

## *In vitro* Inhibition of Soilborn Phytopathogens Treated With Swine Wastewater

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### Abstract

Swine wastewater (SWW) is a residue from pig farming that presents a high load of nutrients and organic matter. The appliance of organic matter in soil alters the microbial dynamic and may suppress soilborn phytopathogens. This study aimed at evaluating the inhibition on mycelial growth of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* *in vitro* under SWW doses. Hereupon, three kilograms of a soil classified as red dystroferic latosol was collected and sieved. Half of it was autoclaved. SWW was incorporated at doses of 0 mL, 2.5 mL, 5 mL, 10 mL and 20 mL in both soil conditions, autoclaved and not autoclaved. Afterwards, 130 grams of each soil was separately put into Petri plates above what a thin layer ( $\cong$  5 mL) of Water-Agar (2%) medium was carefully spread over. Above this agar layer, one disk (6 mm diameter) of pure mycelium from each fungal grown in Potato Dextrose Agar medium was individually placed on the center of each plate. Daily evaluations on mycelial growth measuring were taken and ended when mycelium in control plates (without SWW addition) reached plate borders. Results indicated that in autoclaved soil condition, the inhibition was proportional to the dose, what is to say that the higher the dose the less the mycelial growth. In not autoclaved soil there was no significant difference among treatments, suggesting stimuli on suppression effect for both pathogens caused by SWW. In addition, the confirmed potential of SWW as a suppressor of *S. sclerotiorum* and *S. rolfsii* leads to promising investigations on other phytopathogens hard to control.

**Keywords:** liquid pig farming manure, sclerodium, soil supressiveness

### 1. Introduction

Brazilian pig farming is an important agribusiness sector for national economy and south region correspond to 66.9% of total production. Compliant with IBGE (2017), in the first trimester of 2017, 10.46 million pigs were slaughtered, representing 2.6% increase compared to the previous year. Eastern region of Paraná stands out due to the biggest animal breeding squad of the state. However, this activity became worrying about environmental problems caused by large swine wastewater (SWW) production and its inappropriate dumping on lands (Smanhotto et al., 2010).

Final destination of SWW in soil must consider environmental protection practices once incorrect destination may cause problems as river contamination (eutrophication), soil pollution (nitrogen and phosphorus) and air contamination (Kunz et al., 2005; Orrico et al., 2009; Zheng et al., 2014).

Liquid manure contains organic matter and many nutrients from animal diet (Diesel et al., 2002). The most current manure management in Brazil consists of its conservation in lakes or tanks for further appliance as biofertilizer in croplands (Kunz et al., 2009). Adequate appliance may supply nutrients and water for plants, reduce fertilizer use and inhibit phytopathogens in soil through stimulation on native microbial (Erthal et al., 2010). Microbial biomass synthesizes organic matter in soil up to a stage of humic state, tough degradable (Bailey & Lazarovits, 2003).

Tropical-temperate climates in Brazil turn easier fungal diseases development in plants by constant temperature and humidity variance (Hoeltz, 2009). Fungal root diseases highlight in disturbed soil, partially as consequence

of agriculture system adoption, which simplify ecology in very large areas turning them more susceptible to pathogenic establishment (Michereff et al., 2005).

Controlling soilborn pathogenic fungi is a difficult task because they stay in soil for long through resistance structures as sclerotium, formed by *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, what requires integrated management for improving control, such as biological and cultural methods.

Biological control relies on interactions among pathogenic microorganisms and biological control agents, as antibiosis, antagonism, competition and parasitism (Howell, 2003). Dumping vegetal rests allows microorganisms aiding in sclerotium decay (Henning et al., 2014). Certified seeds use and seed treatment with fungicides from benzimidazole group reduce the transmission of dormant mycelium, scaling down initial inoculum. Crop rotation helps in natural degradation of resistance structures (Leite, 2005).

Other controlling strategy is the soil suppressiveness, a natural phenomenon that prevents, inhibits or avoids pathogens establishment and their activity in soil (Bettiol et al., 2009), reduces inoculum density and disease intensity even when there is high inoculum incidence.

