IMPROVING FORMULATION OF *ARTHROBOTRYS DACTYLOIDES*, A NEMATODE-TRAPPING-FUNGUS, IN KAOLIN-ALGINATE GRANULES (*PERBAIKAN FORMULASI* Arthrobotrys dactyloides, *JAMUR PERANGKAP NEMATODA, DALAM BUTIRAN KAOLIN-ALGINAT*)

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ABSTRACT

This research aimed at evaluating performance of *Arthrobotrys dactyloides* in improved kaolin-alginate granular formulation. *A. dactyloides* was formulated in kaolin-alginate granules enriched with three types of organic matters (saw dust, rice hay, and corn cob), each with two concentrations (1% and 3% (w/v)). A formulation of granule without organic matter was also provided as a control. For each formulation, one half of granule was air-dried directly and the other half was given additional treatment, re-fermentation. The granules were used in experiments to observe performance of *A. dactyloides* in the formulations both in agar medium and in soil tests. All experiments were conducted with completely randomized design and data were analyzed with analysis of variance. Results showed that neither types nor concentrations of organic matters had any significant effect on the performance of *A. dactyloides*. Re-fermentation of granules, however, significantly improved weight of granules, number of propagules in granules, vigor of the fungus, growth of the fungus in soil, spore and ring production in soil.

Key words: Formulation, *Arthrobotrys dactyloides*, kaolin-alginate, organic matters

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi performa Arthrobotrys dactyloides *dalam formulasi butiran kaolin-alginat yang diperkaya.* A. dactyloides *diformulasikan dalam butiran kaolin-alginat yang diperkaya dengan tiga macam bahan organik (serbuk gergaji, jerami padi, dan tongkol jagung), masing-masing dengan dua konsentrasi (1% dan 3% (b/v)). Formulasi tanpa penambahan bahan organik disiapkan sebagai kontrol. Untuk tiap formulasi, setengah dari butiran langsung dikering-anginkan dan sisanya diberikan perlakuan tambahan, re-fermentasi. Butiran digunakan pada percobaan-percobaan untuk mengetahui performa* A. dactyloides *dalam formulasi, baik pada pengujian di media agar maupun di dalam tanah. Semua percobaan dilaksanakan dengan rancangan acak lengkap dan data dianalisa dengan analisis keragaman. Hasil penelitian menunjukkan bahwa tipe bahan organik atau konsentrasinya tidak berpengaruh nyata terhadap performa* A. dactyloides. *Sebaliknya, re-fermentasi butiran secara nyata meningkatkan berat butiran, jumlah propagul dalam butiran, vigor jamur, pertumbuhan jamur di dalam tanah, produksi spora dan cincin perangkap di dalam tanah.*

Kata kunci: Formulasi, Arthrobotrys dactyloides*, kaolin-alginat, bahan organik*

INTRODUCTION

Several reports (Jaffee *et al*., 1992; Stirling and Mani, 1995; Stirling and Smith, 1998; Stirling *et al*., 1998; Sudirman, 1997) have suggested that *Arthrobotrys dactyloides*, a nematode trapping fungus, is a promising biological control agent against root-knot nematodes. However, biological control agents are of no commercial value unless they can be supplied to growers in a form that can be easily used. Development of a formulation, which is efficacious, economic to produce, and capable of maintaining the fungus in a viable state, is one of the most important steps in furthering the development of biological controls for nematodes.

The use of alginate-kaolin to encapsulate hyphal biomass is one promising development in the formulation of fungal biological control agents. The technique has been used to formulate several biological control agents with or without an additional

nutrient source (Walker and Connick, 1983; Fravel *et al*., 1985; Lewis and Papavizas, 1985, 1987; Knudsen and Bin, 1990; Knudsen *et al*., 1990). Basically, the technique involves incorporating an antagonistic organism into an alginate solution and dripping the mixture into aqueous calcium gluconate. Once the fungus has been encapsulated, the biological control agent grows from granules, contacts target propagules and parasitises them (Knudsen *et al*., 1991).

