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Note

A simple *in-vitro* 'wet-plate' method for mass production of *Phytophthora nicotianae* zoospores and factors influencing zoospore production

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Abstract

A simple *in-vitro* 'wet-plate' method for mass-producing *Phytophthora nicotianae* zoospores at $\geq 1.0 \times 10^6$ zoospores/ml is described. Temperature critically affected zoospore production; 22 °C was optimum, while 36 °C was completely inhibitory. Zoospores being the most important propagule of *P. nicotianae*, temperature of recycled irrigation water may be manipulated to reduce diseases in irrigated nursery crops.

Keywords: *Phytophthora*; Recycled irrigation water; Zoospore production

Zoospores are the most important propagule for development of *Phytophthora* and *Pythium* disease epidemics in crop production, particularly in recycled water irrigation systems (Thomson and Allen, 1974; Neher and Duniway, 1992; Thinggaard and Andersen, 1995; Stanghellini et al., 1996; van der Gaag et al., 2001). Hence, the zoospore stage is intensely investigated in many laboratory and greenhouse studies aimed at understanding the biology, epidemiology and development of control methods for these pathogens. Currently, the favored method for producing zoospores of *Phytophthora nicotianae* Breda de Haan (=*P. parasitica* Dastur), a pathogen causing diseases of many herbaceous and woody plants (Erwin and Ribeiro, 1996), is the 'cut-agar pieces' method (Hong et al., 2002, 2003; Kong et al., 2003; Banko et al., 2006). In this method, matured V8-agar culture (2 to 3-week old) is cut into small pieces, submerged in 20 to 25 ml of 1% chilled sterile soil water extract (SSWE) and incubated under fluorescent light for 3 to 4 h to produce a spore suspension (Kong et al., 2003; Banko et al., 2006). However, unlike micro-conidia of many fungi that can readily be produced in millions per milliliter using solid agar media, the concentration of zoospores of *P. nicotianae* (like many other *Phytophthora* sp.) produced using this method rarely exceeds 2.0×10^4 zoospores/ml. This poses an enormous challenge to some studies, particularly those that require large volumes of concentrated zoospore suspension, or studies requiring a highly concentrated stock solution of DNA or RNA from zoospores. Usually, to obtain concentrations of zoospores higher than 1×10^4 for DNA extraction, 1 ml of original zoospore suspension in 1.5 ml microcentrifuge tubes are centrifuged at 10,000 ×*g* and the pellets re-suspended in smaller volumes (Kong et al., 2003). This is not efficient and the success of concentrating the zoospore suspension to the desired concentration is never guaranteed. In an ongoing irrigation water decontamination study in our laboratory, over 60 l (at >20 l per experimental day) of 3000 spores/ml zoospore suspension is required for each experiment. For this type of research, it would be a daunting task (if not impossible) to depend on the 'cut-agar pieces' method of zoosporic inoculum production. It was therefore essential to identify an enhanced method of producing this important biological entity.

Initially, we tested several methods that did not result in the desired level of zoospore production. Some of those methods targeted at increasing the number of zoosporangia produced included: culturing the pathogen on 5% clarified V8-agar compared with 10% and 20% V8-agar; using 5% or 20% V8-agar/

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broth modified with 1% SSWE; and growing the pathogen in 250 ml of 20% V8-broth contained in a 500 ml flask in standing or shaking position for 1 or 2 weeks before removing the V8-broth from the mycelial mass, and adding pre-chilled de-ionized water or 1% SSWE for zoospore production. Another method that was tried which eventually led to the method described in this paper is growing the pathogen in a 500 ml flask containing only 25 ml of 20% V8-broth in standing position (not shaken) for 1 or 2 weeks, rinsing the mycelial mass with de-ionized water before incubating for zoospore production in chilled de-ionized water for 3 h. With this method, most of the isolates tested produced several zoosporangia after two weeks and consequently up to 4.5×10^4 zoospores/ml.

