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EFFECT OF NUTRITION AND PHYSICAL FACTORS ON MYCELIAL GROWTH AND SPORE PRODUCTION OF *Curvularia prasadii*, A MYCOHERBICIDE AGENT FOR BARNYARD GRASS (*Echinochloa crus-galli*) IN RICE

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ABSTRACT

Echinochloa crus-galli is an important weed in rice paddies. The optimum cultural and physical conditions for production of *Curvularia prasadii* (CP01, CP02, and CP03 isolates), a biocontrol agent for the rice weed, *E. crus-galli*, were determined. Culture media, light regime, pH, incubation temperature, and aeration were tested for their effect on the mycelial growth and spore production of the fungal biocontrol agent. Showed to be the best medium for the linear growth of the three isolates of the fungus was malt extract agar (MEA). Maximum yield of spores of the three isolates was obtained on potato dextrose agar (PDA). Contentious darkness was the most suitable light regime for the mycelial growth and sporulation of the CP01 and CP02 isolates, while contentious or diurnal light were best for CP03 growth. However, all degrees light regime were sutable for the spore production of the isolate CP03, with no significant differences among them. The pH levels of 6, 7, and 8 induced the highest mycelial growth of the three isolates. However, the latter two pH levels (7 & 8) were best for the sporulation of all three isolate. The best mycelial growth was obtained at incubation temperature of 30°C (for CP01 and CP03) and 35°C (for CP02). For spore production, the optimum level of temperature for isolates CP01 and CP02 was 35°C, while it was 30°C for CP03. Aeration level (as negatively correlated with the number of Parafilm® layers around the culture plate edge) was also tested. There was no effect of the number of Parafilm® layers (aeration level) on the CP02 mycelial growth. However, two layers of sealing culture plates with Parafilm® provided optimum level of aeration for best mycelial growth for isolates CP02 and CP03 and highest spore production for all three isolates.

Keywords: Biological control, mycoherbicide, *Curvularia prasadii*, cultural conditions, physical factors, rice, *Oryza sativa*, *Echinochloa crus-galli*.

INTRODUCTION

Rice (*Oryza sativa* L.) is a vital food crop for more than half of the world population (IRRI, 2012; Luo & Yin, 2013). In Egypt, rice comes the second major cereal crops following wheat, and the annual cultivated area is about 1.42 million feddan, produces nearly 5.89 million tons of paddy rice. The average of production is 4.15 t/fed, which ranks as one of the highest average yield in the world (RRTC, 2012).

Weeds are considered the most serious factor affecting rice production because they compete for nutrients, light, and water. About 350 species of 150 genera and 60 families have been reported as rice weeds throughout the world (Hill *et al*., 1990; Safari, 2010). Normally the loss in rice yield due to weeds infestation ranges between 15-20%. However, in severe cases the

yield losses can be more than 50%, depending upon the species and intensity of weeds (Mamun, 1990; BRRI, 2006).

Echinochloa crus-galli is one of the most serious weeds affecting rice in all methods of rice establishment. Biological control of weeds is an alternative approach to chemical control, which causes hazards to the environment and pose risk to human health in addition to evolution of resistant weeds. Much research on the development of new mycoherbicides has been conducted during the two past decades worldwide. Several weeds have been targeted for microbial control (Boyette & Walker, 1985; TeBeest *et al*., 1992; Schroeder, 1993; Johnson *et al*., 1996; Imaizumi *et al*., 1997; Thomas *et al*., 1998; Montazeri & Greaves, 2002), but little attempts has been reported for barnyard grass. Applying mycoherbicides is the most essential measure in controlling weeds with biological backgrounds, particularly in the sustainable agriculture because they target ecosystem much less than controlling factors such as herbicides. In addition, they perform quite selectively and cause minimum damages to crops (Charudattan, 2001; 2005).

