic fungi. Its optimum value for most of them is between 25 and 30°C [5]. Soil temperature and organic matter are amongst the most important factors influencing viability of conidia in soil [6]. Because of the extreme temperatures in which insect hosts can live, it is desirable to find entomopathogenic fungi capable of growing at a wide range of temperatures [7]. Temperature also affects the virulence of *Metarhizium anisopliae* [7]. Conidium germination and fungal development on the insect cuticle are closely influenced by temperature [5,7]. Temperature optimum for growth may be different from the optimum for insect infection [7]. However, the former may be used as a screening criterion for strain selection together with other parameters. Other environmental parameters such as pH have also been found to affect both growth and physiology of nematophagous and entomopathogenic fungi [8].

The insect cuticle, the main barrier to infection, is mainly composed of protein, chitin and lipids [9]. Insect pathogens such as entomopathogenic fungi produce extracellular enzymes that degrade the chemical components of the cuticle [9]. Although pathogenicity is a multicomponent process, the production of cuticle-degrading enzymes can help in the infection process [9,10].

One of the most important factors to take into account when selecting antagonistic fungi is their compatibility with commonly used agrochemicals such as insecticides or fungicides [11]. Rosato *et al.* [12] studied the effect of triadimephon and copper oxichloride on *Verticillium lecanii*. They found growth inhibition at high concentration of the chemicals as well as the formation of sclerotia. The effect of benomyl on *V. lecanii* strains parasitic on nematode eggs has also been studied [13].

Finally, any characterization of insect pathogens must include estimations of their pathogenicity. Virulence of entomopathogenic fungi can be measured in bioassays with a given number of pathogen-free standard insect hosts. These are exposed to a known inoculum of the pathogen in standard conditions and the host mortality with time is assessed [14].

In this paper, we present data on the characterization of eight strains of *V. lecanii*. Four of these have been isolated in Spain from several sources (Table 1), the rest are included for comparison. *V. lecanii* is a well-known entomopathogen against important insect pests such as aphids [15-19]. Many experiments of control with the fungus in controlled environments such as glasshouses have been carried out successfully in many countries [17, 20]. We have studied the effect of temperature and pH on the

Table 1. *Verticillium lecanii* strains used in this paper together with their origin and place of isolation. Abbreviations: CBS (Centraalbureau voor Schimmelcultures, Dutch Fungal Collection), SCRI (Scottish Crop Research Institute).

Strain number	Host or origin	Place of isolation
41	Unknown	Unknown
50	Unknown	Unknown
55	Moss with mites	Garden of CBS, Baarn, The Netherlands
57	Aphid	SCRI, Dundee, Scotland
131	Trialeurodes sp.	Cabrils, Barcelona, Spain
132	Bemisia sp.	Cabrils, Barcelona, Spain
139	Saissetia oleae	Denia, Alicante, Spain
141	Forest soil	Font Roja, Alicante, Spain

growth on solid media of the strains. Physiological factors closely related to pathogenesis such as germination (including self-inhibition of conidia) also have been studied. Extracellular enzyme production, sporulation, conidial size as well as compatibility with commonly used agrochemicals are also given. We have finally studied the pathogenicity of the *V. lecanii* strains on larvae of the lepidopteran *Galleria mellonella* and discuss the results obtained.

MATERIALS AND METHODS

Entomopathogenic fungi. For experiments *V. lecanii* strains belonging to the Collection of the Phytopathology group, Departamento de Ciencias Ambientales y Recursos Naturales of the Universidad de Alicante were used. A list of the strains used in this paper together with their origin and place of isolation is given in Table 1.

Effect of temperature and pH on fungus growth. The effect of temperature on fungus growth was studied on 90 mm diameter Petri dishes with corn meal agar (CMA, Oxoid). Plates were inoculated in the centre with a 5 x 5 mm fragment from the edge of *V. lecanii* colonies from seven-day-old colonies on CMA. These plates were then incubated at 4, 7, 25, 30, 40 °C in the dark. Ten replicate Petri dishes per strain and temperature were prepared. For 25 days the diameter of the fungal colonies was recorded every two days.

The effect of pH on fungus growth was studied on 90 mm diameter Petri dishes with a continuous pH gradient on CMA. One molar citric acid and 2M disodium phosphate solutions were made and sterilized. CMA was prepared in two equal portions and sterilized. After autoclaving, to one half of the medium, sterile 1M citric acid was added (5 ml per 45 ml sterile medium). Sterile 2-molar disodium phosphate was added likewise to the other half of the medium. CMA citric acid with was poured in petri dishes (10 ml per plate). The petri dishes were then tilted so that the medium formed a slope which covered the plate. When CMA with the citrate solidified, the medium containing 2M disodium phosphate (5 ml per 45 ml sterile medium) was added to the plates. By doing this a continuous gradient from pH 3 to pH 7 is formed [21]. On the underside of the plates a line was marked along the diameter [pH gradient] of the plates. The plates were then inoculated with conidia of the fungi by uniform streaking along the line. Three plates per strain were inoculated and incubated at 25 °C in the dark [8]. The growth of the fungus colony was measured for 25 days in two points of the gradient located at 1 cm from the edge of the plate.