Following the results of the initial trials described above, the 'wet-plate' method was developed as a Petri dish based method employing 20% clarified V8-broth as the culture medium. It was hypothesized that a thin layer broth-Petri dish method would result in improved zoospore production and efficiency, since growing the pathogen in 25 ml of broth in 500 ml flask resulted in a significantly higher number of zoospores than in 250 ml broth in the same flask. At the early stage of the development, nine isolates of *P. nicotianae* were used, and the plates were observed daily under the microscope after pathogen inoculation to determine when the first few sporangia are produced. The first few sporangia were observed on the sixth day in most of the isolates, so broth was removed from the cultures on the sixth day. Then, the wet-plate cultures were grown for another 2 days before adding de-ionized water/1% sterile soil water extract to the plates and incubating for 3 to 4 h for zoospore release. Of the three best isolates (26E7, 31A3 and 18C7) which resulted in between 5.1×10^3 and 7.5×10^3 zoospores/ml, an A2 mating type isolate, 31A3, isolated in 2004 from *Petunia* sp. in Virginia, USA was selected for further refinement of the method. Major areas focused on were method of V8-broth inoculation/age of culture use for inoculation, the time and method of V8-broth removal from culture, length of further incubation after V8-broth drain-off from culture and temperature and length of incubation for zoospore release.

The final 'wet-plate' method developed is described as follows: From an autoclaved 20% clarified V8-broth stored in the refrigerator (for easy access at all times), 10 ml is dispensed into a 10-cm diameter plastic Petri dish. From a matured and sporulated V8-agar culture (at least two weeks old) 3 discs of 3-mm diameter agar plugs (made with a 3-mm cork borer) are picked up in turn with an inoculating knife/needle to inoculate the entire 10 ml V8-broth in the Petri dish by scrubbing the agar plug through the plate surface before placing it at the center (Fig. 1). This inoculation method results in a multi-colony non-flooded mycelial mass (Fig. 1) when the unsealed Petri dish is incubated at 25 °C in the dark for 1 week (at which time a few zoosporangia have been produced). A half-full Petri dish lid of de-ionized water (20 to 25 ml) at room temperature is carefully added to the culture plate, gently swirled and the liquid drained-off without disturbing the mycelial colonies which are mostly well attached to the plate (not floating, and not submerged in the 20% V8) because of the shallow depth of V8-broth. The wet (or moist) culture plate with the lid on, but without sealing is further

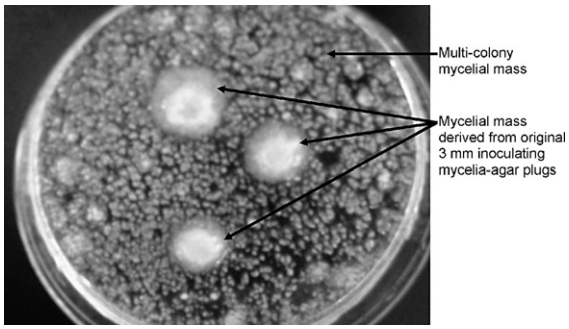

Fig. 1. One-week old 'wet-plate' culture of *P. nicotianae* isolate 31A3 ready for further incubation at 25 °C for high zoosporangia production.

incubated at 25 °C in the dark for at least another 3 days or, better still, 1 to 2 weeks for high numbers of zoosporangia to be produced. Under these conditions, high humidity, but without flowing liquid (that may create an environment conducive to the unwanted release of zoospores), along with adequate aeration (because it is not sealed) are maintained. Each of the many colonies continues to grow and firmly attach to the plate as in solid media plate culture and produce several zoosporangia. In this condition the plates can remain moist without drying for up to two weeks or more. If the plate begins to dry, it may be kept longer by remoistening it with de-ionized water. For zoospore production, 20 ml of de-ionized water is added to the culture plate directly from the tap (not necessary to chill in a refrigerator) followed by incubation at 22 °C for 2 to 3 h. The plate is gently swirled and the zoospore suspension which is almost completely free of mycelial fragments is filtered through a layer of miracloth (Cat# 475855 Calbiochem®, CA 92039-2087 USA) to eliminate any remaining mycelial fragments. Usually, the concentration of the zoospore suspension is between 7×10^5 and 2×10^6 spores/ml depending mostly on age of the wet-plate culture. This highly concentrated zoospore suspension is normally diluted 10× to allow for easier and more accurate estimation of the number of spores/ml using a hemacytometer under a light microscope at magnification of 100×. Samples are transferred into 1.5 ml micro-centrifuge tube in three replicates, vortexed to force the zoospores to encyst, and from the encysted zoospore suspension, 10 µl are added to both chambers of a hemacytometer (Fisher Scientific Hemacytometer, Cat # 0267110) using a micro-pipette. All zoospores in each of the four 1-mm^3 corner grid square (Factor = 10,000) are counted and averaged per square; replicates of this count are then averaged again and multiplied by both the grid factor (10,000) and the dilution factor (10) to obtain number of zoospores/ml. We observed that the same culture plate may be reused with even higher efficacy for zoospore inoculum production several times with at least a 1-day interval for as long as it can be maintained.

