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Development of Chlorate Resistant Mutants in the Entomopathogenic Fungus Verticillium lecanii

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Abstract

Background: Strain improvement in fungi is based on the knowledge of biochemical and molecular basis of pathogenicity. Successful application of genetic manipulation techniques relies on the formation of mutants. The present study aimed at strain improvement for enhancement of biocontrol efficiency of Verticillium lecanii. **Methods:** Two potent fungicides were developed as markers in the V. lecanii isolate: Benomyl and Ketoconazole. Chlorate Resistant Mutants (ChlR) of V. lecanii were isolated and classified according to their phenotype containing one of the four nitrogen sources: 10mM Nitrate, nitrite, ammonium, glutamate. Also, vegetative growth of the mutant isolates at different temperatures was checked. Also, pathogenicity assay was performed with varied doses of conidial suspension. The conidial germination rates of mutant and wild type strains were tested. **Findings:** It was concluded that V. lecanii was highly sensitive to Benomyl and its growth was strictly inhibited even at low concentration of 2 µg/ml whereas it could tolerate up to 20 µg/ml concentration of Ketoconazole. Of the five chlorate resistant mutants purified, two were unable to utilize nitrate, they were considered as niaD- mutants. Remaining three showed inability to utilize nitrite and were considered niiA mutants. Vegetative growth of the mutant isolates at different temperatures was found to be significantly faster as compared to that of wild type isolates. Also, wild type strain of V. lecanii was less virulent than the mutants. Rate of germination of spores from mutants of V. lecanii was faster and exhibited thermotolerance with respect to conidial germination as compared to that of wild type. **Application:** Hence, development of mutants in Verticillium lecanii could enhance its effectiveness as an improved biocontrol agent.

Keywords: Biological Control, Chlorate, Entomopathogenic, Mutants, Resistant

1. Introduction

Entomopathogenic fungi are economically important agents for integrated pest management programs of insect pests[1,2]. Insecticides such as viruses, bacteria, fungi, protozoa and nematodes can offer effective alternatives to replace chemicals under IPM programme[3]. However, these fungi are affected by environmental factors, such as sunlight, humidity, temperature[4] and artificial factors, such as fungicide applications[5]. Negative effects of temperature, such as heat stress that exceeds 35°C can have serious deleterious effects on conidial germination and persistence, vegetative growth, sporulation and/or the infection process in these entomopathogenic fungi[6–8]. This may result in reduced effectiveness of these fungi in biological control, especially in hot seasons, tropical and subtropical regions or in glasshouses with elevated temperatures. One of the major challenges in biological control is the development of a genetic technique by which desirable traits can be combined to produce superior strains. Techniques for random mutagenesis and targeted gene manipulation have been adapted and developed as efficient tools for investigating gene function in fungi. Enhancement of thermotolerance by artificial selection, transformation[9] or mutagenesis using ultraviolet B (UV-B)[10] is one of the potential method to overcome this problem[11,12].

2. Genetics of Nitrate Assimilation in Fungi

Nitrate is a major source of nitrogen for most algae,

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bacteria, fungi and higher plants and it is the nutrient that most frequently limits their growth[13–15]. The first step in the assimilation of nitrate is the influx of nitrate into cells, which is an active process, because it can occur against an electrochemical potential gradient[16] followed by the catalytic activities of nitrate reductase and nitrite reductase that sequentially produces nitrite and ammonium, the latter being converted to organic nitrogen for cellular growth.

