

Mass Production of Gummy Stem Blight Spores for Resistance Screening

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In recent years, much work has been done in breeding cucurbits for resistance to gummy stem blight caused by *Didymella bryoniae*. Researchers have screened for useful sources of resistance in watermelon (*Citrullus lanatus*) (2), squash (*Cucurbita spp.*) (9), melon (*Cucumis melo*) (10), and cucumber (*Cucumis sativus*) (7). Germplasm screening often involves a combination of field and greenhouse inoculations, requiring many spores to ensure proper disease intensity. However, researchers have found different spore concentrations to be useful in screening cucurbits for resistance to gummy stem blight among experiments and species: 10^5 spores/ml in watermelon (1), squash (9), and melon (10); 10^6 spores/ml (3, 4, 6, 8) or 10^7 spores/ml (5) in cucumber. The production of a high number of spores of gummy stem blight often results in the preparation, infection, and harvest of hundreds of Petri plates per hectare of field to be inoculated.

In our study, we tested a new container to be used as an alternative to Petri plates for mass production of spores of gummy stem blight. We also tested two different methods of infection of the culture medium: 1) transfer of a plug of agar from a fungal culture, and 2) preparation of an inoculum solution from a previous culture and using that to flood the medium.

Methods: The experiments were conducted in the Horticultural Science pathology laboratory at North Carolina State University. We used a strain of *D. bryoniae* isolated in 1998 from diseased cucumber tissues harvested from naturally infected plants in Charleston, SC. The experiment was a split-plot treatment arrangement in a randomized complete block design, with two types of containers (20 Petri plates vs. 1 Nalgene autoclavable pan), two infection methods (potato dextrose agar plugs vs. inoculum solution), and four replications.

For all treatment combinations, *D. bryoniae* was grown on 50% potato dextrose agar (PDA) and incubated for 4 weeks at $24 \pm 2^\circ\text{C}$ under alternating periods of 12 hours of fluorescent light (40 to 90 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ PPF) and 12 hours of darkness.

Two different types of containers were used: plastic Petri plates (90 mm internal diameter) and autoclavable Nalgene autoclavable pans (420x340x120 mm). We poured the PDA into the Petri plates after sterilizing it in autoclave, while we autoclaved it directly into the final container for the Nalgene autoclavable pans. The Nalgene autoclavable pans were covered with aluminum foil for sterilization in autoclave and inserted in a sterile plastic, sealable, and transparent bag (Tower Self-Seal TSM, 24x30 inch or 61 x 76 cm, Allegiance Healthcare Corporation, McGaw Park, IL) after infection.

In our experiment each plot included 20 Petri plates or 1 Nalgene autoclavable pan, each containing 1 liter of PDA. We infected the Petri plates and Nalgene boxes following two different methods: 1) we transferred 5mm square plugs of PDA infected by gummy stem blight from previous cultures, and 2) we harvested spores from one Petri plate from previous cultures using a sterile glass rod and deionized water and then flooded the fresh medium with the inoculum solution (the inoculum solution was adjusted to a volume of 200 ml with sterile deionized water). The initial inoculum for each plot was prepared from a single Petri plate (20 plugs of PDA or 200 ml of inoculum solution). When Petri plates were infected using the solution rather than a PDA plug, we poured the solution into one Petri plate, recollected it in a sterile beaker, and then poured onto the next Petri plate until all 20 Petri plates of the plot were infected. With the Nalgene autoclavable pan, we poured the solution into the box and then recollected it in the beaker and disposed of it, since one pan was one plot.

At the end of the 4 week subculture, we harvested the spores from all plots separately: each Petri plate was flooded with 5 ml of sterile deionized water using a sterile repipette, while each Nalgene autoclavable pan received 100 ml of sterile deionized water (corresponding to 5 ml x 20 Petri plates) using a sterile graduated cylinder. The PDA surface was scraped with a sterile L-shaped glass-rod and the solution was then filtered through 4 layers of sterile

Table 1. Mean squares for spores production of gummy stem blight (total time, stock concentration, and total inoculum produced).^z

Sources of variation	D F	Total time (seconds)	Stock conc. (1,000 sores/ml)	Total inoculum (liters)
Replication	3	148.6 ns	1580600039.0 ns	38.5 ns
Type of container	1	525262.6 **	2979112852.0 ns	250.7 *
Inoculation method	1	2047.6 *	10566555039.0 **	200.9 *
Container x method	1	40100.1 **	108550352.0 ns	9.3 ns
Error	9	373.9	955730456.0	26.3

ns, *, ** indicates F value non-significant or significant at a p-value <0.05 or p-value <0.01, respectively.