This new method was compared with the cut-agar pieces method (Hong et al., 2002, 2003; Kong et al., 2003; Banko et al., 2006) for zoospore production efficiency using the same *P. nicotianae* isolate-31A3. Both the plates designated for the 'wet-plate' and the 'cut-agar pieces' methods were inoculated from a 3-week-old culture of isolate 31A3 and grown for

Table 1
Comparison between the 'wet-plate' method and the 'cut-agar pieces' method for *Phytophthora nicotianae* zoospore production efficacy

Method	Number of zoospore/ml ($\times 10^3$) [a, b, c, d]					
	Experimental day-1		Experimental day-2		Experimental day-3	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
Wet-plate	1429.2	53.12	1475.0	22.25	1720.8	42.81
Cut-agar pieces	14.1		10.0		15.9	

[a] Experiment was repeated twice in three consecutive experimental days using different culture plates each time.
[b] Ages of culture used on each experimental day were the same for both methods, but were 21, 22 and 23 days for experimental days, 1, 2 and 3, respectively.
[c] 20 ml of zoospore suspension was recovered per plate.
[d] Three replicates were used to calculate the mean.

3 weeks for zoosporangia production. Three Petri dish cultures (replicates) were evaluated per method per day on three separate days. For the 'cut-agar pieces' method, 10 plates of cut-pieces of agar plugs submerged in 20 ml of pre-chilled 1% SSWE were made from each V8-agar plate and the resulting zoospore suspensions were pooled as a replicate. There was no dilution of the original zoospore suspension and total number of zoospores on the big square (factor = 1111) of the hemacytometer was counted. Mean concentration of the zoospore suspension from the wet-plate method was 1.4×10^6, 1.5×10^6 and 1.7×10^6 zoospore/ml for experimental day-1, day-2 and day-3, respectively (Table 1). In contrast, mean concentration of the zoospore suspension from the cut-agar pieces method was 1.4×10^4, 1.0×10^4 and 1.6×10^4 zoospore/ml for experimental day-1, day-2 and day-3, respectively (Table 1).

To confirm earlier observations made during the initial trials that led to the development of the 'wet-plate' method for *P. nicotianae* zoospore production, the importance of using chilled 1% SSWE and incubating plates under white fluorescent light for zoospore production (Hong et al., 2002, 2003; Kong et al., 2003; Banko et al., 2006) was evaluated using the newly developed wet-plate method. There was no significant difference in the number of zoospores/ml of harvested zoospore suspension between using chilled 1% SSWE or chilled de-ionized water for incubating culture plate for zoospore production in neither of two consecutive experimental days (Table 2). Similarly, in either of two separate experimental days, the number of spores/ml of harvested zoospore suspension did not differ ($P>0.05$) when the wet-plate culture was incubated for zoospore production with chilled de-ionized water (4 °C) or de-ionized water (17 °C) taken directly from the tap (Table 2).

Another factor that may affect the number of zoospores released from zoosporangia in culture plates was thought to be temperature during incubation of culture plates for zoospore production. Consequently, the effect of temperature on the number of zoospores released from zoosporangia was tested at 10 to 38 °C, including, 10 °C, 15 °C, 22 °C, 25 °C, 30 °C, 33 °C, 36 °C and 38 °C. Plates were incubated for zoospore production at the different temperature regimes for 3 h. The eight temperature regimes were separated into three experimental groups and evaluated on three different experimental days with each temperature regime replicated three times and 22 °C included in each experimental group (or day) as a control. Results showed that the number of zoospores released from wet-plate cultures when incubated for zoospore production was critically dependent ($P<0.001$) on the incubation temperature (Table 2). There were significantly lower numbers of zoospores/ml of harvested zoospore suspension at 25 °C (4.2×10^5) and 33 °C (7.0×10^3) compared with at 22 °C (1.25×10^6), at 10 °C (4×10^3) and 30 °C (1.1×10^4) compared with at 22 °C (7.42×10^5), and at 15 °C (2.64×10^5) compared with 22 °C (7.38×10^5). At 36 °C or 38 °C, there were no zoospores produced compared with 7.38×10^5 zoospores/ml of zoospore suspension harvested from plates incubated at 22 °C (Table 2). The 36 °C and 38 °C incubation temperature treatments were repeated on another day, and the result was the same (Table 2). However, when the wet-plate culture incubated for zoospore production at 36 °C or 38 °C was removed and incubated at 22 °C for 3 h zoospores were produced in amount comparable with incubation at 22 °C (data not shown).