The genetic analysis of nitrate assimilation has been greatly facilitatated by having a positive selection method for nitrate non utilising mutants, based on the findings that cells lack nitrate reductase are resistant to the toxic analogue, chlorate (Table 1). The genetics and biochemistry of nitrate assimilation in fungi have made outstanding contributions to the knowledge of enzymology, gene expression and gene regulation. Strain improvement now embodies a more rational approach in respect to the strategies used for screening the mutated population of cells. Hence, in the present study, chlorate resistant mutants of Verticillium lecanii were developed which showed increased virulence and thermotolerance, thus improved biocontrol mechanisms.

$$NO_3^- \xrightarrow[\text{Permease}]{crnA-} NO_3^- \xrightarrow[\text{Nitrate reductase}]{niaD-} NO_2^- \xrightarrow[\text{Nitrite reductase}]{nitA-} NH_4^+$$

3. Materials and Methods

3.1 Effect of Fungicides on Wild Type of Verticillium lecanii

Benomyl and Ketokenozole (0 to 20 µg/ml) were employed as anti fungal for antibiotic marker isolation from Verticillium lecanii. These plates were spot inoculated with axenic culture of Verticillium lecanii and incubated at 28 ± 1°C for 7 days. At the end of incubation period, Minimum Inhibitory Concentration (MIC) was observed and noted.

3.2 Isolation of Mutants of Verticillium lecanii

NiaD- mutants of Verticillium lecanii were generated according to a procedure developed by Cove (1979)[17], modified by Puhalla (1985)[18] and Joaquim and Rowe (1990)[19]. Minimal Chlorate Medium (MMC) amended with 5% potassium chlorate was used to generate niaD-mutants. Mycelial plugs were placed on the chlorate media either at the center or at 3-6 pts. on petriplates (9 cm diameter) and incubated at 28 ± 1°C. Chlorate resistant sectors, evident after 10-15 days, were transferred to minimal medium amended with $NaNO_3$, $NaNO_2$ and hypoxanthine respectively at 10 mM concentration used for partial phenotyping of chlorate resistant mutants[20].

3.3 Effect of Temperature on Vegetative Growth of Mutant and Wild-Type Isolates

Thermotolerant mutants and the wild-type isolates were subcultured on SDA for 4–5 days at 25 ± 1°C. Mycelial plugs, about 4-mm in diameter, from each isolate were then placed at the centre of petri dish containing approx. 30 ml SDA. These plates were incubated at 15°C, 20°C, 25°C, 30°C, 37°C ± 1°C for seven days, after which time the mean colony diameter was determined. Two replicates were taken per temperature for each isolate. The mean colony diameter of each mutant, at each temperature, was compared statistically to that of the wild type from which it was derived by Mann–Whitney U test[21].

3.4 Virulence of Mutant and Wild-Type Isolates

Pathogenicity assay was performed in disposable cups with varied doses of conidial suspension. For each dose, 10 mosquito larvae were tested each time. For the bioassay, different concentrations of conidial suspension were pipetted evenly over fourth in star larvae to make up the total volume of water to 100 ml. Control set of larvae were kept in 100 ml sterile distilled water. After inoculation, all disposable cups were incubated at 28 ± 1°C. Larval mortality was determined daily by counting the number of infected versus non-infected larvae per concentration of conidial suspension. Dead larvae

Table 1. Chlorate resistant mutants defective in assimilation of different nitrogen sources

Gene Mutation	Chlorate Resistance	Utilization of sole nitrogen source						Summary
		A	B	C	D	E	F	
niaD-		-	+	+	+	+	+	Nitrate reductase structural gene
crnA-		+	+	+	+	+	+	Encode for nitrate uptake in young cells

were checked for sporulating Verticillium lecanii after incubation on SDA media for 3 days microscopically. The percentage mortality due to fungal infection was arcsine square-root transformed and then the mean mortalities, at each day after inoculation, were statistically analysed using ANOVA followed by Tukey's HSD test and Student's t-test for multiple comparisons and single comparisons, respectively[21].