General pathogen suppression is directly related to a high degree of soil fungistasis that involves more than a single microorganism or specific group of microorganisms to reach general suppression (Cook & Baker, 1983). Specific suppression requests particular effects of antagonistic organisms on pathogen at some point of its life cycle. In this context, suppressiveness of any given soil ranges from highly conducive to suppressive soils, having crop health as major goal. For this to happen, it is necessary to develop tools to manage soil biotic and abiotic factors in order to increase soil suppressiveness against phytopathogens (Janvier et al., 2007).

This study aimed at evaluating the growth of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* under SWW doses added in artificial culture medium. We hypothesize that SWW may reduce phytopathogens growth significantly.

## 2. Method

*Sclerotium rolfsii* and *Sclerotinia sclerotiorum* isolates used are from Phytopathology Laboratory of Universidade Federal do Paraná, Setor Palotina. Isolates were cultured in Petri plates with PDA (potato dextrose agar), stored into BOD at 27±2 °C under 12-hour photoperiod.

Swine wastewater was collected from a one-year-old biogas production system in a farm of Palotina city, Paraná state.

For mycelia growth test, three kilograms of a soil classified as red dystroferric latosol (Embrapa, 2013) was sieved. Half of this soil was autoclaved at 120 °C, 1 atm for 30 minutes. It was deposited 130 grams of the soil at the bottom of each Petri plate followed by swine wastewater incorporation at the doses of 0 mL, 2.5 mL, 5 mL, 10 mL and 20 mL. The same procedure was done for not autoclaved soil. These doses correspond to proportions applied in fields of 20, 40, 80 and 160 m<sup>3</sup> per hectare. Each treatment had five repetitions.

Afterwards, a thin layer (≅ 5 mL) of Water-Agar (WA) 2% medium was carefully spread over it. Above this agar layer, one disk (6 mm diameter) of pure mycelium containing sclerotium of each pathogen grown in PDA medium was placed, individually, on the center of each plate. Then, plates sealed with Parafilm were stored into BOD at 27±2 °C under 12-hour photoperiod until full growth of mycelia.

For growth diameter, mycelia area was taken daily with a ruler by measuring two orthogonal axes (diametrically opposite measures) and stopped when control plates exhibited full growth. Growth inhibition was determined comparing the growth between each treatment to its respective control, using the following equation (Edgington et al., 1971):

$$PIC = \left( \frac{Dc - Dn}{Dc} \right) \times 100 \quad (1)$$

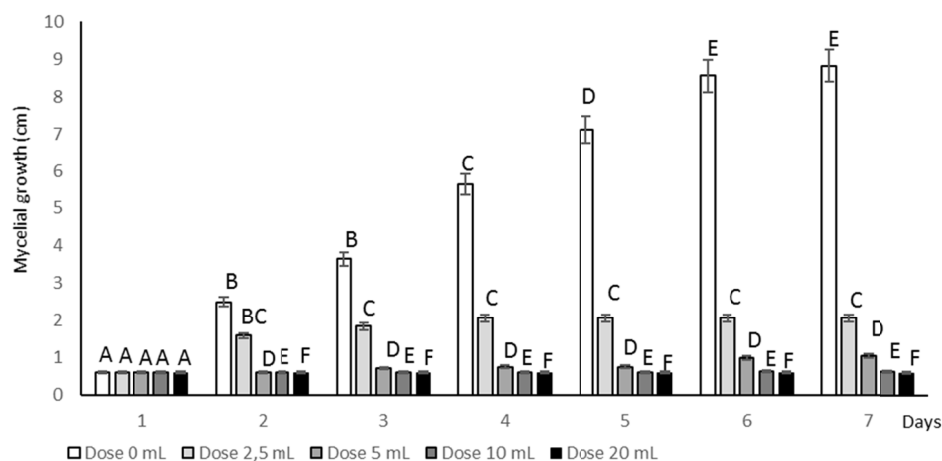
where, PIC = Percentage inhibition of mycelial growth; Dc = Control diameter; Dn = treatment diameter. Data was expressed in percentage.