Self-inhibition and germination of V. lecanii conidia. Conidia from a seven-day-old colony of *V. lecanii* (strain 50) grown on CMA were collected in sterile distilled water (SDW). Numbers of conidia were estimated and 90 cm diameter Petri dishes with cool (45 °C) CMA were inoculated with 5, 10, 25, 50, 150, 300 or 500 conidia per plate. Ten replicate Petri dishes per conidial inoculum were prepared. Once inoculated, plates were mixed by hand shaking, left to set and incubated at 25 °C in the dark. When conidia germinated, percentages of germination were calculated and maximum conidial dose per plate showing no self-inhibition was assessed. Using this parameter, experimentally established germination rates of *V. lecanii* strains were calculated on CMA as before. Three replicate plates per strain were prepared.

Extracellular enzyme production by V. lecanii strains. The production of proteolytic, chitinolytic, lipolytic and amylolytic extracellular enzymes by *V. lecanii* strains was determined on solid media by estimating the degradation of the corresponding substrates of these enzymes included on the media [22]. Three plates per enzymatic activity and strain were prepared. These were inoculated with mycelial discs and incubated as explained before. Enzymatic activity was expressed as the ratio between colony diameter and the diameter of the halo caused by substrate degradation [18]. Except on plates for detection of amylolytic activity (destructively sampled 10 days after inoculation), colony and halo diameters were scored until the latter merged with the edge of the plate.

Sporulation and conidium size of V. lecanii strains. Dialysis membrane fragments (5 x 5 cm) preweighed and sterilized were placed onto Petri dishes with CMA. Three replicate plates per *V. lecanii* strain were prepared. Conidia were obtained as explained above. Each plate was inoculated with 50 conidia and incubated at 25 °C in the dark for 12 days. Afterwards, membranes were extracted, dried at 50 °C for 30 min and weighed. Fungal biomass on the membranes was then estimated gravimetrically.

Conidia on membranes were then collected by shaking on 0.02 % Tween 20 for 20 min at 215 rpm and counted using standard techniques. Conidial size length and width was scored using an Olympus BH-2 microscope with an ocular micrometer (CWHK 10X/18L). Three sets of 50 conidia each from a 7-day-old colony on CMA for each strain were prepared. Conidium area was calculated by assuming ellipsoid form.

Effect of agrochemicals on the growth of V. lecanii strains. To test the effect of commonly used pesticides in agriculture on *V. lecanii*, selected agrochemicals (Table 2) were included in 90 mm diameter Petri dishes with 10 ml of autoclaved CMA. Agrochemicals were included as 1.25, 2.5, 5, 12.5, 25, 50 and 83 µg (active ingredient) ml⁻¹ of medium. Plates with agrochemicals were inoculated with mycelia of *V. lecanii* (strain 50) and incubated as already explained. Three replicate plates per dose of active ingredient and control (medium with no agrochemicals) were prepared. Colony diameter was scored every two days approximately for 25 days.

Pathogenicity of V. lecanii on insects. Pathogenicity of *V. lecanii* strains on insects was estimated in bioassays using *G. mellonella*. *G. mellonella* adults (Carolina Biological Supply Company, USA) were fed on an artificial diet freshly prepared as in Alves *et al.*, [14]. Insects were reared at 25 °C, 60% relative moisture and a photoperiod of 14 h light-10 h dark [11]. In these conditions insects were able to complete their life cycle in 3-4 weeks. However, only 4th stage larvae (L4) were used for experiments. Entomopathogenic fungi were grown on

Table 2. Agrochemicals used in experiments described in this paper. Abbreviations: W.P.= Wettable powder. E.L.= emulsifiable liquid. All compounds except Dimilin (Insecticide) are fungicides.

Commercial Name	% Active Ingredient	Formulation
Benlate	50% Benomyl	W.P.
Dimilin 25	25% Diflubenzuron	W.P.
Tiotox	80% Thiram	W.P.
Alerte	80% Fosetyl-Al	W.P.
Tecto-45	45% Thiabendazole	E.L.
Ventine	80% Mancozeb	W.P.