As populations of aquatic monocot weeds resistant to registered herbicides appear, there is increasing interest in the use of naturally occurring pathogens of weeds as potential biological control agents (Charudattan, 1985; 1991; Morin, 1993). Hence, the aim of the present work was to know the basic knowledge about the actual weed problem in rice fields and the possibilities to integrate potential mycoherbicides into the farming system in Egypt. An attempt was done to formulate and, thereafter to test efficacy of safe and effective bioherbicides to control weeds in rice instead of using toxic chemical substances. The specific objective of the current research was to determine the optimum cultural and physical conditions for production of *Curvularia prasadii*, a biocontrol agent for barnyard grass, a serious rice weed.

MATERIALS AND METHODS

Source of the fungal biocontrol agent:

Surveys for diseases on rice weeds were done in Egypt in three governorates (Dakahleia, Kafr El-Sheik, and El-Gharbeia) in three consecutive growing seasons (from 2012 to 2014). Leaves of weeds showing disease symptoms were collected in polyethylene bags and kept on ice until processed in the laboratory the same day.

All fungal isolates obtained from the surveys over three years were tested for their pathogenicity against barnyard grass weed plants. By fulfilling Koch's postulates, only three isolates proved to be pathogenic on the weed causing leaf spots and blight resulting in reduction in the growth of weed seedlings in the greenhouse. Those isolates were identified as *Curvularia prasadii* through the Department of Survey and Identification of Fungi, Agricultural Research Center, Giza, Egypt.

Effect of Nutrition and Physical Factors on Mycelial Growth and Spore Production of the Biocontrol Agents:

Effect of culture media:

To determine the effect of nutrients on mycelia growth and sporulation of the three fungal biocontrol isolates (CP01, CP02, and CP03), four culture

media were tested. The media used were PDA (Bio Life Italiana s. r. l. viale Monza, Milan, Italy), malt extract agar MEA) (25g malt extract + 30g agar/L), carrot agar (CA) (200g fresh carrot + 20g agar/L), and Czapek-Dox agar (CDA). Five-millimeter-diameter discs from a 10-day-old culture on PDA were placed in 9-cm Petri dishes containing 20 ml of the medium and incubated at 28°C in darkness for 30 days. Eight replicates (plates) for mycelial growth and sporulation were used for each treatment (medium).

The diameter of the colonies was measured 2, 4, and 6 days after seeding. Spore production was quantified 30 days after inoculation. The spores were flooded with 10 ml sterile water, diluted as 1: 10 with sterile water, stirred in a Vortex mixer and the number of spores/ml (ns/ml) in three replicates was enumerated with a haemacytometer (Shabana, 2000).

Effect of pH:

The pH of the PDA medium was adjusted to 4, 5, 6, 7 and 8, using 1N and/or 50% NaOH and 1N HCL before autoclaving. The pH was re-adjusted after autoclaving to the same values. Five-millimeter-diameter discs from a 10-day-old culture on PDA were placed on the surface of PDA medium in a 9-cm-diameter Petri dish (20 ml medium/plate) and incubated at 28°C in the dark for 30 days. Eight replicates for mycelial growth and sporulation were used for each treatment.

The colony diameter was measured 2, 4, and 6 days after seeding. Spore production was quantified 30 days after inoculation. The spores were flooded with 10 ml sterile water, diluted as 1: 10 with sterile water, stirred in a Vortex mixer and the number of spores/ml in three replicates was enumerated with a haemacytometer as previously described (Shabana, 2000).

Effect of temperature:

The cultures were prepared in the same manner as described above. Inoculated Petri dishes were incubated at 15, 20, 25, 30, and 35°C. Eight replicates were used for each treatment.

The colony diameter And the amount of spores were measured as described above.

Effect of light regime:

The cultures prepared as described above were incubated at 28°C under three light regimes: continuous darkness (CD), continuous light (CL) and diurnal light (DL). Eight replicates were used for each treatment. The colony diameter And the amount of spores were measured as described above.

Effect of sealing of the culture plates:

Cultures prepared as described above were sealed with 0, 2, 4, 6, and 8 layers of Parafilm (American National Can, Greenwich, CT, USA) and incubated in darkness at 28°C. Eight replicates were used for each treatment. The colony diameters and the quantification of spore production were measured as mentioned above.