In this study, *A. dactyloides* was incorporated into alginate-kaolin with the addition of saw dust, rice hay or corn cob. The aim was to supply *A. dactyloides* with its carbon needs from these incorporated nutrients and determine whether this had an effect on its predacious activity. Besides investigating whether organic nutrients incorporated into granules had beneficial effects on granule performance, this study also aimed to find the best formulation for further research on the behaviour of *A. dactyloides* in soil.

MATERIALS AND METHODS

This research was done in Laboratory of Microbiology, Faculty of Agriculture, University of Mataram in 2006, with materials and methods prepared as the following description.

Nematophagous fungus, *Arthrobotrys dactyloides*

The fungus used in this research was *Arthrobotrys dactyloides* isolated from vegetable plantation in Ampenan. The fungus was maintained on corn meal agar (CMA) in 9 cm diameter Petri dishes. When the whole surface of the dish was covered by mycelia, the agar was cut into squares (6 mm x 6 mm) and stored in bottles containing sterile distilled water at 27ºC. When fungus was required for formulation, one square from these water cultures was placed on CMA in a 9 cm diameter Petri dish and plates were incubated at 27ºC for 6 - 7 days before use.

Mass Production of Mycelia

The fungus was mass produced in 250 ml Ehrlenmeyer flasks containing 100 ml Glucose Peptone yeast (GPY) broth (15 g glucose, 2 g peptone, 5 g yeast, 1 g asparagine, 0.5 g K_2HPO_4, 0.25 g $MgSO_4.7H_2O$, 0.001 g thiamine HCl,, 1 L H_2O). Flasks were inoculated with two 5 mm-diameter discs taken from an actively growing colony on CMA as described previously and were incubated at 27ºC on a rotary shaker at 120 rpm. After 10 days incubation, about 0.007 g dry wt biomass/ml was produced. Before it was used, the mycelial suspension was homogenized for 15 seconds with blender.

Production of Formulations

Saw dust, rice hay, or corn cob (milled to pass through a 0.5 mm sieve) were each separately incorporated into kaoline-alginate at two concentrations (1 % and 3 % (w/v)). Formulation ingredients were autoclaved separately prior to use. 100 g kaolin (MP Biomedical Inc, Ohio, USA) and 10 g sodium alginate were added to 1 L water. After autoclaving, 80 ml of the blended and sterilized kaolin-alginate mixture was mixed with 20 ml of mycelial suspension prepared as described previously. Batches of this mycelium-kaolin-alginate mixture were separately amended with 1 g or 3 g of either saw dust, rice hay or corn cob. These mixtures were respectively designated as 1SD and 3SD for the mixture containing 1% and 3% saw dust, 1RH and 3RH for those containing 1% and 3% rice hay, and 1CC and 3CC for those containing 1% and 3% corn cob.

The mixture of each organic nutrient and mycelium-kaolin-alginate was then mixed with a magnetic stirrer in a 1 L Ehrlenmeyer flask and dripped through a Pasteur pipette into a continuously shaken aqueous suspension of 0.1 M Ca-gluconate. The drops gelled upon contact with the Ca-gluconate. In order to maintain a homogenous spherical form of granules, the distance between the tip of the Pasteur pipette and the surface of the Ca-gluconate suspension was kept at about 1 cm. Granules were harvested on a sieve and one-half of them were spread on a wire mesh tray and allowed to dry in the laminar air flow cabinet in the laboratory for approximately 60 hours. These were termed non-re-fermented (nrF) granules. The other half of the granules were kept sterile and transferred to Ehrlenmeyer flasks containing 100 ml GPY broth and incubated in shake culture at 27ºC. After 3 days, the re-fermented granules were harvested and dried on a sterile wire mesh as previously described. These were termed re-fermented (rF) granules. With the same procedure one batch of granules was produced without addition of organic nutrient and these were designated as no organic matter (NOM).