CMA at 25 °C in the dark. Conidia for bioassays were collected from 10-day-old cultures with sterile distilled water (SDW). Each insect larvae (L4) was inoculated with a 20 µl SDW drop containing 4 x 10⁴ conidia using a micropipette. Three Petri dishes each containing 10 larvae were inoculated for each strain (Table 1). Insects inoculated with fungi were incubated at 25 °C scoring every two days the numbers of the dead ones until mortality reached 50 % of the larvae in each plate. Pathogenicity of fungal strains was estimated as their average lethal time (LT50) on insect larvae [23].

RESULTS

Effect of temperature and pH on growth of strains of V. lecanii. Strains differed in growth depending on temperature (Figure 1). Of all temperatures tested, 25 °C (Figure 1c) was the best for growth of *V. lecanii* strains. At 40 °C none of the strains could grow. At 4 and 7 °C, most strains could grow (except strains 41 and 131, Figure 1a,b) although colony diameter was minimal. At 30 °C all fungi could grow (except for strain 41). Strains 131 and 139 had the highest growth at this temperature (Figure 1d).

V. lecanii had best development at pH values close to 7 (F = 27,64, P < 0,01) (Figure 2). Strains 41, 50, 57 and 141 did not show big differences in growth with pH. On the contrary, strain 131 had significantly less growth at pH 3 than at pH 7, but produced pigments at pH close to 3, nine days after inoculation. The rest of the strains studied did not show pigment production.

Self-inhibition and germination of V. lecanii conidia. Figure 3 shows decline (self-inhibition) of germination of *V. lecanii* (strain 50), when more than 0.78 conidia. cm⁻² (50 conidia/90 mm diameter Petri dish) were plated on CMA. Germination of conidia using this inoculum (empirically established) was scored for all *V. lecanii* strains. Three days after inoculation, percentage germination was close to 100% for all strains (Figure 4) except for strain 141 isolated from a forest soil (Table 1), with a germination rate of 50%.

Extracellular enzyme production by V. lecanii strains. Production of extracellular enzymes by *V. lecanii* strains is shown in figure 5. Enzymatic activity is expressed as the ratio (C/H) between colony diameter (C) and the diameter of the halo (H) caused by substrate degradation [18]. Therefore C/H values close to one indicate extracellular enzyme activity reduced or absent. Most strains showed high proteolytic activity (Figure 5a) this was followed by lipolytic and chitinolytic activities. Amylolytic activity was scarcely present in the strains studied, except for strain 139 which displayed the lowest C/H ratio (highest activity, value close to 0.4; Figure 5d). Extracellular enzyme production in relation to time by *V. lecanii* strains, was again highest for proteolytic activity reaching the highest values (low C/H ratios) at only

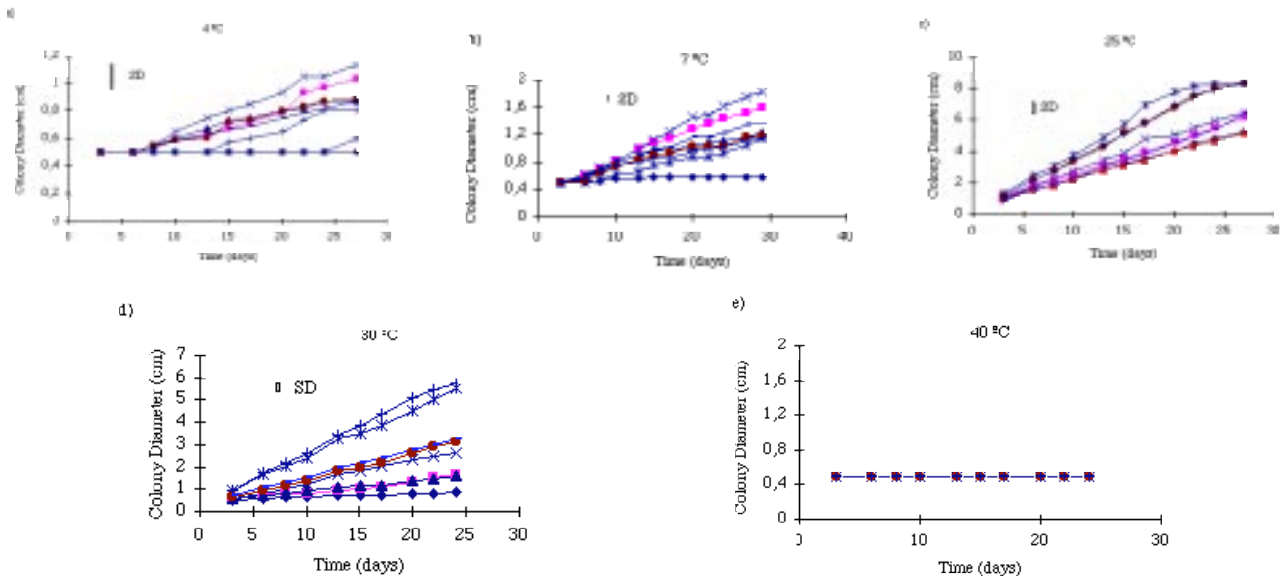


Figure 1. Effect of temperature on the growth of *V. lecanii* strains, ◆ strain 41, ■ strain 50, ▲ strain 55, × strain 57, * strain 131, ● strain 132, + strain 139 and ■ strain 141 (see Table 1 for origin of strains) on CMA. Values represent the radial growth (colony diameter). SD = Standard Deviation.

