BIOLOGICAL CONTROL

Selection of Culture Media and *In Vitro* Assessment of Temperature-Dependent Development of *Nomuraea rileyi*

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Seleção do Meio de Cultura e Avaliação *in vitro* do Desenvolvimento de *Nomuraea rileyi* Dependente da Temperatura

RESUMO - O desenvolvimento *in vitro* de isolados de *Nomuraea rileyi*, obtidos de larvas de *Anticarsia gemmatalis* Hübner e de Plusiinae, foi estudados em quatro meios de cultura: Sabouraud, maltose, agar e levedura - SMAY; SMAY com extrato de arroz - SMAYR; meio completo para *N. rileyi* - CMNr; e maltose, agar, levedura com extrato de batata - MAYP. Seu crescimento radial em MAYP foi analisado sob cinco temperaturas. Foi estudada a esporulação em relação à temperatura, e a produção de conídios nos quatro meios testados. Dois modelos matemáticos foram aplicados para descrever as taxas de desenvolvimento radial dependentes da temperatura *in vitro* e *in vivo*. MAYP permitiu a maior taxa de crescimento, porém SMAY provocou as taxas mais baixas para os isolados testados. As temperaturas ótimas estimadas para o crescimento do micélio *in vitro* variam de 22°C a 26°C. A esporulação não variou entre 20°C e 26°C. Às temperaturas de 12, 16 e 30°C observou-se pouca ou nenhuma esporulação. A produção relativa dos conídios por biomassa de fungo foi muito variável, de 0,5 a 16 conídios por centigrama de micélio, não sendo considerada um critério adequado para escolher o meio de cultura. Com base nos presentes resultados, um meio à base de extrato de batata ou fatias de batata enriquecidas poderiam ser usados para a produção experimental e, eventualmente, massal de *N. rileyi*. Dadas as similaridades entre exigências térmicas *in vitro* e *in vivo* aqui descritas, as características térmicas da micose poderiam ser simplesmente estimadas com base na temperatura ambiente.

PALAVRAS-CHAVE: Fungo entomopatogênico, crescimento radial, modelo matemático, controle biológico

ABSTRACT - *In vitro* development - radial growth and sporulation - of *Nomuraea rileyi* isolates from *Anticarsia gemmatalis* Hübner and Plusiinae larvae was studied on four culture media: Sabouraud, maltose, agar and yeast - SMAY; SMAY plus rice extract - SMAYR; complete medium for *N. rileyi* - CMNr and maltose agar yeast with potato extract - MAYP. Their development on a selected medium (MAYP) was analysed at five temperatures. Two mathematical models were fitted to *in vitro* and *in vivo* temperature dependent radial growth rates and thermal requirements were estimated. The medium with potato and yeast extract induced the highest growth rate in most cases, while SMAY induced the lowest rates for the tested isolates. Estimated optimum temperatures for mycelium *in vitro* growth ranged from 22ºC to 26ºC. No differences between the proportion of sporulation of colonies maintained at 20ºC and 26ºC were detected. Few or no colonies sporulated at 12, 16 and 30ºC. The relative production of conidia per fungal biomass was very variable, ranging from 0.5 to 16 conidia per centigram of mycelium. Therefore, this was not a useful criterion for selecting a culture medium. Based on present findings, a medium based on potato extract, or enriched slices could be used for *N. rileyi* experimental and eventually mass production. Because of the similarities found between *in vitro* and *in vivo* thermal requirements, thermal traits of the mycosis could be simply estimated on the basis of the environmental temperature.

KEY WORDS: Entomopathogenic fungus, radial growth, mathematical model, biological control

The possibilities of using naturally occurring entomopathogens within the IPM context in agroecosystems will rely on a better knowledge of the environmental and biological factors that govern epizootics. Entomopathogens are important regulatory factors in insect populations and many of them are used as biological control agents of insect pests. However, the reliance on the natural occurrence of entomopathogens for management of pest insects is risky due to the unpredictability of factors that drive epizootics. Integrating an organism for microbial control in a pest management strategy requires basic studies such as isolation, culturing, biological testing and prediction of its effects on the pest population and the environment. A greater adoption will require, among other important aspects, a predictable performance under challenging environmental conditions, for example cool or warm weather, and a higher production efficiency (Lacey *et al*. 2001).