The experimental design was completely randomized. Obtained data were subjected to variance analyses (ANOVA). In the case of significant results ( $P < 0.05$ ), Tukey test (5% error probability) was employed to compare averages using SISVAR 5.6<sup>®</sup> program (Ferreira, 2011).

## 3. Results

Control plates of both isolates exhibited full growth in seven days, therefore measuring evaluations stopped at seventh experimental day.

There was significant difference among treatments of *S. sclerotiorum* in autoclaved soil, presenting an exponential growth for 0 mL dose, the control treatment (Graph 1). Mycelial growth inhibition was proportional to the dose applied. For the 2.5 mL dose, mycelia grew until third day, stopping right after. Plates treated with 5 mL presented initial mycelia growth only at fifth to sixth day. There was complete growth inhibition for 10 mL and 20 mL.



Graph 1. Mycelial growth of *Sclerotinia sclerotiorum* in autoclaved soil under effect of different SWW doses incorporation

*Note.* Means followed by the same letter did not differ significantly from each other by Tukey test, at 5% error probability.

Visually, the inhibition was caused by antagonistic effect of microorganisms present in SWW. They established faster than phytopathogens, overcoming *S. sclerotiorum* growth. This behavior occurred in all treatments, but in absence of SWW (Figure 1).

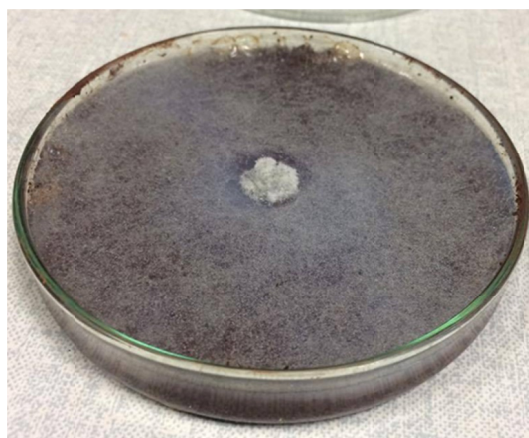
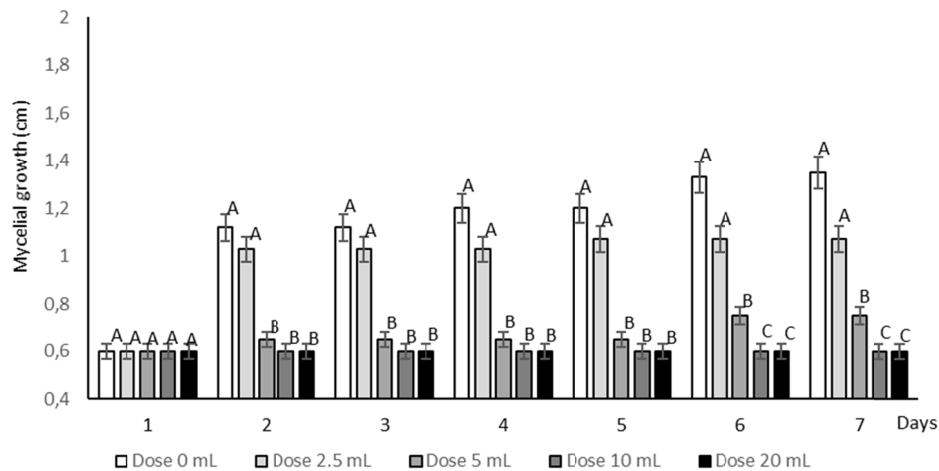


Figure 1. Microorganism from SWW inhibited *S. sclerotiorum* growth

However, *S. sclerotiorum* presented divergent behavior in not autoclaved soil. There was no significant difference among treatments because none of them manifested growth.