RESULTS

Effect of culture media:

Maximum linear growth of all three isolates (CP01, CP02, and CP03) was obtained on MEA medium. The second best medium was carrot agar medium (CA) or PDA (for CP01); CA (for CP02); and PDA (for CP03) (Table 1). However, maximum yield of spores was obtained on PDA for all three isolates (CP01, CP02, and CP03) (Table 1).

Table 1: Effect of culture media on the linear growth of mycelium and spore yield of the three isolates of the biocontrol agent *Curvularia prasadii.*

Medium[w]	*Curvularia prasadii* (isolate)								
	CP01			CP02			CP03		
	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^{6x}
(CA)	6.30±1.39	1.05 b[y]	80.60 b	6.50±0.71	1,08 b	96.90 a	6.31±0.46	1.05 c	62.32 b
(CDA)	5.50±0.96	0.92 b	47.92 c	5.00±0.46	0.83 d	30.10 c	6.36±0.81	1.06 c	63.00 b
(MEA)	8.94±0.17	1.49 a	61.77 bc	9.00	1.50 a	57.11 b	9.00	1.50 a	74.48 b
(PDA)	5.59±0.47	0.93 b	152.84 a	5.55±0.34	0.93 c	101.51 a	7.94±0.75	1.32 b	135.57 a

[w].CA=fresh Carrot Agar; CDA= Czapek-Dox Agar; MEA= Malt Extract Agar; PDA= Potato Dextrose Agar

[x] Mean number of spores/ml per plate 30 days after inoculation.

[y] Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range test (*P* = 0.05).

Effect of light regime

Contentious darkness was the most suitable light regime for the mycelial growth and sporulation of CP01 and CP02 isolates, while contentious or diurnal light was best for CP03 linear growth (Table 2). However, all light regimes were proper for the spore production of the isolate CP03, with no significant differences among them, while continuous light produced maximum linear growth of this isolate (Table 2).

Table (2): Effect of light regime on the linear growth of mycelium and spore production of the three isolates of the biocontrol agent *Curvularia prasadii.*

Light[x]	*Curvularia prasadii* (isolate)								
	CP01			CP02			CP03		
	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^{6y}
(CD)	3.91±0.17	0.65 a[z]	176.10 a	4.33±0.23	0.72 a	161.80 a	4.89±0.30	0.81 c	92.30 a
(DL)	3.58±0.27	0.60 b	119.10 b	3.84±0.38	0.64 ab	52.10 b	5.73±1.07	0.95 b	109.55 a
(CL)	3.51±0.45	0.59 b	63.10 c	3.55±0.72	0.59 b	57.10 b	7.21±0.62	1.20 a	100.90 a

[x] CD= Continuous Dark; DL = Diurnal Light; CL = Continuous light;.

[y] Mean number of spores/ml per plate 30 days after inoculation.

[z] Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range test (*P* = 0.05).

Effect of pH:

Maximum mycelial growth of the three isolates was obtained when the pH level of the culture medium was adjusted at 6, 7, and 8. However, the latter two pH levels (7 & 8) have induced the maximum yield of conidia of all three isolate (Table 3). Thus, this fungus prefer alkali medium for growth and sporulation.

Table (3): Effect of pH on the linear growth of mycelium and spore production of the three isolates of the biocontrol agent *Curvularia prasadii.*

pH[x]	*Curvularia prasadii* (isolate)								
	CP01			CP02			CP03		
	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^{6y}
4	7.31±0.75	1.22 b[z]	20.18 b	8.13±0.51	1.35 b	31.88 ab	4.81±0.66	0.80 c	36.62 bc
5	7.35±0.54	1.23 b	22.26 b	7.68±0.38	1.28 c	22.00 b	6.06±0.79	1.01 b	27.79 c
6	9.00	1.50 a	22.16 b	8.95±0.11	1.49 a	28.23 b	8.75±0.27	1.46 a	15.76 d
7	8.99±0.04	1.50 a	49.29 a	9.00	1.50 a	44.95 a	9.00	1.50 a	46.69 a
8	9.00	1.50 a	41.02 a	9.00	1.50 a	44.06 a	9.00	1.50 a	38.91 ab

[x].pH=pH levels were tested;.