Natural epizootics caused by the mitosporic fungus *Nomuraea rileyi* (Farlow) Samson frequently arise in field populations of lepidopteran pests (Thorvilson & Pedigo 1984). In Argentina, Brazil and Uruguay, it mainly infects larvae of *Anticarsia gemmatalis* Hübner, *Spodoptera frugiperda* (J.E. Smith), *Colias lesbia* (F.), *Spilosoma virginica* (F.), and Plusiinae subfamily, including *Rachiplusia nu* (Guenée), *Chrysodeixis includens* (Walker) and *Plusia* spp. (Gazzoni *et al.* 1994, Rizzo & La Rossa 1994, H.F. Rizzo pers. comm.), which are very difficult to identify in field scouting.

Commercial products based on entomopathogenic fungi, including *N. rileyi*, are currently in use or under development. For the sake of fungal mass production, simple media with few and low price elements should be designed. Although highly variable growth was recorded, *N. rileyi* was routinely cultured for assays and conservation in Sabouraud maltose agar with yeast (SMAY) alone or with soluble starch (Getzin 1961, Kish *et al*. 1974, Bell 1975, Goettel & Inglis 1997). Also, rice and sorghum with yeast extract added, boiled or crushed, were tested as media (Sosa Gómez *et al*. 1990, Vimala Devi 1994). Faster growth of *N. rileyi* was observed in media containing potato and maltose (Valadares *et al*. unpublish.), but no comparisons to other media were performed or published. Due to high sensitivity of *N. rileyi* to nutritional conditions (Goettel & Roberts 1991), compared to other entomogenous fungi, no optimum culture medium has been developed yet.

Temperature has been extensively proved to affect mycelium development (Thomas & Blanford 2003). Fargues *et al.* (1992, 1997) and Ouedraogo *et al.* (1997) found different temperature-dependent *in vitro* growth patterns in isolates of several entomopathogenic fungus species. Particular responses to thermal stimuli were reported by these authors depending on the *N. rileyi* isolate. Most of the different isolates were obtained from different hosts.

Mathematical models can be used to describe fungal growth under given, frequent and extreme, environmental conditions. Lamb (1992) applied an explicit regression model with approximately normal distribution to insect developmental rates. Similarly, the optimum temperature for radial hyphal extension of *Metarhizium flavoviridae* Gams & Rozsypal Thomas & Jenkins (1997) and *Erynia neoaphidis* Remaudière and Hennebert (Zygomycetes: Entomopthorales) (Stacey *et al*. 2003) was estimated by an asymmetric equation. However, models have rarely been used to relate *in vitro* fungal growth to temperature, particularly in the case of entomopathogenic fungi.

The objectives of the current study were both to select a culture medium for experimental production of *N. rileyi* and to determine the fungal thermal requirements for *in vitro* development on a selected medium.

Materials and Methods

Media and *N. rileyi* Isolates. Four solid media were tested: (1) Sabouraud Maltose Agar + Yeast (SMAY); (2) Complete Medium for *N. rileyi* (CMNr) (modified from El-Sayed *et al*. 1992, Lecuona 1996); (3) SMAY plus rice extract (SMAYR); and (4) Maltose Agar Yeast, with potato extract added (MAYP). The extracts were prepared by autoclaving either pealed potato (170 g) or rice (100 g) in water, using the resulting liquid suspension to prepare the SMAYR and MAYP media. The four media contained agar (15 g), maltose (40 g) and yeast extract (15 g) as common ingredients. Both SMAY and SMAYR contained 10 g of peptone. CMNr contained also potassium phosphate (KH_2PO_4, 0.4 g), sodium phosphate (Na_2PO_4, 1.4 g), potassium chloride (KCl, 1 g), magnesium sulphate ($MgSO_4$, 0.6 g) and ammonium nitrate (NH_3NO_4, 0.7 g). The sterilised media were plated into petri dishes (5.2 cm diameter). The *N. rileyi* isolates from the collection at the Laboratorio de Hongos Entomopatógenos (IMYZA, INTA Castelar) were original from *A. gemmatalis* larvae (Nr 27 and Nr 32) and from an unidentified Plusiinae individual (Nr 34), collected in Manfredi (Córdoba, Argentina) and tested after a maximum of two *in vitro* passages. Suspensions of approximately 1 x 10^5 conidia of each isolate were inoculated with a wire loop in the centre of a reversed plate, in order to get a single colony in each experimental unit. The colonies were cultured at 26 ± 0.5ºC. The four media and the three isolates were displayed in experimental units according to a factorial experimental design with 10 replicates.