Wet weight of a granule was determined soon after production. Immediately, after drying in the laminar air flow, ten granules from each formulation were weighed and their moisture content was determined by drying in an oven at 105 ºC.

Estimation of Number of Propagules in Granules

To determine the number of propagules (i.e. the number of colony-forming units (CFU)) in formulations, 1 g of each batch of freshly prepared alginate granules or 0.1 g of each batch of dried granules were soaked overnight in vials containing 9.0 ml or 9.9 ml, respectively, of sterile phosphate buffer (11.8 g KH_2PO_4 and 4.2 g Na_2HPO_4 in 1 L water at pH 6.4). The vials were then shaken vigorously on a wrist-action shaker to disperse the granules. Since the dried fermented granules failed to disperse because they were held together by hyphae, they were also macerated gently with a sterile glass rod. The suspensions were then diluted serially in sterile water and 0.1 ml of each dilution was plated onto CMA containing 50 mg/L streptomycin sulphate. Five replicates were prepared for each batch of granules. The numbers of colonies of *A. dactyloides* were counted after 5 days incubation at 27°C. Data were expressed as $\log_{10}$ of numbers of CFU before being subjected to analysis.

Sterility, Viability and Vigor

For sterility test, three replicates of 10 granules from each formulation were plated on CMA and incubated at 27°C. The viability and vigor of each formulation was determined by placing five replicate samples of ten granules on tap water agar (TWA) and incubating the plates at 27°C for 5 days. Viability was determined by counting the number of granules which produced mycelial growth. Each granule was also rated for vigor on a 1-4 scale (1 = sparse mycelial growth; 2 = mycelial growth covering less than 50% of the granule; 3 = mycelial growth covering 50% - 90% of granule; 4 = mycelial growth covering the whole granule with very dense mycelia).

Growth of Mycelia from Granules in Soil

The growth of mycelia from granules of each formulation was assessed using the standard slide test (Sudirman, 1997). A Granule was placed at marked position on a glass slide in the bottom of a 9-cm diameter Petri dish. The granule was covered with a piece of nylon mesh (the same size as the glass slide) with 100 μm apertures and the Petri dish was then filled with 60 g of soil, moistened with water to approximately field capacity. The Petri dish was placed in a moist chamber (an air-tight plastic container in which humidity was maintained by covering the base and top with 2 layers of moist paper) and incubated at 27°C. On the day of observation, the soil and nylon mesh were carefully removed so that all granules and mycelia growing from the granules stayed in position. To achieve this, a little pressure was applied with the tip of a fine forceps at the point where a granule was located, and the edge of nylon mesh was lifted in such a way that granules remained in position with minimal disturbance to the mycelia. The mycelia were separated from granules by using a very sharp tip of a forceps to cut around the granules. The granules were then removed. This latter step was done very carefully to avoid mycelia from being disarranged or dislodged with the granule. The slide was then flooded with lactoglycerol cotton blue, a cover-slip was applied and the surface of the slide was observed under microscope. Mycelia on the glass slide were observed under a microscope at a magnification of 100 X, and the length of radial growth (the distance between mycelial tips and granule) was measured from four random directions.

Twelve replicates of Petri dishes each containing a glass slide with one granule were prepared for each formulation. Three replicate dishes were assessed each time of observation after 5, 10, 15 and 20 days.

Production of Spores and Rings in Soil

The production of spores and traps in soil was assessed using the standard slide test described previously. Twelve replicate Petri dishes were prepared for each formulation and three of these were taken for observation after 5, 10, 15 and 20 days. Three granules were placed on one glass slide for the observations on day 5 and day 10, and two granules were used per slide for the observations on day 15 and day 20. On days of observations, three randomly selected microscope fields around each granule were assessed to determine the number of spores or rings per mm^2. The total numbers of rings per granule for each formulation was also determined by scanning all hypha for the presence of rings. Since the numbers of rings per mm^2 corresponded to the total number of rings per granule, and the possibility that not all rings were counted at day 15 and 20 due to outgrowth of mycelia on the slide, only the numbers of rings per mm^2 were subjected to analysis.

