Development of a semi-synthetic medium for production of azygospores of *Zoophthora radicans* (Brefeld) Batko, a pathogen of rice leaf folder

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ABSTRACT

Resting spores were obtained *in vitro* of *Zoophthora radicans* culture using carbon and nitrogen sources. Yeast extract and peptone as the nitrogen source and sunflower oil and dextrose as the carbon source were utilized at different combinations. Among the different C:N ratios tested for the production of azygospores, 4:8 yeast extract and sunflower oil medium combination was found to yield abundant resting spores when compared to other combinations.

Key words: Azygospores, *Zoophthora radicans*, C: N ratio, yeast extract, peptone, sunflower oil, dextrose

INTRODUCTION

Most of the entomopathogenic fungi including *Zoophthora radicans* (Brefeld) Batko in the order Entomophthorales produce two types of reproductive spores viz., conidia and resting spores. The relatively short lived conidia are actively ejected from cadavers and are normally infectious. Within late instars of larvae killed by *Z. radicans*, azygospores are formed when individual hyphal bodies coil and form a thick double wall. Azygospores are normally dormant after production during early summer but germinate to form infectious germ tube during season. The resting spores used for field introductions are collected either through the cadavers or through soil samples containing high load of the resting spores. Unfortunately both of these methods for obtaining and releasing. Z.*radicans* resting spores appear problematic and not amenable for large scale applications (Hajek,1997). Many entomopathogenic fungi are reported to produce resting spores *invitro*. Species of *Conidiobolus* (Gustaffson,1965; King,1977: Latge and Perry 1980; Keller, 1991) produce resting spores *in vitro* and *Z. radicans* also form the resting spores (Batko,1964). Efforts were hence made to synthesize azygospores from *Z.radicans* with different combinations of carbon and nitrogen sources in the medium chosen.

Taking these points into consideration, Mohamed Nizam and Narayanasamy (2006) made a maiden attempt to produce azysospores of *Z. radicans*, an entomogenous fungus infecting rice leaf folder, *Cnaphalocrocis medinalis* Guenee. Utilization of such resting spores in combating the insect pests of agricultural crops appears interesting as they are able to survive during unfavourable climatic situations and become infectious when favourable conditions prevail. The concept of using the resting spores of entomogenous fungus in combating insect pests of crops appears new and interesting. In the context explained above the present attempt was made to synthesize and characterize resting spores of *Z. radicans*.

MATERIALS AND METHODS

In an attempt to detect an appropriate *in vitro* sporulation medium for *Z. radicans*, an experiment was conducted with twenty combinations between two nitrogen (N) and two carbon sources (C) in liquid medium. Two each of carbon sources and nitrogen sources utilized in experiment were sunflower oil, dextrose and yeast extract, peptone respectively. The five different proportions of the nutrients tested for sporulation were *viz*., 1% N + 2%C, 1% N + 4%C, 2%N + 4%C, 2% N + 8% C and 4%N + 8% C. Four replicates were used in each.

Preparation of sporulation medium

Each semi defined medium was prepared with a concentration of 10 g per 1000 ml of medium at one percent of the corresponding carbon or nitrogen source utilized. The sunflower oil was added at the rate of 30ml for 1000 ml of the medium. In each replicate, 30ml of the medium was taken in 250ml. Erlenmeyer flask was adjusted to the PH 6.5 before autoclaving at 1.15 kg/cm^2 for 15 minute.

Preparation of the fungal pathogen

The flasks with semi defined medium were inoculated with 25 ml of the *Z. radicans* culture grown in Sabouraud-Egg-Milk-Agar (SEMA) broth at 25 °C for five days showing good growth and not sub cultured more than four times. The experimental cultures were grown at 25 ± 2 °C on a reciprocating shaker with 100 oscillations per minute.

Quantification of the resting spores

Quantifications of the resting spores were done for each replicate at tenth day after initial day of inoculation. The resting spores formed were recovered by filtration and

washing with sterile distilled water through the top of stack of sieves (123, 63 and 20 mm sieve openings) to discard the mycelial debris. The fraction collected over 20 mm sieve was resuspended in distilled water and used for the estimation of sporulation rate through determining the number of spores per ml with an improved Neubaur's Haemo cytometer. Number of cells were counted as resting spores and were detected as double walled and contained oil globules (Kogan and Hajek, 2000).