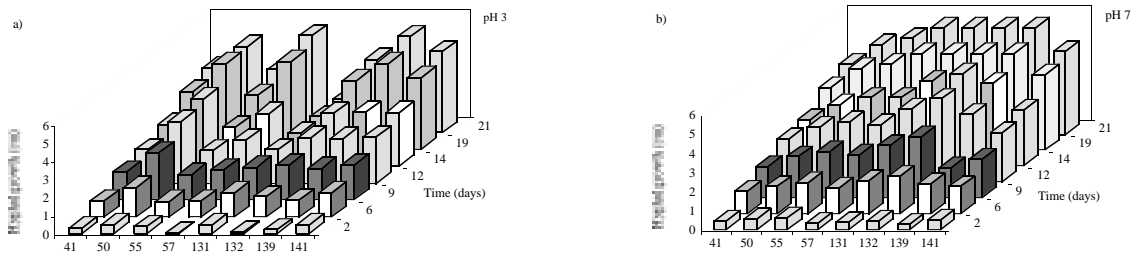


Figure 2. Effect of pH on the growth of eight *V. lecanii* strains (Table 1). The graphs represent the time course of colony growth in two points (pH 3 (a) and (b)) on a continuous pH gradient.

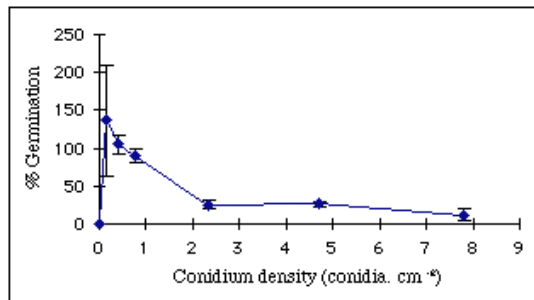


Figure 3. Effect of conidial density on germination of *V. lecanii* (strain 50) conidia (Table 1) plated on CMA. Graph shows decline (self-inhibition) of germination above 0.78 conidia · cm⁻² (50 conidia/90 mm diameter Petri dish). SD = Standard Deviation.

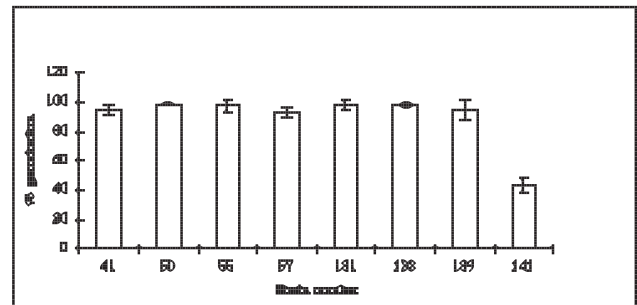


Figure 4. Percentage of conidial germination for eight *V. lecanii* strains (Table 1) on Petri dishes with CMA after inoculating 50 conidia per Petri dish (see Figure 3). Values shown are the average of three replicates. Bars representing standard deviations.

two days after inoculation on the plates. Of all strains tested, number 139 displayed the highest values (C/H ratios close to 0.4) of activity for most extracellular enzymes tested (all except lipase, see Figure 5b).

Sporulation and conidial size. In the assays of sporulation *V. lecanii* strains 55 and 141 showed the highest conidial densities respect to the rest. On the contrary, strains 57 and 131 showed the lowest conidial production per microgram of dry mycelium (Figure 6). There were significant differences ($P < 0.05$) between strains 55 and 57 ($F = 2.84$) and also between strains 55 and 141 ($F = 3.37$). Respect to conidial size (Figure 7), all strains studied virtually had the same value (approximately $2 \mu\text{m}^2$).