^z Data are means of 4 replications, 2 types of container (Nalgene box vs. Petri plates), and 2 inoculation methods (infected PDA plugs vs. inoculum solution).

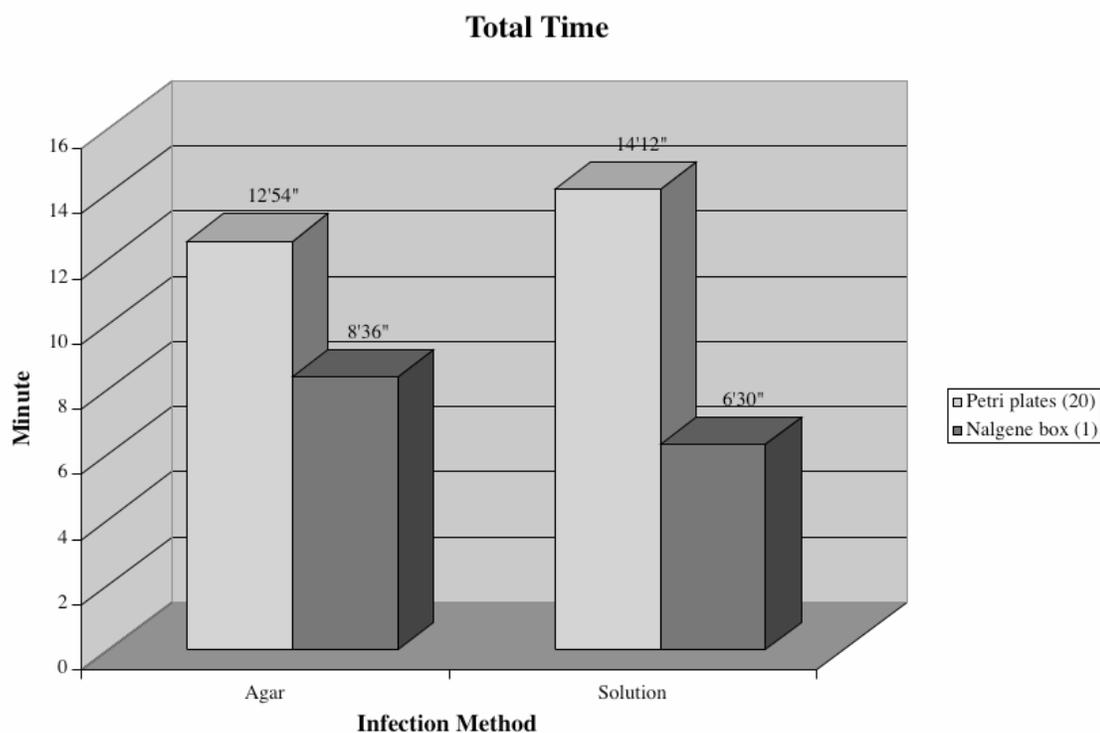


Figure 1. Total time needed from starting the culture to stock solution production.