RESULTS AND DISCUSSION

Carbon and nitrogen sources on quiescent spore production in *Z. radicans*

Results showed that there was a drastic rise in the sporulation (2.3×10^5 to 6.37×10^5 spores/ml) rate with yeast extract as nitrogen source and sunflower oil as the carbon source in the sporulation medium with increase in the N:C ratio variable from 1:2 to 4:8. Downward trend of the sporulation was evident with nitrogen source peptone and the carbon source sunflower oil in the sporulation medium with increase in the N:C ratio of 1:2 to 4:8 combinations. A gradual increase in the sporulation of the fungus was evident with dextrose as carbon and yeast extract as nitrogen sources except at the N:C range of 2:8.The carbon source dextrose had brought minimum sporulation with peptone and no sporulation was found at all the N:C ratios of 2:4 to 4:8 (Table 1). Highest mean quiescent sporulation (2.323×10^5 spores/ml of medium) was witnessed with yeast extract as nitrogen source and was found on a par with sunflower oil as the carbon source in the composition of the medium (2.88×10^5 spores/ml). The yeast extract based medium followed by peptone had a mean sporulation of 0.717 $\times 10^5$ spores/ ml of the medium. While the dextrose as a carbon source had mean sporulation of 0.15×10^5 spores /ml (Table 2).

Table 1. Influence of various carbon and nitrogen sources on azygospores production by *Z. radicans* in liquid shake culture

Carbon sources	Nitrogen sources	N:C Ratio	No. of resting spores ($\times 10^5$)/ml
Yeast extract	Sunflower Oil	1:2	2.35(1.53)^e
		1:4	3.75(1.93)^d
		2:4	4.62(2.14)^c
		2:8	4.68(2.16)^b
		4:8	6.37(2.52)^a
Yeast extract	Dextrose	1:2	0.12(0.34)^n
		1:4	0.17(0.41)^m
		2:4	0.30(0.54)^l
		2:8	0.50(0.70)^j
		4:8	0.37(0.60)^k
Peptone	Sunflower Oil	1:2	2.3 (1.51)^f
		1:4	2.2(1.48)^g
		2:4	1.9(1.37)^h
		2:8	0.6(0.77)^i
		4:8	0.1(0.31)^o
Peptone	Dextrose	1:2	0.05(0.22)^p
		1 :4	0.02(0.14)^q
		2:4	0.00(0.00)^r
		2:8	0.00(0.00)^s
		4:8	0.00(0.00)^t
		CD	0.25
		SED	0.12

Each value is a mean of four replications
Figures in parentheses are square root transformed
In a column means followed by a common letter are not significantly different (P=0.05) by DMRT

Results indicated that 2:8 and 4:8 percent combinations of yeast extract and sunflower oil as well as 1:2 and 1:4 percent combination of peptone with sunflower oil exerted *Z. radicans* to sporulate tremendously. However, there was an increasing trend in the sporulation of *Z. radicans* with yeast extract and sunflower oil from 1:2 to 4:8 combinations. There was a gradual decrease in the sporulation with peptone and sunflower oil from 1:2 to 4:8 combinations while nitrogen source peptone and carbon source dextrose were seen less supporting for sporulation of the test fungus. The sporulation was maximum with nitrogen sources namely yeast extract and peptone and carbon sources sunflower oil (>1-5x10^5 spores/ ml). The sporulation was poor with dextrose as carbon source and peptone as nitrogen source (less than 1×10^5 spores/ml) (Table 3).

These studies indicate yeast extract sunflower oil mixture was found to be an efficient medium for the quiescent spore production *in vitro* in liquid shake culture. It is thus clear that N.C ratios of 2:8 and 4:8 were found optimum for the yeast extract and sunflower oil based sporulation medium. The N:C ratio of 1:2 to 2:4 was optimum for peptone and sunflower oil based sporulation medium.

Earlier several authors reported the azygospores formation of entomopathogenic fungi. Kogan and Hajek (2000) produced azygospores of *Entomophaga maimaiga* Humber, Shimazu and Soper (3×10^4 spores/ml) with a medium comprising Graces insect tissue culture medium (95%) and Fetalbovine serum (5%). Blastopores of two isolates of *Paecilomyces fumosoroseus* Wize were produced *in vitro* by shake culture in Jackson's liquid medium (JLM) and their infectivity evaluated against *Spodoptera frugiperda* Smith through injection (Altre and Vandenberg, 2001).

Table 2. Effect of various carbon and nitrogen sources on azygospores production by *Z. radicans* in liquid shake culture

Nitrogen sources	Carbon Sources		Mean Sporulation	
	Sunflower oil (ml)	Dextrose (gram)	Per nitrogen source ($x10^5$ spores/ml)	
Yeast extract	2.35	0.12	0.247	2.323
	3.75	0.17	0.392	
	4.62	0.30	0.492	
	4.68	0.50	0.518	
	6.37	0.37	0.674	
Peptone	2.3	0.05	0.235	0.717
	2.2	0.02	0.222	
	1.9	0.00	0.190	
	0.6	0.00	0.060	
	0.1	0.00	0.001	
Per carbon source ($x10^5$ spores /ml)	2.88	0.15		
			CD	0.2521
			SED	0.1273

Each value is a mean of four replications
Figures in parentheses are square root transformed
In a column means followed by a common letter are not significantly different (P=0.05) by DMRT

Entomophthrous fungi produce various types of resistant or resting structures: single-celled chlamydospores, thick walled zygospores (fusion of mycelial fragments or hyphal bodies without meiosis), thick walled hyphal bodies or hyphae and resting spores (Latge, 1976; Latge *et al.*, 1982). *Entomophthora floridana* Weiser is an exception to this statement, which over winters (50 °C) as conidia does not form spores (Brandenburg and Kennedy,1981).