Effect of agrochemicals on the growth of *V. lecanii* strains. Most of the fungicides tested inhibited, to some extent, radial growth of *V. lecanii* (strain 50) on CMA. Conversely, thiram and the insecticide (diflubenzuron) had no effect on the growth of the fungus (Figures 8c&8f). Benomyl was the most inhibitory fungicide (Figure 8a) followed by thiabendazole (Figure 8d) and mancozeb (Figure 8b) which were less effective in reducing fungus growth. For instance, low concentrations of benomyl ($1.25 \mu\text{g} \cdot \text{ml}^{-1}$) caused growth inhibition whereas higher concentrations of mancozeb ($83 \mu\text{g} \cdot \text{ml}^{-1}$) were required to obtain the same effect. Fosetyl-Al (Figure 8e) inhibited growth of *V. lecanii* at concentrations above $25 \mu\text{g} \cdot \text{ml}^{-1}$. Thiram even promoted fungal growth (Figure 8c).

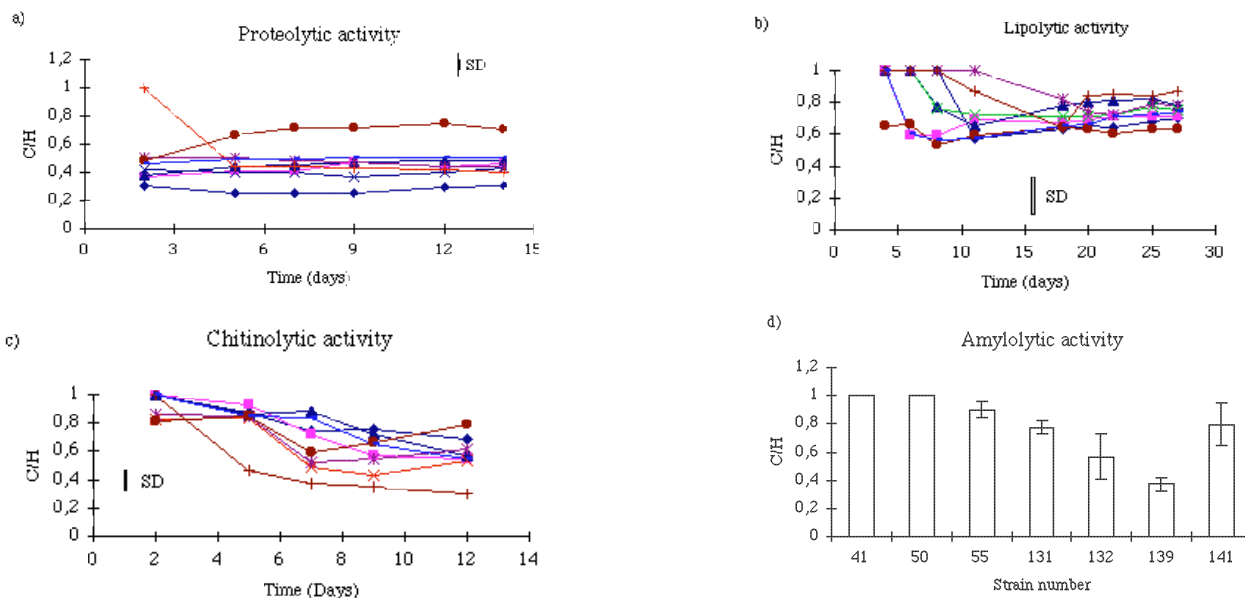


Figure 5. Time course of extracellular enzyme production by eight *V. lecanii* strains (◆—strain 41, ■—strain 50, ▲—strain 55, ✕—strain 57, *—strain 131, ●—strain 132, +—strain 139 and ▣—strain 141 (Table 1) on solid media containing the corresponding substrates. Enzymatic activity is expressed as the ratio (C/H) between colony diameter (C) and the diameter of the halo (H) caused by substrate degradation (Jackson *et al.*, 1985). Proteolytic (a), lipolytic (b), chitinolytic (c), and amylolytic activities (d). SD = Standard Deviation.

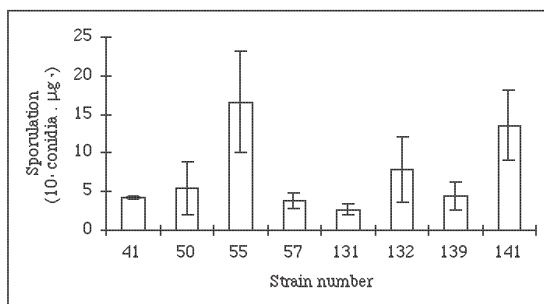


Figure 6. Sporeulation (conidial density) of eight *V. lecanii* strains (Table 1) 12 days after inoculation on CMA plates. SD = Standard Deviation.

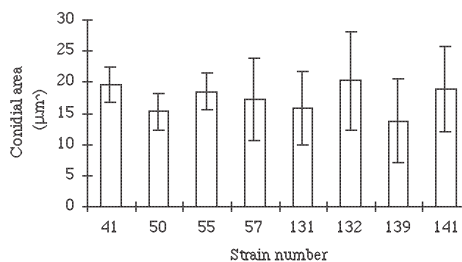


Figure 7. Conidial surface of eight *Verticillium lecanii* strains (Table 1). Values are the average of three estimations. Conidial area was calculated by assuming ellipsoid form.