Statistical Analysis

All experiments were conducted using a completely randomized design and conducted twice. Data were analyzed by analysis of variance and the effect of treatments was determined. When the variance ratio (F) was significant, means for each treatment were separated using a least significant difference test.

RESULTS AND DISCUSSION

Results of analysis showed that neither the type of organic matter nor their concentration had a significant effect on wet weight, dry weight and moisture content of dried granules. However, re-fermentation significantly influenced these parameters. The wet and dry weights of rF-granules were significantly higher than nrF-granules. In contrast, the moisture contents of rF-granules were significantly less than nrF-granules (Table 1).

Table 1. Wet and dry weight of granules and moisture content of dried granules containing different types, concentrations of organic matters, and with or without re-fermentation.

Tabel 1. Berat basah dan berat kering butiran dan kadar air butiran kering yang mengandung bahan organik dengan tipe dan konsentrasi yang berbeda dengan atau tanpa fermentasi ulang

Treatments*	Wet weight of one granule (mg) **	Dry weight of one granule (mg) **	Moisture content of dried granules (%)**
Organic matter			
SD	35.37	4.31	2.81
RH	35.36	4.32	2.81
CC	35.37	4.30	2.81
NOM	35.36	4.30	2.80
Concentration			
1%	35.36	4.31	2.81
3%	35.37	4.30	2.80
Fermentation			
nrF	35.07a	4.27a	2.85a
rF	35.63b	4.37b	2.76b
$LSD_{0.01}$	0.08	0.04	0.02

* SD = saw dust, RH = rice hay, CC = corn cob, NOM = no organic matter, nrF = non-re-fermented, and rF = re-fermented.

** Numbers followed by different letters in the same column are significantly different.

Table 2. Number of colony-forming units (CFU) in wet or dried granules containing different types, concentrations of organic matters, and with or without re-fermentation.

Tabel 2. Jumlah unit pembentuk koloni pada butiran basah atau kering yang mengandung bahan organik dengan tipe dan konsentrasi yang berbeda dengan atau tanpa fermentasi ulang

	Log_{10} numbers of CFU	
Treatments*	Wet granules (per 10 g) **	Dried granules (per 1 g) **
Organic matter		
SD	7.19	6.69
RH	7.08	6.68
CC	7.09	6.71
NOM	7.03	6.69
Concentration		
1%	7.01	6.69
3%	7.00	6.70
Fermentation		
nrF	6.95a	6.46a
rF	7.07b	6.92b
$LSD_{0.01}$	0.014	0.015

* SD = saw dust, RH = rice hay, CC = corn cob, NOM = no organic matter, nrF = non-re-fermented, and rF = re-fermented.

** Numbers followed by different letters in the same column are significantly different.

Neither the type of organic matter nor their concentration had any significant effect on the number of propagules entrapped in granules. However, significantly higher propagule densities were obtained when granules were returned to GPY for additional fermentation (Table 2).

Seven days after incubation, granules from all formulations were found to be free from contamination. All formulations were found to be 100% viable. Analysis showed that there was no significant effect of organic matter or their concentration on the viability and vigor of granules. The vigor of re-fermented granules was higher than non-re-fermented granules (Table 3).

Mycelia grew from granules in all directions with a growth rate of about 0.95 mm per day. There were no significant differences in radial growth from granules containing different types and concentrations of organic matter (Table 4).

Table 3. Vigor of granules containing different types and concentrations of organic matters with or without re-fermentation.

Table 3. Vigor butiran yang mengandung bahan organik dengan tipe dan konsentrasi yang berbeda dengan atau tanpa fermentasi-ulang

Treatments*	Vigor of granules (index 1 - 4) **
Organic matter	
SD	3.12
RH	3.13
CC	3.11
NOM	3.12
Concentration of organic matter	
1%	3.13
3%	3.11
Fermentation	
nrF	2.82a
rF	3.42b
$LSD_{0.01}$	0.09

* SD = saw dust, RH = rice hay, CC = corn cob, NOM = no organic matter, nrF = non-re-fermented, and rF = re-fermented.