Under *in vitro* conditions, the formation of hyphae is by the coalition of protoplasts to form spheres or spherical cells that enlarge to become pro hyphal spheres, which germinate to form hyphae. Some develop thick walls to form resting spores (Nolan,1985). Chlamydospores develop from small portions of hyphae or hyphal bodies that develop thick walls(MacLeod, 1963). These spores germinate readily under favourable conditions. Zygospores are produced when two specific hyphal bodies fuse (Latge, 1976). Hajek and Humber (1997) stated that resting spores are formed when individual hyphal bodies round up and form a thick double wall. Hajek (1997) reported that *in vivo* produced or field collected azygospores from soil samples or cadavers are not amenable for large scale applications and it necessitates *in vitro* production.

A variety of factors which were found to promote the development of resting spores in entomopathogenic fungi include host age or inappropriate host (Wilding and Laukner, 1974; Steinkraus and Kramer, 1989), host (Benze'ev and Uziel,1979), temperature (Shimazu,1979) and available nutrients (Latge,1980). Darkness was mentioned as a possible factor for inducing resting spore formation, rather than conidiophores and conidia, in entomophthorales pathogenic to Lepidoptera (Li and Humber, 1984).

Based on the above reports from the various authors, the present attempt was made to generate azygospores of *Z .radicans* and a novel sporulation medium with yeast extract and sunflower oil (4:8) mixture was formulated for the production of azygospores of *Z.radicans.* Our findings are in corroboration with the Mohamed Nizam and Narayanasamy (2006) who reported that 4:8 Yeast extract and Sunflower oil combination was effective in the production of resting spores of *Z. radicans* and Latge (1980) also reported that higher sporulation rates were obtained consistently on a medium with yeast extract.

The concept of utilizing resting spores against pest problems like leaf folder will be more appropriate mainly because in some fungi, there is an alternation of conidial forms and resting spore forms in insect populations. In *Erynia bullata* Thaxter and Macleod that infects adult *Sarcophaga aldrichi Parker,* one generation of conidia alternates with one generation of overwintering resting spores. In *Erynia phytonomi* Arthur ,

Table 3. Effect of various carbon and nitrogen combinations on production of azygospores by *Z. radicans*

Nitrogen Sources	Nutrient concentration (%)		Carbon sources	
	Nitrogen	Carbon	Sunflower oil*	Dextrose*
Yeast extract	1.0	2.0	2.35(+)	0.12(-)
	1.0	4.0	3.75(+)	0.17(-)
	2.0	4.0	4.62(+)	0.30(-)
	2.0	8.0	4.68(+)	0.50(-)
	4.0	8.0	6.37(++)	0.37(-)
Peptone	1.0	2.0	2.3(+)	0.05(-)
	1.0	4.0	2.2(+)	0.02(-)
	2.0	4.0	1.9(+)	0.00(-)
	2.0	8.0	0.6(-)	0.00(-)
	4.0	8.0	0.1(-)	0.00(-)

*As per the scale of Sporulation by Latge *et al* (1978)

\+ 1-5 x 10^5 Spores /ml

++ 5-10 x 10^5 Spores/ml

– < 1 x 10^5 Spores/ml

the conidia are produced in second instar larvae and resting spores in third instar larvae of the alfalfa weevil (Watson *et al.*,1981).

Resting spores generally appear towards the end of the active season of the insect i.e., late summer or winter as experienced in the case of *Triplosporium fresenii* Olivier in which zygospores germinated in the spring in synchronization with the build-up of *Aphis spiraecola* Patch on citrus trees in Israel (Bitton *et al.*,1979). Similarly, in populations of *Pseudoplusia includens* Walker infected with *Tarichium gammae* Weiser, the number of individuals with conidial forms decreased and that of individuals with resting spore forms increased as the epizootic progressed. Of course, this is not caused by short photoperiod or low temperatures but is correlated with larval size since conidial forms are predominant in small larvae and the resting spores in large larvae. It becomes therefore clear that the role of resting spores of pathogens like *Z. radicans* will be supplementing the conidial forms in suppressing the leaf folder of rice while not letting off the different instars of the larva. This is the best strategy wherein both conidia and resting spores are made use of together in containing the pest at all its infestive stages.

Therefore, it is concluded that the pathogen, *Z .radicans* by virtue of its ability to develop resting spores and the normal conidial forms appear to be an effective candidate pathogen to check the leaf folder infestation and also shows promise for formulation as Mycoinsecticide for the pest.

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