Radial Growth Rates. Once the colonies reached a minimum of 1 mm (approx. 3 days after inoculation), two orthogonal diameters per colony were measured daily, for six days until sporulation begun. Colony growth rates ($mm \times day^{-1}$) were estimated by linear regression of individual diameter per colony on time of observation. Isolate growth rates were compared among the culture media by a Kruskal-Wallis analysis of variance and a Dunn test.

Effect of Temperature on Mycelium Growth. The radial growth rates of the isolates Nr 32, 27 and 34 were measured on MAYP medium and assessed under different thermal conditions. Ten petri dishes were kept in each of five climatic chambers, set each at one temperature of 16, 20, 26, 28 and 30 (± 0.5)ºC. The thermal range was selected following thermal limitations reported by Fargues *et al*. (1992).

Thermal Requirements for Mycelium Radial Growth of *N.*

rileyi. The median growth rates at every temperature were submitted to least squares regression analysis. The thermal requirements for the radial growth were estimated through the following equations:

$$R(T) = \frac{\left(exp\left(T/a\right)\right)}{\left(b + exp\left(T/c\right)^2\right)} \quad (2)$$

$$R(T) = R_m \cdot exp\left[-1/2\left(\frac{(T - T_m)}{T_\sigma}\right)^2\right] \quad (1)$$

(Lamb 1992, Thomas & Jenkins 1997, respectively), where $R(T)$ is the temperature dependent growth rate; R_m is the maximum growth rate at temperature T_m; T is actual temperature and, T_s, is a range or dispersion parameter from the T_m point; and, a, b and c are fitting constants. The estimated parameters were compared by a Z test with a limiting condition of Z = 2.576 using a Bonferroni correction of bilateral a level of errors.

In order to have reference levels about temperature requirements, data from Getzin (1961), Boucias *et al.* (1984), and Fargues *et al.* (1992) were also applied to equation 1.

Sporulation of Cultures

Proportion of Sporulated Colonies. Fifteen colonies of the three *N. rileyi* isolates were cultured at 26ºC to estimate the proportion of colonies that could sporulate in the four tested media. Also, the effect of temperature on this proportion was analysed by growing the different isolates of the fungus on MAYP medium at 12, 16, 20, 26 and 30ºC and compared by an Irwin-Fisher test (a = 0.05).

Conidia Production. The production of conidia per unit of biomass in the different media was recorded in six colonies per medium. These petri dishes were scratched and cleaned with 10 ml of aqueous-Tween 80 0.01% suspensions. Each colony was collected in glass vials and stirred for 4 min at 15 z. This suspension was diluted one hundred times with water. The density of conidia was counted under microscope at a magnification of 400, with a Neubauer haemocitometer from a sample of 100 ml from each vial, as a mean from five fields. The suspensions were centrifuged at 10.000 rpm and 15ºC for 30 min. The water was poured and the excess was extracted by a vacuum device with a 5 cm nitro-cellulose filter. The fresh and dry weights were measured with a precision scale and the relative productions of conidia per fungal biomass for each isolate among the four media were compared by a Friedman test.

Results

Radial Growth Rates. Median values of radial growth rates ranged from 0.65 to 1.43 mm×day^{-1}. The isolates Nr 34 and Nr 32 showed the highest and lowest values, respectively. The highest rates were recorded on MAYP medium. However, for Nr 27, radial growth on MAYP did not differ significantly from that on SMAYR (Table 1).

Table 1. Radial growth rates (in mm×day^{-1}) of three isolates of *N. rileyi* cultured on four solid media.