[y] Mean number of spores/ml per plate 30 days after inoculation.

[z] Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range test (*P* = 0.05).

Effect of incubation temperature:

The best mycelial growth was obtained at incubation temperature of 30°C (for CP01 and CP03) and 35°C (for CP02) (Table 4). For spore production, the optimum level of temperature for isolates CP01 and CP02 was 35°C, while it was 30°C for CP03 (Table 4).

Table (4): Effect of incubation temperature on the linear growth of mycelium and spore production of the three isolates of the biocontrol agent *Curvularia prasadii.*

Temp.[x] (ºC)	*Curvularia prasadii* (isolate)								
	CP01			CP02			CP03		
	Mean colony diameter (cm)	Growth rate (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rate (cm day^{-1})	Conidia per ml x 10^6	Mean colony diameter (cm)	Growth rate (cm day^{-1})	Conidia per ml x 10^{6y}
15	2.45±0.37	0.41 d[z]	69.75 c	2.21±0.22	0.37 d	54.96 d	2.81±0.37	0.47 e	55.15 d
20	3.24±0.57	0.54 c	62.45 c	3.80±0.93	0.63 c	141.92 cd	5.31±0.84	0.89 d	92.45 cd
25	3.48±1.036	0.58 c	185.10 b	4.68±1.08	0.78 b	175.64 bc	7.71±0.79	1.29 b	171.60 b
30	5.05±0.67	0.84 a	174.15 b	5.14±0.78	0.86 ab	28.20 ab	8.57±0.26	1.43 a	256.30 a
35	4.26±0.87	0.71 b	250.40 a	5.76±0.49	0.96 a	303.36 a	6.54±0.59	1.09 c	135.10 bc

[x] incubation temperature dgree

[y] Mean number of spores/ml per plate 30 days after inoculation.

[z] Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range test (*P* = 0.05).

Effect of sealing of the culture plates

Aeration level (as negatively correlated with the number of Parafilm® layers sealing the culture plate) was also tested. There was no significant effect of the number of Parafilm® layers (aeration level) on the mycelial growth of CP02 (Table 5). However, two layers of sealing culture plates with Parafilm® provided optimum level of aeration for maximum mycelial growth for isolates CP02 and CP03 and maximum conidial yield for all three isolates (Table 5). Also, the unwrapped cultures of isolates CP01&CP03 induced maximum level of sporulation (Table 5).

Table (5): Effect of number of Parafilm® layers on the linear growth of mycelium and spore production of the three isolates of the biocontrol agent *Curvularia prasadii.*

No. of Parafilm layers	*Curvularia prasadii* (isolate)								
	CP01			CP02			CP03		
	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^{6} [y]	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x10^{6}	Mean colony diameter (cm)	Growth rates (cm day^{-1})	Conidia per ml x 10^{6}
0	9.00	1.5a[z]	152.79 a	8.37± 0.23	1.39a	99.95 bc	9.00	1.5a	135.99 a
2	8.50	1.4b	146.25 a	8.43±0.17	1.4a	200.36 a	9.00	1.5a	125.81 a
4	8.50	1.4 b	74.17 b	8.43±0.17	1.4a	65.55 b	8.27±0.43	1.37c	94.27 b
6	8.012±0.035	1.3c	99.14 b	8.50	1.4a	97.29 cb	8.50	1.4b	38.00 d
8	8.37±0.23	1.39b	108.52 b	8.43±0.17	1.4a	120.34 b	8.00	1.3d	67.06 c

[y] **Mean number of spores/ml per plate 30 days after inoculation.**

[z] **Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range test (*P* = 0.05).**

DISCUSSION

The specific objective of this research was to determine the optimum cultural and physical conditions for production of *C. prasadii*, (CP01, CP02, and CP03 isolates), a potential bioherbicide for barnyard grass