3.5 Effect on Conidial Germination of Mutant and Wild-Type Isolates

This experiment was done following the method of Leng et al. (2011)[9] with some modification as described below. For each mutant and wild-type isolate, conidial suspension (1×10^7 conidia mL^{-1}), prepared, were placed conical flasks and incubated in water bath at 45 ± 1°C. After 0 h, 1 h, 2 h and 3 h, 50 µL samples were taken from each flask and inoculated in each of three Petri dishes containing SDA media. These plates were incubated at 25 ± 1°C for 24 h, after which time the mean germination rate was determined. The mean germination rates for each treatment, at each incubation time, were statistically analyzed using ANOVA followed by Tukey's Honestly Significant Difference (HSD) test and Student's test for multiple comparisons of means and single comparisons of means, respectively[21].

4. Results and Discussion

4.1 Effect of Fungicides on Wild Type of Verticillium lecanii

4.1.1 Dominant Selectable Markers for V. lecanii

One of the most important factors to take into account when selecting antagonistic fungi is their compatibility with commonly used fungicides[22]. Hence, two potent fungicides were developed as markers in the V. lecanii isolate during the present investigation: Benomyl and Ketoconazole

Benomyl: It is a systemic benzimidazole fungicide that is selectively toxic to micro-organisms

and to invertebrates, especially earthworms[23]. The effect of Benomyl on the V. lecanii isolate was studied at concentrations ranging from 2 µg to 50 µg/ ml. It was concluded that the test organism (V. lecanii) was highly sensitive to Benomyl and its growth was strictly inhibited even at concentration of 2 µg/ ml (Table 2). Lopez Llorca et al. (1999)[24] reported that Benomyl was the most inhibitory fungicide and even low concentrations of Benomyl (1.25 µg/ ml) caused growth inhibition of V. lecanii. The effect of Benomyl on V. lecanii strains parasitic on nematode eggs has also been studied by Meyer et al. (1992)[25] who deduced similar results. Abdel- Fattah et al. (2001)[26] found Benomyl to be inhibitory to mycelial growth of Phytophthora infestans. Ketoconazole {cis-1-acetyl4(4)2-(2-4dichlorophenyl)-2(1H imidazol-lylmethyl)-1-3-dioxalan-4-methoxy (phenyl)piperazine}: It is active in vitro against dermatophytes, yeasts, dimorphic fungi, zygomycetes and various other fungi[27]. In the present study, the effect of Ketoconazole upon growth and sporulation of the V. lecanii isolate was studied at concentrations ranging from 2 µg/ ml to 50 µg/ ml and it was concluded that V. lecanii could tolerate upto 20 µg/ml concentration of Ketoconazole and hence showed radial growth on agar. Beyond 20 µg/ ml concentration of Ketoconazole, growth of V. lecanii was inhibited (Table 2). Estrella et al. (2005)[28] determined MICs of Ketoconazole on Candida sp

Table 2. MIC of Benomyl and Ketoconazole and their effect on growth of V. lecanii

Concentration of anti fungal (µg)	Benomyl		Ketoconazole	
	Radial Colony Diameter	Growth Response (+/-)	Radial Colony Diameter	Growth Response (+/-)
2	0.51	+/-	1.98	+
4	0.00	-	1.87	+
6	0.00	-	1.71	+
8	0.00	-	1.52	+
10	0.00	-	1.24	+
20	0.00	-	1.01	+
30	0.00	-	0.53	+
40	0.00	-	0.31	+/-
50	0.00	-	0.00	-
Control	2.26	+	2.32	+

4.2 Isolation of Mutants of Verticillium lecanii

4.2.1 Chlorate Resistant Mutants of Verticillium lecanii

In the present investigation, the Chlorate Resistant

Mutants (ChlR) of V. lecanii were isolated and classified according to their phenotype on media containing one of the four nitrogen sources: 10 mM nitrate, nitrite, ammonium and glutamate respectively. It was observed that out of the five chlorate resistant mutants purified, two were unable to grow and utilize nitrate, they were considered niaD-mutants (Table 3, Plate 1). Remaining three colonies showed inability to utilize nitrite and were considered niiA-mutants. Sugimoto et al. (2003)[29] isolated auxotrophic mutants of V. lecanii based on chlorate resistance and deficient in nitrate reductase activity. Also, they classified the mutants on the basis of utilization of different nitrogen sources and placed them in three phenotypic classes. Novas and Cabral (2002)[30] isolated niaD and cnx mutants from Aspergillus flavus. They obtained approx. 90% of niaD, 8% of nirA and 2% *cnx* mutants.