** Numbers followed by different letters in the same column are significantly different.

Table 4. Radial growth of mycelia of *A. dactyloides* growing in soil from granules containing various types and concentrations of organic matters.

Tabel 4. Pertumbuhan radial miselia A. dactyloides di dalam tanah dari butiran yang mengandung berbagai tipe dan konsentrasi bahan organik

Treatments*	Radial growth (mm) of mycelia
Organic matter	
SD	19.09
RH	19.10
CC	19.08
NOM	19.09
Concentration of organic matter	
1%	19.17
3%	19.09

* SD = saw dust, RH = rice hay, CC = corn cob, NOM = no organic matter

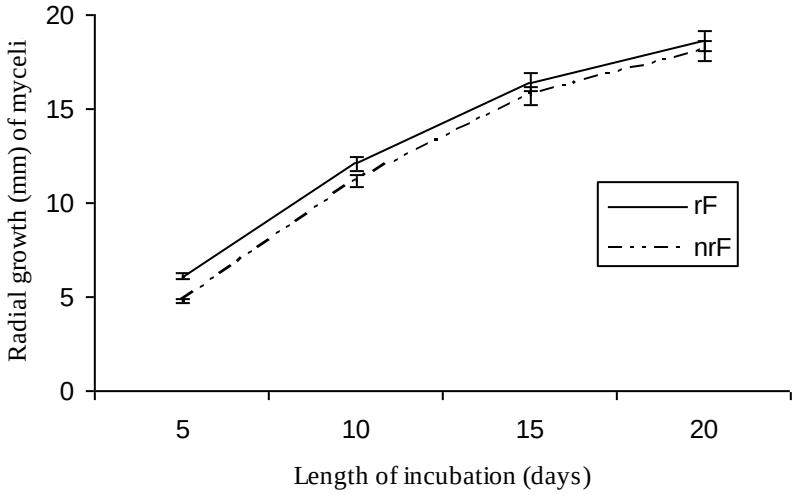

Figure 1. Radial growth of mycelia *A. dactyloides* in soil from re-fermented (rF) and non-re-fermented (nrF) granules introduced after various incubation periods. Bars are the values of $LSD_{0.05}$.

Gambar 1 Pertumbuhan radial miselia A. dactyloides di dalam tanah dari butiran yang difermentasi- (rF) dan tidak difermentasi-ulang (nrF) yang diberikan setelah berbagai periode inkubasi. Bar adalah nilai $BNT_{0,05}$.

Time of observation and re-fermentation of granules significantly influenced the radial growth of mycelia in soil and there was a significant interaction between these variables. During the first 10 days, the re-fermented granules produced significantly greater radial growth of mycelia that non-re-fermented granules (Figure 1). As the time of observation increased to 15 and 20 days, there were no significant differences in radial growth produced by re-fermented or non-re-fermented granules.

Mycelia grew well from granules and produced spores and rings. The highest and lowest numbers of rings per granule (596 and 176) were produced at day 5 by re-fermented and non-re-fermented granules, respectively. Neither the type of organic matter nor its concentration had any significant effect on numbers of rings and spores (Table 5).

Table 5. Number of rings and spores produced in soil by *A. dactyloides* growing from granules containing various types and concentrations of organic matters.

Tabel 5. Jumlah cincin dan spora yang terbentuk di dalam tanah oleh A. dactyloides yang tumbuh dari butiran yang mengandung berbagai tipe dan konsentrasi bahan organik.