Table 2
Factors affecting *Phytophthora nicotianae* zoospore production from zoosporangia

Incubation medium/ condition for zoospore production	Number of zoospore/ml ($\times 10^3$) [a, b]					
	Experimental day-1 [c]		Experimental day-2 [d]		Experimental day-3 [e]	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
(a) 1% sterile soil water extract (SSWE)/de-ionized water (dH_2O)						
1% SSWE	1020.8	67.06	1141.7	85.65	–	
dH_2O	1016.7		1179.2			
(b)Room temperature de-ionized water (dH_2O)/chilled dH_2O (Chd-dH_2O)						
Chd-dH_2O (4 °C)	1050.0	121.66	754.2	59.66	–	
dH_2O (17 °C)	1291.7		783.3			
(c) Illumination						
White light	1170.8	100.69	1016.7	105.37	1037.5	54.00
Dark	1050.0		1362.5		950.0	
(d) Temperature [f]						
22 °C	1254.2					
25 °C	420.8	73.91	–		–	
33 °C	7.0					
22 °C			741.7			
10 °C	–		3.9	48.30		
30 °C			10.8			
22 °C					737.5	
15 °C	–		–		264.2	
36 °C					None	
38 °C					None	

[a] 20 ml of zoospore suspension was recovered per plate.
[b] Three replicates were used to calculate the mean.
[c] Age (in days) of culture used was 14, 16, 16 and 21 for (a), (b), (c) and (d), respectively.
[d] Age (in days) of culture used was 15, 12, 18 and 12 for (a), (b), (c) and (d), respectively.
[e] Age (in days) of culture used was 20 and 14 for (c) and (d), respectively.
[f] 36 °C and 38 °C were repeated and the same results as presented here were obtained.

The wet-plate zoospore production method described in this paper is a simple and efficient *in-vitro* method that can be reliably used to produce zoospores of *P. nicotianae* in concentrations as high as 100 fold (Table 1) over those that can be obtained with the currently used cut-agar pieces method. Just by picking up a wet-plate culture and adding 20 ml of de-ionized water, 2 to 4 l of 1×10^4 spores/ml zoospore inoculum can be produced in 3 h. With this level of efficiency, it becomes easy to run experiments requiring large volumes of concentrated zoospores. In addition, unlike the cut-agar pieces method, where it is difficult to obtain a pure zoospore suspension that is free of fine mycelial fragments and agar pieces that may affect results of quantitative DNA assays based on number of zoospores, the wet-plate zoospore production method results in a clean zoospore suspension even before filtering through miracloth. This method may therefore have significant impact on improving quantitative DNA or RNA assays such as quantitative real-time PCR assays that are dependent on number of *P. nicotianae* zoospores. The efficiency of this new *P. nicotianae* zoospore production method and the quality of zoospore suspension produced will potentially increase the number and quality of studies on this pathogen.

The findings in this study implicating 36 °C or 38 °C as completely inhibiting to zoospore release from zoosporangia indicate the need for further studies that may bring a practical and environmentally benign solution to infestations in recycled irrigation water by *P. nicotianae* and possibly other *Phytophthora* species. Further studies focused on evaluating the efficacy of temperature regime of this range (up to 38 °C) in inhibiting zoospore production of *Phytophthora* and *Pythium* species in recycled irrigation water and how such temperature can be generated and efficiently maintained in recycled irrigation water tanks are needed. At this temperature regime, cooling may not be required before using the water to irrigate crops (Brockwell and Gault, 1976), therefore the problem/cost of having to cool irrigation water after heating to 95 °C and above (Runia et al., 1988; Russell et al., 1992; McPherson et al., 1995; Runia, 1995; Ehret et al., 2001; Poncet et al., 2001) can be eliminated. In addition, this temperature regime may enhance the growth of biocontrol bacterial species in recycled irrigation water, giving them competitive advantage over pathogenic Oomycetes and fungi thereby increasing control efficacy.

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