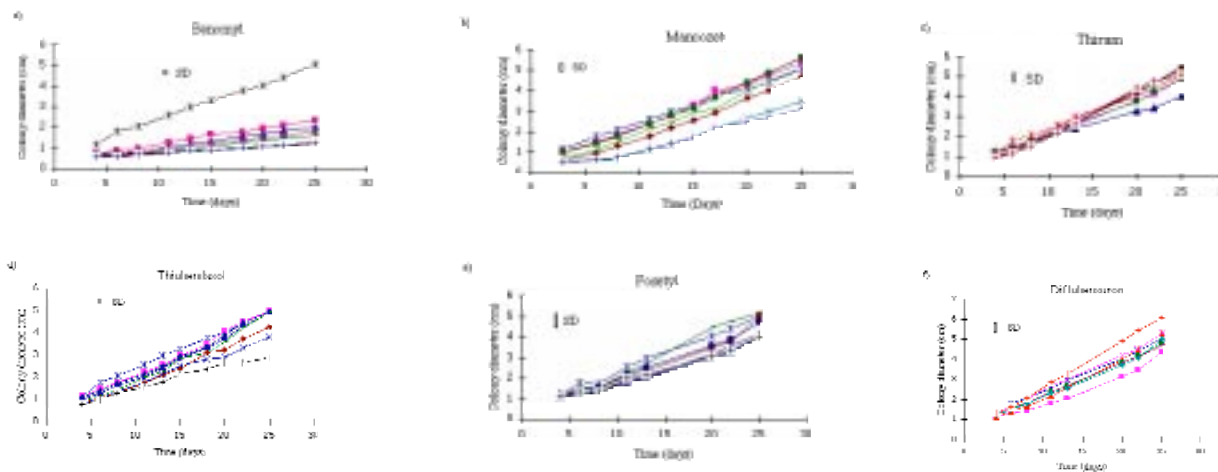


Figure 8. Effect of agrochemicals on the growth of *V. lecanii* strain 50 (see Table 1). Values are the average colony diameter (three estimates) of fungi growing on CMA control plates (with no chemicals) or medium with increasing concentrations of *fungicides or an **insecticide (—* 0 µg.ml⁻¹, —■— 1.25 µg.ml⁻¹, —▲— 2.5 µg.ml⁻¹, —●— 5 µg.ml⁻¹, —▣— 12.5 µg.ml⁻¹, —◆— 25 µg.ml⁻¹, —✕— 50 µg.ml⁻¹ and —+— 83 µg.ml⁻¹ active ingredient per ml of medium). *Fungicides: Benomyl (a), Mancozeb (b), Thiram (c), Thiabendazole (d), Fosetyl-Al (e). **Insecticide: Diflubenzuron (f). Note: For commercial names of the agrochemicals see Table 2. SD = Standard Deviation.

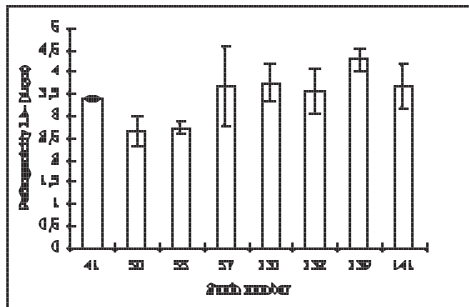


Figure 9. Pathogenicity of eight *V. lecanii* strains on *Galleria mellonella* larvae (L4). Values are lethal time 50 (LT50) in days.

Pathogenicity of *V. lecanii* on insects. Strains of *V. lecanii* varied in pathogenicity when tested against larvae of the lepidopteran *G. mellonella* (Figure 9). Strains 50 and 55 were the most pathogenic ones with LD50 of 2.66 ± 0.33 and 2.73 ± 0.15 days respectively. The pathogenicity of these strains was significantly ($P < 0.05$) higher than the rest, except for strain 41. The least pathogenic strain was number 139, with an LD50 of 4.27 ± 0.25 days.

DISCUSSION

The *V. lecanii* strains studied are mesophilic like most entomopathogens [7]. Their temperature range for growth is in fact rather broad (8–30 °C). The optimum for growth is between 15–30 °C which is also the best for growth of many entomopathogenic fungi [7]. There is also a direct relationship between the maximum and minimum temperature and the climatic conditions of the original environment where the fungus was isolated from [7].