Treatments*	Numbers of rings/mm^2	Numbers of spores/mm^2
Organic matter		
SD	9.33	1.055
RH	9.34	1.065
CC	9.33	1.059
NOM	9.33	1.058
Concentration of organic matter		
1%	9.33	1.059
3%	9.34	1.060

* SD = saw dust, RH = rice hay, CC = corn cob, NOM = no organic matter

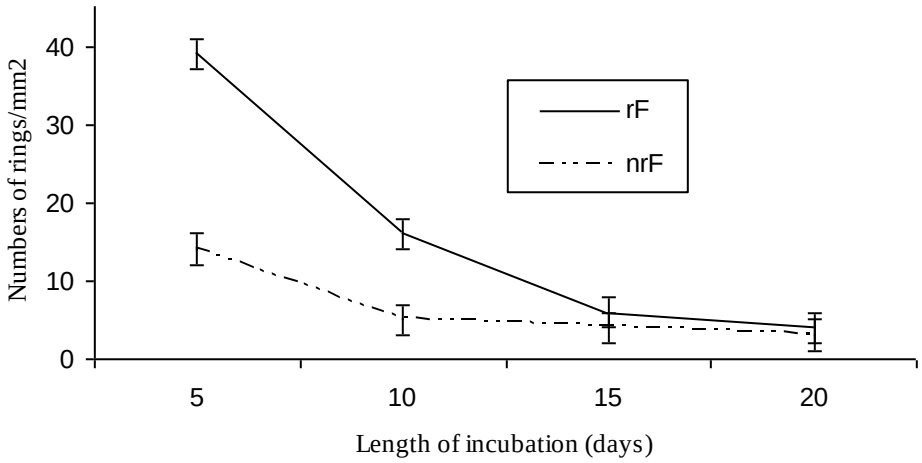

Figure 2. Number of rings produced in soil by *A. dactyloides* growing from re-fermented (rF) and non-re-fermented (nrF) granules introduced after various incubation periods. Bars are the values of $LSD_{0.01}$.

Gambar 2.Jumlah cincin yang dibentuk di dalam tanah oleh A. dactyloides yang tumbuh dari butiran yang difermentasi- (rF) dan tidak difermentasi-ulang (nrF) yang diberikan setelah berbagai periode inkubasi. Bar adalah nilai $BNT_{0,01}$.

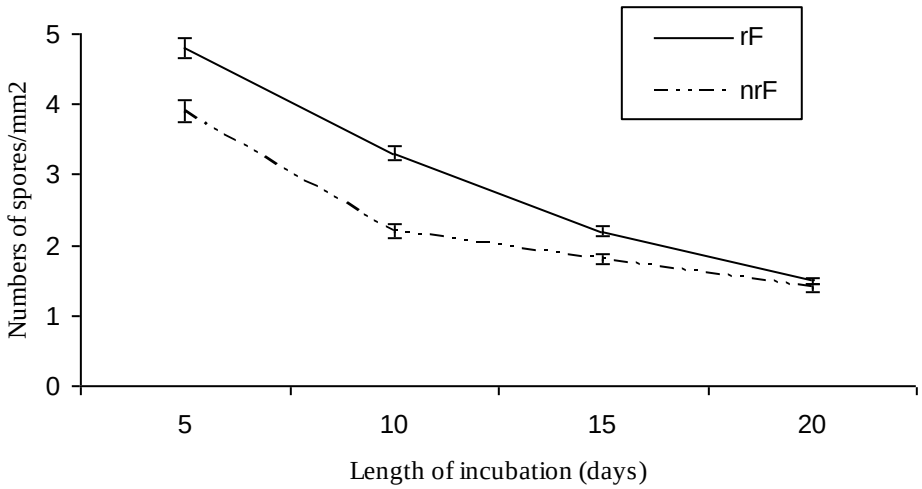

Figure 3. Number of spores produced in soil by *A. dactyloides* growing from re-fermented (rF) and non-re-fermented (nrF) granules introduced after various incubation periods. Bars are the values of $LSD_{0.01}$.

Gambar 3.Jumlah spora yang dibentuk di dalam tanah oleh A. dactyloides yang tumbuh dari butiran yang difermentasi- (rF) dan tidak difermentasi-ulang (nrF) yang diberikan setelah berbagai periode inkubasi. Bar adalah nilai $BNT_{0,01}$.