Isolate	Media	Median	H[2]	
Nr 27	CMNR	0.82	24.64	A[3]
	SMAY	0.75		A b
	MAYP	1.08		c
	SMAYR	1.18		c
Nr 32	SMAY	0.65	22.15	A
	SMAYR	0.67		A b
	CMNR	0.66		A bc
	MAYP	0.80		d
Nr 34	SMAYR	0.95	23.31	A
	SMAY	1.02		A b
	CMNR	1.19		bc
	MAYP	1.43		d

[1]Standard Error of the mean; [2]Kruskal-Wallis statistic estimation; [3]Distinct letters indicate significant differences among media for each isolate (Dunn test, P ≤ 0.05)

Effect of Temperature on Mycelium Growth. Growth rates at all the tested temperatures were between 0.25 (± 0.03) mm×day^{-1} and 1.27 (± 0.04) mm×day^{-1}. The isolates were able to grow at 16ºC and different rates were measured depending on the isolate. The highest growth rate was estimated for Nr 34 (1.27 ± 0.04 mm×day^{-1}) close to Nr 27 at 26ºC (1.13 ± 0.1 mm×day^{-1}). The isolate Nr 32 showed a distinct temperature dependent development pattern, requiring lower temperatures to reach its highest rates but it did not grow at 30ºC.

Thermal Requirements for Mycelium Radial Growth of *N. rileyi*. Both of the models tested significantly fitted to *in vitro* radial growth rates of *N. rileyi* (P < 0.001; Table 2). The coefficients of determination and F-ratios were higher for the Lamb's model, with the exception of Nr 27, whose growth rates were slightly better explained by Thomas and Jenkins'. Equation 1 represented as well our three *N. rileyi* isolates as the temperature effects on the *in vitro* growth rates reported by Getzin (1961) and Fargues *et al.* (1992) (Table 2). No significant differences were found among the optimum temperatures (T_m) and thermal ranges (T_s) for mycelium *in*

Table 2. Comparison of two models fitted to data of *N. rileyi* radial growth rates.

	Equation 1[1]		Equation 2[2]	
Isolate	R^2	F-ratio	R^2	F-ratio
Nr 32	0.930	13.35	0.906	9.60
Nr 27	0.989	43.48	0.999	2287.24
Nr 34	0.958	22.56	0.924	12.24
All strains	0.663	10.82	0.641	9.84

[1]Lamb (1992); [2]Thomas & Jenkins (1997)

Table 3. Thermal parameters (maximal rate, optimal temperature and thermal range) for mycelium radial growth of *Nomuraea rileyi* colonies.

Isolate[1]	R_m[2]	(S.E.)[3]	T_m[4]	(S.E.) ns[5]	T_s[6]	(S.E.) ns	R^2 (%)[7]
NR 4	1.37	(0.178) A	24.10	(0.590)	4.55	(0.601)	92.13
NR 5	0.95	(0.079) B	23.60	(0.369)	4.83	(0.431)	96.21
NR 6	1.17	(0.139) C	23.47	(0.563)	4.94	(0.580)	92.80
NR 7	0.79	(0.138) A	24.75	(1.123)	5.47	(1.126)	78.32
NR 8	0.68	(0.082) A	24.39	(0.582)	4.87	(0.607)	93.21
NR 9	0.54	(0.044) B	24.44	(0.411)	4.81	(0.432)	95.90
GET-VITRO	1.02	(0.103) B	25.11	(0.579)	5.05	(0.577)	95.76
GET-VIVO	0.16	(0.020) C	23.37	(0.890)	6.29	(0.971)	92.39
BOU-VIVO	0.15	(0.027) C	25.88	(1.690)	8.44	(2.775)	67.64
Nr 27	1.21	(0.092) B	23.90	(0.256)	3.98	(0.302)	98.86
Nr 32	1.05	(0.102) B	22.59	(0.493)	5.64	(0.695)	93.03
Nr 34	1.25	(0.096) B	25.78	(0.705)	5.93	(0.828)	95.75