The same happened to *S. rolfisii*, but in autoclaved soil. Only for 5 mL dose mycelia started growing at fifth to sixth day (Graph 2). Although, plates treated with 0 mL and 2.5 mL displayed distinct growth, there was no

statistical difference. There was significant difference between 5 mL and 10 mL doses at sixth and seventh days. Just as happened to *S. sclerotiorum*, higher doses also caused full inhibition on mycelial growth of *S. rolf sii*.



Graph 2. Mycelia growth of *Sclerotium rolf sii* in autoclaved soil under effect of different SWW doses incorporation

*Note.* Means followed by the same letters did not differ significantly from each other by Tukey test, at 5% error probability.

For not autoclaved soil, mycelia grew only in dose 0 mL, revealing a weak suppressive effect of this soil against *S. rolf sii*, while SWW addition caused suppressive effect in soil.

Microorganisms from SWW turned unviable sclerotium germination of *S. rolf sii* in autoclaved soil. Hyphae started germinating but stopped, developing in opposite direction from antagonistic mycelium (Figure 2).



Figure 2. Inhibited germination of *S. rolf sii* sclerotium by the presence of microorganisms from SWW

Related to inhibition percentage in autoclaved soil, there was a reduction of 76.56% and 20.74% for *S. sclerotiorum* and *S. rolf sii*, respectively, in 2.5 mL dosage. Doses of 10 and 20 mL presented the highest mycelial inhibition percentages to both phytopathogens (Table 1). For both fungi, mycelial inhibition percentage was caused proportionally to the doses.

Table 1. Mycelial inhibition of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* in autoclaved soil with incorporation of SWW in different doses

Swine wastewater doses	Mycelial inhibition percentage (%)	
	<i>Sclerotinia sclerotiorum</i>	<i>Sclerotium rolfsii</i>
2.5 mL	76.53 a	20.74 a
5 mL	88.15 b	44.44 b
10 mL	92.85 c	55.55 c
20 mL	93.19 d	55.55 c

Note. Means followed by the same letters in column did not differ significantly from each other by Tukey test, at 5% error probability.

In not autoclaved soil, the inhibition of *S. sclerotiorum* was 100% and less than 30% for *S. rolfsii* (Table 2), suggesting that SWW stimulated native microbial giving the soil a suppressive status.

Table 2. Mycelial inhibition of *S. sclerotiorum* and *S. rolfsii* in not autoclaved soil under effect of different SWW doses incorporation

Swine wastewater doses	Mycelial inhibition percentage (%)	
	<i>Sclerotinia sclerotiorum</i>	<i>Sclerotium rolfsii</i>
2.5 mL	100 a	28.57 a
5 mL	100 a	28.57 a
10 mL	100 a	28.57 a
20 mL	100 a	28.57 a

Note. Means followed by the same letters in column did not differ significantly from each other by Tukey test, at 5% error probability.

#### 4. Discussion

In agricultural areas, suppression in soil may occur naturally, however, organic compounds addition leads to the predominance of biological suppression mechanism, although chemical and physical factors can also affect. To improve the efficiency of disease control it is possible to inoculate materials with biological control agents, handling anaerobic biodigestion process and characterize the profile of microbial community (Pereira et al., 1996; Martin & Brathwaite, 2012). Our results provided interesting pathogenic control from SWW appliance, an organic material.

Microbial diversity in SWW community depends upon anaerobic process. Ducey and Hunt (2012) identified the interactions of microbial ecosystem of four SSW-anaerobiosis lakes through the next-generation sequencing technology, extraction and DNA sequencing, biochemical and microbiological analyses of SWW. They found a large number of anaerobic gram positive phylotypes that reduce sulfate, cycle nitrogen and sulfur, ferment and produce stink. However, there was variation in microbial diversity among lakes, implying in the interference caused by environmental conditions.

Moura et al. (2016) analyzed bacterial community alteration in soil passed eight years of SWW continuous use. Elements such as zinc, copper, phosphorus and nitrogen were found to be important for microbial population growth maintenance. Unlikely, manganese and iron negatively affected microbial population. Microbial community in soil varies according to the SWW dose and time, once its continuous appliance in high doses (200 and 300 m<sup>3</sup> ha<sup>-1</sup>) induced lower diversity and favored the permanence of given microbial groups.