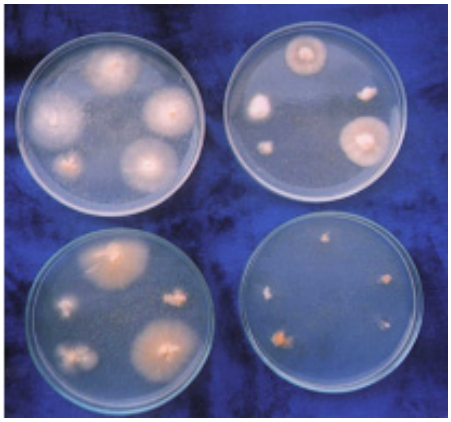

Plate 1. niaD- mutants of Verticillium lecanii showing growth on different nitrogen sources.

4.2.2 Effect of Temperature on Vegetative Growth of Mutants and Wild Type Islolates

Thermo tolerant mutants were successfully isolated by screening at 38°C which completely inhibited vegetative growth of the wild-type isolates. Five mutants were isolated from the wild-type isolate. Vegetative growth of the mutant isolates at different temperatures was compared to that of the wild-type isolates. Of the mutants, at each temperature from 25°C to 36°C, two also grew significantly faster than the wild type. In contrast, third mutant isolate had an upper thermal limit of 40°C, higher than that the wild type, although this mutant grew significantly slower than the wild type at the optimal temperature for vegetative growth of both mutant and wild-type isolates (Table 4, Plate 2) ($P < 0.05$, Mann–Whitney U test). de Crecy et al. (2009)[11] reported that a M. anisopliae variant generated by natural selection–adaptation was able to germinate and grow well at 37°C; in contrast, while the wild type from which it was derived was able to germinate at 37°C, it failed to subsequently grow. Their findings are similar to our results for vegetative growth in mutant and wild-type isolates.

Table 3. Chlorate resistant mutants of V. lecanii

Mutant No.	Chlorate Resistance	N_2 source utilized				Gene mutated
		NO_3	NO_2	NH_4	Glutamate	
01	R (+)	-	+	+	+	niaD$^-$
02	R (+)	-	+/-	+/-	+	-
03	R (+)	-	+	+	+	niiA-
04	R (+)	-	+	+	+	niaD$^-$
05	R (+)	-	+/-	+/-	+	-

Table 4. Effect of temperature on growth of wild type and mutant isolates of V. lecanii isolates

Isolate	Temperature (°C)				
	15	20	25	30	35
V.lecanii (wild type)	4.3 ± 0.6	15.0± 0.7	23.7 ± 0.3	22.0±0.7	0
Mutant 01	2.7 ± 0.6*	19.3± 1.3	34.2 ± 0.5	27.9±1.0	1.0 ± 0.0*
Mutant 02	4.1 ± 0.2	19.1± 1.0	24.7 ± 0.7	22.3±0.3	3.2 ± 0.4*
Mutant 03	3.4 ± 0.5	18.7±0.0*	26.3± 0.6*	25.2±0.4	3.1±0.2
Mutant 04	2.9 ± 0.7	18.6±0.5*	27.1 ± 0.2*	25.5±0.4	2.9±0.5
Mutant 05	8.7 ± 0.6	14.8 ± 0.2*	23.0 ± 0.0*	23.0±1.0	1.1±0.2

Note: *indicates significant difference in colony diameter between wild-type and each mutant isolates ($P < 0.05$, Mann–Whitney U test)

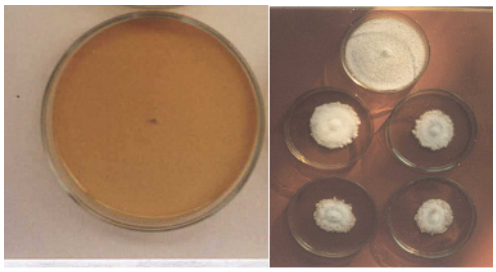

Plate 2. Growth of Verticillium lecanii (wild type and mutant) at 37oC temperature.