[1]NR 4 through NR 9 (Fargues *et al.* 1992), GET-VITRO and GET-VIVO (Getzin 1961), BOU-VIVO (Boucias *et al.* 1984); [2]Estimated maximum rate; [3]Standard error of the estimation; [4]Estimated optimal temperature; [5]ns = non significant; [6]Estimated temperature range; [7] Model fitting; [5] Values in the same column followed by equal letters did not differ significantly (Z test; $\alpha/2 = 0.025$)

vitro radial growth (Table 3). The lowest thermal condition was estimated for Nr 32, which required 22.6 (± 0.5ºC) to grow at its highest rate (1.1 ± 0.1 mm×day^{-1}). In contrast, the isolate original from the Plusiinae larvae, Nr 34, required 25.8 (± 0.7)ºC.

Sporulation of the Colonies

Proportion of Sporulated Colonies. No significant differences were detected among proportions recorded at 20ºC and 26ºC ($P > 0.05$). In contrast, at 12, 16 and 30ºC few or no colonies sporulated (Fig. 1). At 26ºC, most of the colonies were able to produce conidia in the four tested media. However, while all of the Nr 32 colonies sporulated on the four media, just one third of the Nr 34 colonies on SMAYR did ($P < 0.05$).

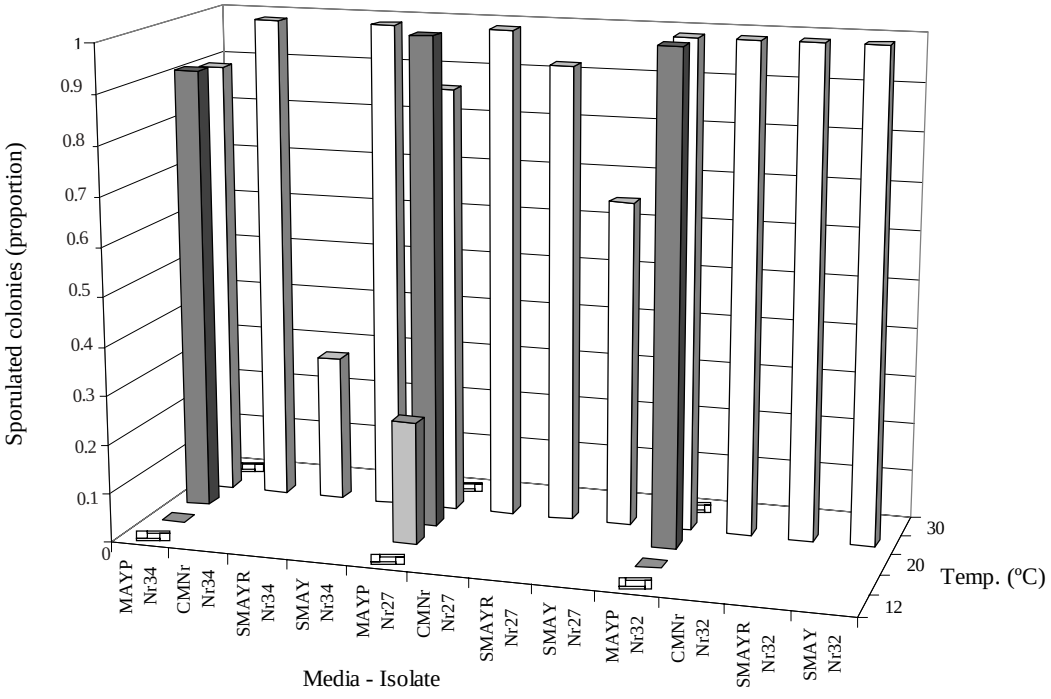

Figure 1. Proportion of sporulated colonies of three isolates of *N. rileyi*, cultured on four solid media. Cultures on MAYP were also maintained at five constant temperatures. (CMNr: Complete medium for *N. rileyi*; SMAY: Sabouraud Maltose Agar with Yeast; MAYP: Maltose Agar with Yeast and Potato extract; SMAYR: Sabouraud Maltose Agar with Yeast and Rice).

Conidia production. The relative production of conidia per unit of biomass of mycelium ranged from 0.5 to 16 conidia per centigram of mycelium. Conidia production of isolates Nr 27, Nr 32 and Nr 34 ranged from 1.4 to 7.5, 4.3 to 15.2 and 0.5 to 16 conidia per centigram, respectively. Due to this high variability, no differences related to the culture media were observed ($P > 0.05$).