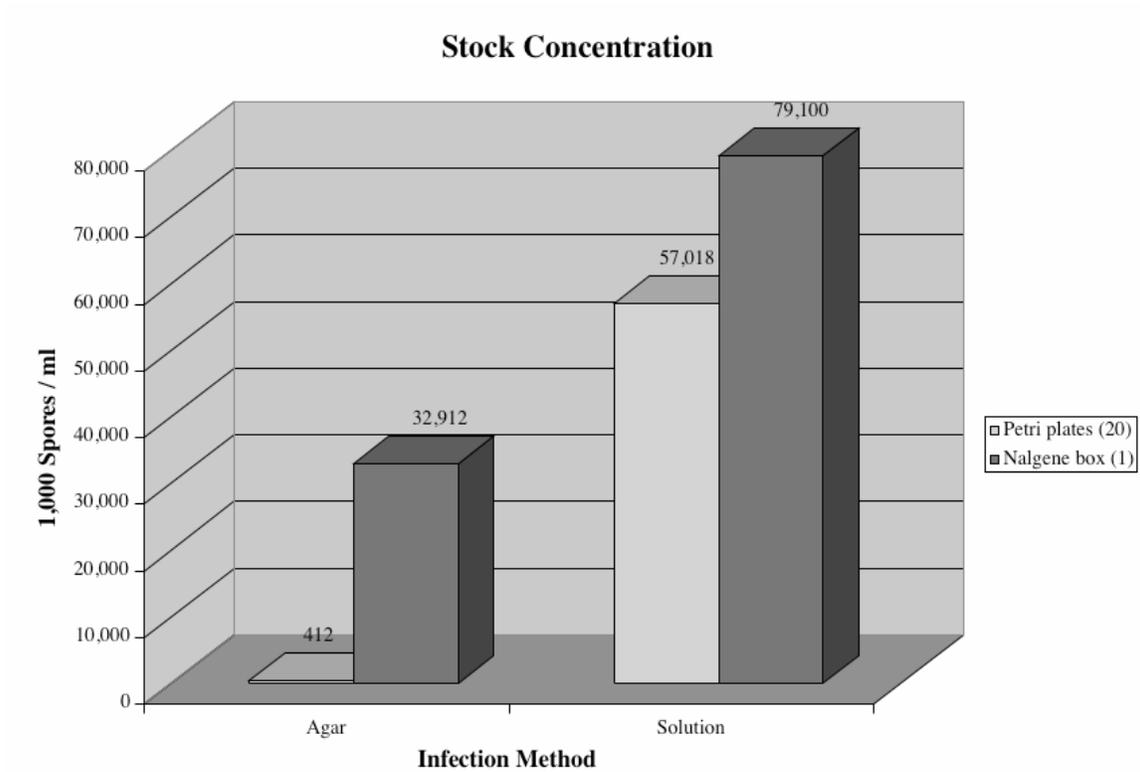


Figure 2. Concentration of the stock solution (1,000 spores/ml).