Alterations in nutrients in culture media used showed promotional effects on the growth of the fungal strain. Our results are in good agreement with Rabbani *et al.*, (2011), who too reported potato dextrose broth medium as the best medium for maximum growth and sporulation of *Drechslera hwaiiensis*, the cause foliar blight pathogen of *Marsilea minuta* L. Also, our results revealed that the best medium for sporulation of the three isolates of the biocontrol agent, *C. prasadii*, was PDA medium. This finding contradict with the funding agree with that of Shabana *et al.* (2000) who stated that the sporulation of *Alternaria eichhorniae* was greatly inhibited on PDA. This disagreement may be attributed to the fact that fungi may differ in their requirements for nutrients for their growth or sporulation.

As temperature affects almost every function of the fungi (Lilly and Barnett, 1951). Our results suggest that the three isolates of *C. prasadii* have their ecological preferences. Low temperature in winter may cause a problem during biological control by influencing the activity of the biocontrol agents

(Magan *et al*., 1988). Our findings support this statement since the growth of our biocontrol agent *C. prasadii* was inhibited by incubation at low temperature (15 and 20^{o}C). Also, these results agree with the findings of Maity and Samaddar (1977) Zhang *et al*. (1996) and Shabana *et al.* (2000) using different fungal agents. Nagesh *et al*. (2007) obtained similar findings, whereas they found that the best temperature for growth of *P. chlamydosporia* was 25-35^{o}C. Also, our results are in line with those of Arevalo *et al*. (2009) who reported that the optimum temperature for growth and spore production for *P. chlamydosporia* isolates ranged between 24 and 28°C and those of Duponnois *et al*. (1995) who observed that optimum growth of *A. oligospora* occurred at 25-30°C. Also, Nagesh *et al*. (2005) demonstrated that the optimum temperature for *A. oligospora* growth, sporulation, conidiospore germination and conidiospore production ranged between 25 and 35°C. The diurnal temperature (30°C) was reported as the optimum temperature for *Aspergillus carbonarius* growth (Mitchell *et al*., 2003; Bellí *et a*l., 2004, 2005; Romero *et al*., 2006).

In this study, the pH levels of 6, 7, and 8 induced the highest mycelial growth of the three isolates. While the pH (7 & 8) was best for the sporulation of all three isolate. This agree with the findings of Nagesh *et al.* (2007) who reported that pH 6.0 and 7.5 were optimum for *A. oligospora* and with those of Kredics *et al*. (2003) who found that the optimum pH for *P. chlamydosporia* was 6.5-7.7. The growth rate of *A. eichhorniae,* however, increased when pH was raised from 4 to 7; the fungus grew well when the initial pH of culture was adjusted in the range of 5.5 to 7.5 and there is often little effect of pH within this range. Our results are in same trend reported by by Shabana *et al.* (2000) and Griffin(1988).

Aeration level (improved by not wrapping the culture plates and negatively correlated with the number of Parafilm® layers sealing the culture plate) was also tested. There was no effect of the number of Parafilm® layers (aeration level) on the CP02 mycelial growth. This contradicts the findings of Shabana *et al.* (2000) who reported that the mycelial growth of *A. eichhorniae*, a bioherbicides for waterhyacinth, increased by improved aeration. They found that the production of the mycelial growth of *A. eichhorniae* was greatly inhibited by increasing the Parafilm® wrappings around the culture plates. Such sealing also suppressed spore production by the fungus (Shabana, 1992). These effects may be due to a reduction of air exchange in wrapped cultures and an increase in the concentration of CO_2 as suggested by Cotty (1987). In our study, two layers of sealing culture plates with Parafilm® provided optimum level of aeration for best mycelial growth for isolates CP02 and CP03 and highest spore production for all three isolates suggesting the inhibitory effect of CO_2 on sporulation (Cotty, 1987).