Discussion

The most frequently used culture medium, SMAY, induced the lowest growth rates for all the isolates. In contrast, the use of MAYP allowed for the highest growth rates in most of the tested isolates. For one of them (Nr 27) the application of rice extract (i.e. SMAYR) yielded equivalent growth rates. Although Holdom & van de Klashorst (1986) and Im *et al*. (1988) observed good mycelium growth by adding yeast extract, this practice was not enough to ensure the fastest radial growth in our trials. Vimala Devi *et al.* (2000) stated that multiplication of *N. rileyi* in media other than SMAY, or an equivalent, is difficult. On the other hand, other nutrients (starch, minerals, mainly K, and Ca, P, Mg, Na, S, Zn, Mn, Al, B, Fe, crude protein and vitamins) could also be needed by the fungus, which would easily be supplied by adding potato extract improving its growth (Durán Hidalgo 1979, FAO 1990). Among all the components of the media tested in this work, the two simplest and most economic ingredients were rice and potatoes. The CMNr used in the present study was similar in composition to the so-called complete medium for *Beauveria bassiana* culture (Lecuona 1996) and to one of the phases in a complex medium proposed by El-Sayed *et al.* (1992) for *N. rileyi* culturing. However, the mycelial growth did not result as fast as it could be expected. Our experimental results support the use of media containing potato extract to promote the highest growth rates of *N. rileyi* colonies. The use of rice, as in Sosa Gómez *et al*. (1990), or its extract could, as tested here, result in similar efficiency for radial growth to the potato one but just for singular isolates.

The relative production of conidia was not a helpful criterion for selecting a culture medium, because none of them allowed high and stable production of conidia. However, MAYP induced a more efficient production of mycelium biomass. Although using agar-based culture media is not a cost efficient mass production system at a reasonable scale (Vimala Devi *et al*. 2000), a medium based on enriched potato slices or pieces could be applied for most isolates of *N. rileyi* in mass production, as same as reported by Arnaud (1927) for *B. bassiana* cultures.

The fungal growth variations due to a driving environmental variable, temperature, were represented and estimated by both of the proposed models. However, their characteristics are fairly different. With Eq. 1, an unrealistic symmetrical shape function is applied but with biologically meaningful parameters. Although Eq. 2 defines an asymmetrically shaped thermal trait, its parameters have difficult biological interpretation. Similar optimum and range of temperatures were estimated with both models for either *in vitro* or *in vivo* conditions. Moreover, for *N. rileyi*, a strong relationship between lethal time, radial growth and temperature could be extracted from data reported by Getzin (1961). A relationship between *in vitro* and *in vivo* development rates was also noted by Stacey *et al*. (2003) for an entomophthoralean fungus. This relationship would be of about 10 times lower for *N. rileyi in vivo* estimations. In the present study, optimum temperatures for mycelium *in vitro* growth were estimated on the basis of statistical modelling, giving more precision to previous *in vivo* estimations of 25ºC (Ignoffo *et al*. 1977) or 26ºC (Boucias *et al*. 1984). Temperatures between 20 to 26ºC allowed for high vegetative radial growth and also a high proportion of sporulation. A limiting condition for sporulation was found at 30ºC, in agreement to Ignoffo *et al*. (1977). The last statement could be important in terms of a low probability of sporulation of cadavers in the field during warmer days (with temperatures higher than 30ºC) and, consequently, few secondary infections. The definition of this simple models are of tactical importance, particularly in the case of biocontrol agents of thermoconformer insects, i.e. lepidopteran larvae (Blanford & Thomas 1999, Thomas & Blanford 2003).

In our study, a genotype-by-environment trait (sensu Thomas & Blanford 2003) was described. A more complex study should be pursued including the relationship genotype-genotype-environment. However, thermal traits of the mycosis (e.g. optimum temperature, incubation times, etc.) could be simply estimated on the basis of environmental temperature. The findings described in this article could be of general importance and applied to tactical systems for field application or monitoring of microbial control of soybean caterpillars.

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