Time of observation and re-fermentation of granules significantly influenced numbers of rings and spores and there was an interaction between these two variables. Numbers of rings (Figure 2) and spores (Figure 3) per mm^2 at day 5 and 10 produced from re-fermented granules were significantly higher than from non-re-fermented granules. As the time of observation increased, the numbers of rings and spores decreased, and there were no significant differences in numbers of rings and spores produced by re-fermented or non-re-fermented granules.

The encapsulation of fungal biomass and organic nutrients within a sphere of kaolin and alginate followed by air drying produced granules

with handling properties that made them ideal for application to soil. Granules were of good form and size and contained at least 10^6 colony forming units (CFU)/g of granules (Table 2). Additional fermentation of granules resulted in a three-fold increase in fungal population density and an increase in the dry weight of granules (Table 1). The drying process was not detrimental to *A. dactyloides*, because the number of viable propagules in granules from all formulations was not markedly reduced following drying.

The results of a series of experiments using formulations prepared by incorporation of biomass with or without organic nutrients such as saw dust, rice hay or corn cob suggested that these organic nutrients did not have any significant effect on the performance of *A. dactyloides*. Tests on agar revealed that organic nutrients incorporated into granules did not affect growth rate and growth vigor. Re-fermentation of granules, on the other hand, significantly improved granule performance. Observations for a period of 20 days on TWA (data not shown) showed that the growth of mycelia from re-fermented granules was faster than that of from non-re-fermented granules. Vigor was also higher, as shown by the density of mycelia growing from granules (Table 3). This vigor was strongly related to the high numbers of propagules observed in re-fermented granules (Table 2). These results support previous findings (Stirling and Mani, 1995) and suggest that for slow growing species such as *A. dactyloides*, additional fermentation can be advantageous, probably because mycelial growth and repair occur following the formulation process.

Since *A. dactyloides* must ultimately grow from granules into soil if the formulation is to be useful in biological control, the granules were tested in soil for their ability to grow, to produce spores and rings. Results showed that in 20 days, mycelia growing from granules dispersed about 19 mm from granules, and produced traps. Organic nutrients did not have any significant effect on the growth of mycelia, the production of spores or rings (Tables 4 and 5). Re-fermentation of granules, however, significantly increased the growth rate of granules (Figure 1), ring and spore production (Figures 2 and 3). The standard slide test proved to be a useful indicator of predacious activity, as traps were invariably produced a few days after granules were added to soil. Despite previous studies with other fungi which have shown both beneficial effects (Lewis and Pavavizas, 1985; Kerry, 1988; Kerry, *et al*., 1993; Daigle and Cotty, 1992) and disadvantageous effects (Knudsen *et al.*, 1990) of incorporating organic nutrients into alginate-kaolin granules, the results of this study demonstrated that incorporation of organic nutrients into alginate-kaolin granules did not benefit *A. dactyloides*. It was hypothesised that *A. dactyloides* would use saw dust, rice hay or corn cob as an initial source of carbon and these nutrients would improve fungal proliferation from granules. The fact that this did not occur may mean that nutrients from the culture broth which was incorporated into granules with the fungus were sufficient for growth and trap production.

Overall, this study has shown unequivocally that *A. dactyloides* can be incorporated into a granular formulation that has good handling and storage properties. The results also confirmed that *A. dactyloides* will produce a network of traps when the fungus is introduced into soil. *A. dactyloides* appears to have relatively weak competitive saprophytic ability and appears to behave in a somewhat similar manner to *Hirsutella rhossiliensis* (Lackey *et al*., 1993) in not requiring an additional source of organic matter in granules. Thus to minimize nutrients that might act as a food source for competitive microorganisms once granules were introduced into soil, it will be better to develop granules without addition of organic nutrient for further studies of *A. dactyloides*.

CONCLUSION

Neither types nor concentrations of organic matters had any significant effect on the performance of *A. dactyloides*. Re-fermentation of granules, however, significantly improved weight of granules, number of propagules in granules, vigor of the fungus, growth of the fungus in soil, spore and ring production in soil.

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