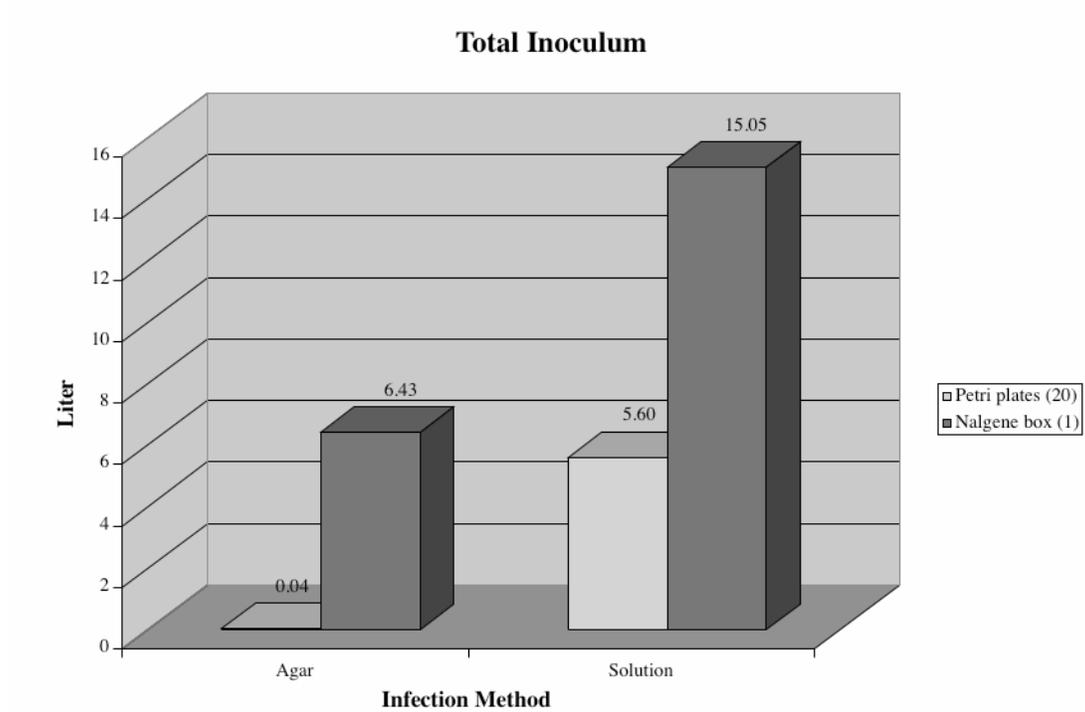


Figure 3. Total inoculum produced at a final concentration of 5×10^5 spores/ml.

cheese-cloth into a 100 ml sterile beaker to remove dislodged agar and part of the mycelium. For each stock solution we measured the volume using sterile graduated cylinders (one per solution), and the spore concentration using a hemacytometer. For each stock solution, we calculated the volume of final inoculum that could be produced, assuming a final concentration of 5×10^5 spores/ml (2).

We timed each of the following stages of spores production: a) preparation of 1 liter of PDA (including pouring into a flask for the Petri plates plots or into the Nalgene autoclavable pans directly), b) pouring of the PDA into the final container after sterilization in autoclave (pouring time = 0 for the Nalgene autoclavable pans), c) infecting each plot (including spore harvest, preparation of the inoculum solution, or preparation of the PDA plugs, and collocation of the Petri plates on the trays constituting each plot), d) harvesting of the spores, e) cleaning of the container after spore harvest (cleaning time = 0 for the Petri plates, being disposable). We did not consider as factors contributing to the total amount of time needed to produce a final inoculum solution from each plot the following: 1) sterilization in autoclave (it does not require labor and varies depending on the size of the autoclave, the temperature at the beginning of the cycle, and the temperature of the inlet water), 2) counting of the spores at the microscope with the hemacytometer (it is fairly consistent from one to another, and depends mostly on the ability of the counter).

Results: The largest source of variation in the analysis of variance was the type of container. Infection method had a smaller effect, and the interaction with type of container was variable. The infection methods had the only large and significant effect on stock concentration and almost the same effect of the type of container on the total volume of final inoculum produced. No effect was due to interaction between type of container and infection method for the two latter traits (stock concentration and total inoculum produced). Replication was not significant for any of the traits (Table 1).

Considering the time requirement, the stock concentration, and the final inoculum volume produced, the best method for spore production of gummy stem blight was the Nalgene autoclavable pan infected with the inoculum solution of spores (Fig. 1, 2, 3). The Nalgene autoclavable pan required

less time than Petri plates and produced more spores and more inoculum, regardless of infection method.

During the 4 weeks of subculture, we observed that the Nalgene autoclavable pans and Petri plates infected with the inoculum solution were ready for spore harvest at 10 days after infection, while the containers infected with the PDA plugs needed 4 weeks or more to colonize the PDA surface with mycelia.

The fastest growth of mycelia on PDA infected with a spore suspension rather than infected PDA plugs might be explained by the higher number of spores delivered with the first method in addition to the more uniform distribution of the spores on the PDA surface. More difficult to explain is the higher efficiency of the Nalgene autoclavable pans vs. the Petri plates: we hypothesize that the higher volume of air present in the box helps to disperse volatile autoinhibitory compounds eventually produced by the fungus. It may also be that the temperature and humidity in the box are more stable than in the Petri plates, due to the larger air volume and to the larger surface area for exchange between the medium and the air. Further studies might be directed to test the same technique on the cultivation of other fungi and on the understanding of the reasons for the higher efficiency in spore production.

Conclusions: Our experiment indicates that the fastest, cheapest, and easiest method for the production of large quantities of gummy stem blight spores and final inoculum should require: 1) the use of Nalgene autoclavable pans, sealed with a sterile transparent bag, instead of Petri plates, and 2) the infection of the PDA with a suspension of spores in sterile deionized water from previous cultures.

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