Neutral pH (close to 7) also was better for growth for most strains than acidic values (pH 3). Some strains responded to adverse conditions by producing diffusible pigments into the medium. These findings are similar to those obtained by López-Llorca *et al.* [8] for nematophagous and entomopathogenic fungi. Acidic requirements for growth and pigment production for the entomopathogenic *Beauveria bassiana* have been reported [24].

Conidia of most fungal species germinate less when present at high densities, even in a medium which would otherwise allow germination [25]. Dormancy can be caused by adverse environmental factors [26], and is due, at least in part, to the presence of metabolites (self-inhibitors) present in ungerminated spores [27]. Although some variability was shown at low conidium density our experiments clearly established a level above which self-inhibition of germination occurred. Self-inhibition was only calculated for one *V. lecanii* strain (strain 50), it was suitable to measure germination rates of other strains since except for one (strain 141), all values were close to 100%.

According to Jackson *et al.* [18] the most virulent strains of *V. lecanii* produced in artificial media more conidia than less virulent strains. Furthermore, fast germination rates were also related with virulence [18].

In our study, most conidia were very similar in size. In any case, conidial size is important since it determines the amount of nutrients contained within a conidium and indirectly sensitivity to mycostasis. The latter has been proved for nematophagous fungi [28] and would be relevant for entomopathogenic fungi in the soil.

Once germination has been achieved, fungal pathogens penetrate their hosts and usually secrete extracellular enzymes [10]. How far these metabolites contribute

to virulence of fungal pathogens is a matter of controversy [29]. Extracellular enzymes degrading compounds present in the insect cuticle [9], such as proteases, chitinases and lipases, are somehow related with virulence of entomopathogenic fungi [18]. However, other studies did not show a correlation between enzymatic activities and pathogenicity [12]. In our study, high proteolytic activity was present in all strains. Chitinases and lipases were also present in lesser amounts. Amylolytic activity was hardly detectable for most of the strains. This could be due to a lack of enzyme production or an intracellular or cell-wall bound localization, because strains grew well in the starch-containing medium. According to Jackson *et al.* [18] there is an inverse relationship between amylolytic activity and pathogenicity. Amylolytic activity may help more saprophytic strains to compete for organic (mostly from plant origin) waste in soil. However, in our study, strains 55 and 141 (isolated from a moss and from forest soil) had less amylolytic activity than strains 57, 132, and 139 which had been isolated from several insect pests.

Respect to the agrochemicals tested, benomyl was the most inhibitory to fungus growth. According to Alves [14], this fungicide completely inhibited mycelial growth of *V. lecanii* above $0.1 \mu\text{g}\cdot\text{ml}^{-1}$. In our study the strain tested was not so sensitive but growth was approximately one third of that of the control. Thiram had little effect on the colony growth of *V. lecanii*. This is in agreement with authors [14] who found even a growth stimulation at concentrations lower than those of our study. Fosetyl-Al had no effect on *Acremonium* [14] but for *V. lecanii* it was inhibitory above $25 \mu\text{g}\cdot\text{ml}^{-1}$. In our study, the chemical had no effect or even promoted growth.

We could finally correlate pathogenicity with physiological traits analysed in this paper. The most pathogenic strains (lower LT50), numbers 50 and 55, were respect to temperature requirements, always in the average values especially from 4 to 25 °C. The strains grew well (as nearly all strains) at neutral pH, but their behaviour was different at acidic values of this parameter. No conclusions could be drawn out of the percentage of germination since they were close to 100% for all strains except for number 141.

The most pathogenic strains produced all extracellular enzyme activities, except amylase, at values (estimated for the C/H ratio) close to the average of the rest of strains. It is somehow puzzling that sporulation was very variable (very high for strain 55 and low for strain 50) in the two most pathogenic strains. One could conclude that tests (i.e. for detection of extracellular enzymatic activities) per se do not provide clear cut data for strain selection of biocontrol fungi such as entomopathogenic fungi. This controversy clearly points out that pathogenicity is a complex process in which the presence, timing and regulation of many factors including the ones covered in this paper and many others, as well as their interactions are probably involved.