4.2.3 Virulence of Mutant and Wild-Type Isolates

The virulence and wild type strain of V. lecanii and that of mutant towards larvae of C. quinquefasciatus were analyzed and it was concluded that wild type strain of V. lecanii was less virulent mutant (Table 5). Also, spores extracted from insect cadavers inoculated with mutant germinated on SMYA at a faster rate than spores of wild type V. lecanii. The relative increase in virulence among the mutant was associated with faster rate of spore germination. Radial growth rate in vitro was high among the mutants. Goettel et al. (1990)[31] transformed the insect pathogenic hyphomycete, Metarhizium anisopliae to Benomyl resistance and observed that the transformants were pathogenic to the hornworm, Manduca sexta. Thakur and Sandhu (2003)[32] tested the virulence in transformant of M. anisopliae towards second instar larvae of S. obliqua and found out that all transformants retained pathogenicity and showed faster spore germination as compared to the wild type strain.

Table 5. Virulence and cultural characteristics of wild type and mutant strains of V. lecanii

S No	Strain	LT50	Radial growth rate (mm / day)
1.	V lecanii (Wild type)	5.8±0.6	1.02±0.8
2.	V lecanii (Mutant 01)	6.1±0.3	1.2±0.7
3.	V lecanii (Mutant 02)	8.4±0.1	1.6±0.4
4.	V lecanii (Mutant 03)	7.9±0.3	1.3±0.5
5.	V lecanii (Mutant 04)	7.2±0.4	1.2±0.6
6.	V lecanii (Mutant 05)	6.9±0.7	1.2±0.4

4.2.4 Effect on Conidial Germination of Mutant and Wild-Type Isolates

Conidia of the wild-type were unable to germinate after 1 h exposure, whereas all the mutants derived from it germinated. Thus, mutant isolates derived from V. lecanii had enhanced thermotolerance in respect to conidial germination, though no mutant isolates survived the longest exposure period (3 h) (Table 6). In a similar study of Leng et al. (2011)[9], the mutant isolates of M. acridum were able to germinate at low levels (< 20%) after 3 h, which was significantly greater than the wild-type isolate and in contrast to the present findings after 3 h exposure. Thermotolerance in conidia of the mutants may represent a considerable advantage for their practical use in biological control because conidial germination is the first step in the infection process of host insects[33].

Table 6. Percent germination (mean ± SD) of wild type isolates of V. lecanii and mutants derived from them after exposure to wet heat stress of 45o C.

Isolate (Wild type /Mutant)	Hours of exposure to wet heat stress			
	0	0.5	1.0	3.0
V. lecanii (wild type)	91.3±0.3a	16.5±2.6[a]	0[a]	0
Mutant 01	92.0±1.5a	62.0 ± 6.6[d]	18.7 ± 1.1c	0
Mutant 02	92.4±2.1a	21.4 ± 2.4[a,b]	6.7 ± 4.3b	0
Mutant 03	94.0±1.1a	43.4 ± 6.2[c]	5.1 ± 1.1b	0
Mutant 04	91.3±1.4a	33.4 ± 4.6[b,c]	7.6 ± 1.8b	0
Mutant 05	91.8±3.2a	15.8 ± 2.7[b,c]	0	0

5. Conclusion

Modern molecular biology techniques offer powerful tools for analyzing interactions between organisms. With increasing understanding of biological phenomena, efficiency of biocontrol agents could be improved by transferring useful genes between micro-organisms. The advent of recombinant DNA technology has opened several avenues of research which can be applied directly to the enhancement of insect pathogens and the development of a new generation of insect control agents.

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