The present results of the light regime study revealed that contentious darkness was the most suitable light regime for the mycelial growth and sporulation of the CP01 and CP02 isolates, while contentious or diurnal light were best for CP03 growth. However, all light regimes were proper with no significant differences for the spore production of the isolate CP03. This finding is in contradiction with that of Shabana, *et al.* (*2000*) who found that

the mycelial growth of *A. eichhorniae* was increased by light. However, our results agree with those of Umalkar and Begum (1976) who reported that the growth rates of *Alternaria brassicicola* (*A. brassicae*) (Mukadam and Deshpande, 1979) and *A. tagetica* (Cotty and Misaghi 1985) were decreased by exposure to light. Sporulation of many fungi is influenced by light intensity and light duration (Tan 1978). However, results of this study have shown that contentious darkness was the most suitable light regime for the sporulation of *C. prasadii*. In this respect, our results agree with Nagraj and Ponnappa (1970) who reported that dark periods are required for maximum production of conidiophores by *A. brassicae,*

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تأثير التغذية والعوامل الطبيعية على نمو وتجرثم الفطر كيرفيولاريا براساديای – المبيد الحيوى لحشيشة الدنيبة في الأرز

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الدنيبة *Echinochloa crus-galli* هي واحدة من أهم وأخطرالأعشاب الضارة فى حقول الأرز. في هذا البحث، تم تحديد الظروف المثلى لإنتاج ثلاثة عزلات لعامل المكافحة البيولوجية *Curvularia prasadii* لحشيشة الدنيبة فى حقول الأرز وهذه العزلات هى (CP01، CP02، وCP03). وقد تم اختبار تأثير أنواع من البيئات الغذائية، ونظام الإضاءة، ودرجة الحموضة، ودرجة حرارة التحضين، ودرجة التهوية على النمو الميسيليومى وإنتاج الجراثيم لفطر المكافحة البيولوجية لحشيشة الدنيبة. وقد أوضحت النتائج أن أفضل بيئة غذائية لنمو العزلات الثلاثة كانت بيئة آجار مستخلص الشعير (MEA)، بينما كانت أفضل بيئة لتجرثم العزلات الثلاثة هى آجار مستخلص البطاطس والدكستروز. كما أوضحت النتائج أن أفضل نظام إضاءة لنمو وتجرثم العزلتين الأولي والثانية كان نظام الإظلام التام المستمر، وكان أفضل نظام إضاءة لنمو العزلة الثالثة هو نظام الإضاءة المستمرة. بينما لم يتأثر تجرثم العزلة الثالثة باختلاف نظام الإضاءة. وفي اختبارات درجة الحموضة أعطت العزلات الثلاث أفضل نمو لها عند درجات حموضة 6 و7 و8، بينما أعطت أفضل إنتاج للجراثيم عند درجتى الحموضة 7 و8. وكانت درجة الحرارة 30 م هي أفضل درجة حرارة لنمو العزلتين الأولي والثالثة، بينما كانت أفضل درجة حرارة لنمو العزلة الثانية هى 35 م. وكانت أفضل درجة حرارة لتجرثم العزلتين الأولي والثانية هى 35 م، بينما كانت 30 م للعزلة الثالثة. وفى تجربة استخدام مستويات مختلفة من التهوية لمعرفة أفضل تلك المستويات علي معدل نمو وتجرثم العزلات، وجد أن افضل مستوي لنمو العزلة الأولي كان التهوية الكاملة (عدم غلق أطباق المزارع بالبارافيلم، أى 0 لفة)، بينما لم يكن هناك اختلاف في معدل النمو للعزلة الثانية مع المستويات المختلفة من التهوية، بينما كان أفضل مستوي لنمو العزلة الثالثة هو التهوية الكاملة (عدم غلق الاطباق بالبارافيلم، او بغلقها بلفتين من البارافيلم) وكان أفضل إنتاج لجراثيم العزلتين الأولي والثالثة عندما كانت التهوية كاملة بينما كان أفضل إنتاج لجراثيم العزلة الثانية عندما تم غلق أطباق المزارع بلفتين من البارافيلم.