References

- Chandler D. The potential of entomopathogenic fungi to control the lettuce root aphid, *Pemphigus bursarius*. *Phytoparasitica* 1992; 20: 11S-15S.
- Andersch W. Production of fungi as crop protection agents. *Pflanzenschutz-Nachrichten Bayer* 1992; 45: 129-142.
- Moorhouse ER, Gillespie AT, Charnley AK. Selection of virulent and persistent *Metarhizium anisopliae* isolates to control Black vine Weevil (*Otiorhynchus sulcatus*) larvae on glasshouse *Begonia*. *J Invertebr Pathol* 1993; 62: 47-52.
- Moore D, Prior C. The potential of mycoinsecticides. *Biocontrol News Infor* 1993; 14: 31-40.
- Hywel-Jones NL, Gillespie AT. Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. *Mycol Res* 1990; 94: 389-392.
- Storey GK, Gardner WA. Vertical movement of commercially formulated *Beauveria bassiana* conidia through four Georgia soil types. *Environ Entomol* 1987; 16: 178-181.
- Fargues J, Maniania NK, Delmas JC, Smits N. Influence de la température sur la croissance in vitro d'hyphomycètes entomopathogènes. *Agronomie* 1992; 12: 557-564.
- Lopez-Llorca LV, Moya M, Llinares A. Effect of pH on growth and pigment production of nematophagous and entomogenous fungi. *Micologia e Vegetazione Mediterranea* 1993; 8: 107-112.
- Charnley AK. Entomopathogenic fungi and their role in pest control. In Wicklow DT, Söderström B (Eds.) *The Mycota IV. Environmental and Microbial Relationships*. Berlin, Springer, 1997: 185-201.
- St. Leger RJ. Biology and mechanism of insect-cuticle invasion by deuteromycete fungal pathogens. In: *Parasites and pathogens of insects*. 1993: 211-229.
- Landa Z, Osborne L, Lopez F, Eyal J. A bioassay for determining pathogenicity of entomogenous fungi on whiteflies. *Biological Control* 1994; 4: 341-350.
- Rosato YB, Messias CL, Azevedo JL. Production of extracellular enzymes by isolates of *Metarhizium anisopliae*. *J Invertebr Pathol* 1981; 38:1-3.
- Meyer SLF. Induction of increased Benomyl tolerance in *Verticillium lecanii*, a fungus antagonistic to plant-parasitic nematodes. *J Helminthol Soc Wash* 1992; 59: 237-239.
- Alves SB, Salguierosa de Andrade CF, Fontana Capalbo DM, et al. *Controle Microbiano de insetos*. Sao Paulo, Editora Manole Ltd. 1986.
- Helyer N, Gill G, Bywater A. Control of *Chrysanthemum* pests with *Verticillium lecanii*. *Phytoparasitica* 1992; 20: S5-S9.
- Etzel RW, Pettitt FL. Association of *Verticillium lecanii* with population reduction of red rice root aphid (*Rhopalosiphum rufiabdominalis*) on aeroponically grown squash. *Florida Entomol* 1992; 75: 605-606.
- Yasem de Romero MG. *Verticillium lecanii* (Zimm.) Viégas, en áfidos de la provincia de Tucumán, Argentina. *Cirpon Rev Invest* 1985; 3: 63-66.
- Jackson CW, Heale JB, Hall RA. Traits associated with virulence to the aphid *Macrosiphoniella sanborni* in eighteen isolates of *Verticillium lecanii*. *Ann appl Biol* 1985; 106: 39-48.
- Feng MG, Johnson JB, Kish LP. Virulence of *Verticillium lecanii* and an Aphid-derived isolate of *Beauveria bassiana* (Fungi: Hyphomycetes) for six species of cereal-infesting Aphids (Homoptera: Aphididae). *Environ Entomol* 1990; 19: 815-820.
- Vestergaard S, Gillespie AT, Butt M, Schreiter G, Eilenberg J. Pathogenicity of the Hyphomycete Fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the Western flower Thrips, *Frankliniella occidentalis*. *Biocontrol Sci Technol* 1995; 5: 185-192.
- Sacks LE, King Jr AD, Schade JE. A note on pH gradient plates for fungal growth studies. *J Appl Bacteriol* 1986; 61: 235-238.
- Hankin L, Anagnostakis SL. The use of solid media for detection of enzyme production by fungi. *Mycologia* 1975; 67: 597-607.
- Biever KD, Josteter DL. Activity of the nuclear polyhedrosis virus of the cabbage looper evaluated up programme temperature regimes. *J Invertebr Pathol* 1992; 18: 81-84.
- Sharma S, Agarwal GP, Rajak RC. Effect of temperature, pH and light on toxin production by *Beauveria bassiana* (Balsam) Vuill. *Indian J Exper Biol* 1992; 30: 918-919.
- Robinson PM. *Practical fungal physiology*. Chichester, John Wiley & Sons, 1978.
- Griffin DH. *Fungal Physiology*. New York, Wiley-Liss, 1993: 379-384.
- Tsurushima T, Ueno T, Fukami H, Irie H, Inoue M. Germination self-inhibitors from *Colletotrichum gloeosporioides* f.sp. *jussiaea*. *MPMI* 1995; 8: 652-657.
- Mankau R. Nematophagous fungi and soil fungistasis. *Phytopathology* 1962; 52: 611-615.
- Mendgen K, Hahn M, Deising H. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Ann Rev Phytopathol* 1996; 34: 367-386.