Studies involving addition of organic matter residues aiming at suppressing phytopathogens activity in soil in different crops have shown promising results. In agreement with Pereira et al. (1996), organic matter addition as a trial to control phytopathogens is older than 100 years, being initiated in Brazil in 80 decade. Biofertilizers obtained by fermentation process contain bacterial, yeasts and bacilli displaying fungicide, nematicide and hormonal effects. When correctly applied it operates as plagues and diseases repellent, protecting plants naturally (Silva et al., 2007). In addition, the high nutrient load and pH have direct effect on soil ecosystem. Our study provided the understanding that such phenomenon also occurs in *in vitro* condition.

Other organic products, such as, liquid slurry from pig farming can control soilborn plant pathogens when incorporated in soil. Silva (2014) tested it against *Phytophthora nicotianae* population in citrus crop, noticing significant reduction on pathogen population. Manteli (2010) also checked efficiency applying the same organic product against *Pythium sp.* in cucumber crop (*Cucumis sativus*).

In this study, growth inhibition of *S. sclerotiorum* observed in soil not autoclaved is probably associated with the huge potential of microbial activation in soil, resulting in competition among fungi already present in soil. Souza et al. (2014) checked similar effect with consecutive SWW appliance stimulating soil microorganisms.

Similar conclusion was obtained by Morales et al. (2007) testing slurry from pig farming against development of neck rotting and damping off diseases in bean seedlings caused by *S. rolfsii*. They observed higher reduction on diseases intensity at the higher dosages. The authors explain that ammonia concentration, copper and zinc levels are the main factors involved.

Tenuta and Lazarovits (2002) and Conn et al. (2005) concluded that chemical composition and concentration in liquid slurry from pig farming influenced on chemical liberation into soil. Ammonia and volatile fatty acids were toxic to micro sclerodium of *Verticillium dahliae*. They also verified that acid pH and nitrite accumulation generated nitrous acid, a great strategy to control soil diseases due to its higher toxicity than ammonia. Thus, we agree that not only edaphic microbial community played an important role at reducing *S. rolfsii* and *S. sclerotiorum*. Very possibly many chemical components of SWW could have contributed to control.

According to Manteli (2010), SWW appliance in soil can cause pH reduction due to organic acids that increase this effect. Cabral et al. (2011) also observed pH reduction applying SWW in soil, but did not find a relation between pH and SWW doses. These authors also detected a linear relation between SWW dose applied in soil and reduction on  $Al^{3+}$  concentration in all soil layers (0-20 cm; 20-40 cm; 40-60 cm). It is valid to point out that soils vary in their buffer capacity (Bedin et al., 2003) what implies in different resistance to pH variation. Therefore, not all times pH alteration is expected but in some cases SWW provides pH reduction, other control mechanism on some soilborn phytopathogens.

Even many positives aspects related to SWW addition in soil are reported, its successive use can cause soil contamination because SWW carries large amounts of not balanced nutrients (Prior et al., 2015), resulting in high concentration as time passes.

Soils diverge in chemical, physical and biological composition affecting directly the SWW absorption. Queiroz et al. (2004) applied SWW at different dosages in a Red-Yellow Podzolic soil (Embrapa, 2013) verifying phosphorus, potassium, sodium and zinc accumulation, manganese and copper decrease and calcium stability in soil. On the other hand, Maggi et al. (2010) applied SWW in a red Dystroferic latosol (Embrapa, 2013) noticing no influence on manganese and nitrogen values and increase on potassium, phosphorus and calcium concentrations as SWW doses increased.

As absorption, the percolation and nutrients supplying vary according to the soil type and SWW dose. Therefore, suppressiveness success on phytopathogens will change in each specific ecosystem where soil and plant nutrition must be considered. Generally, plants cultivated in poor nutritional soils are more susceptible to pathogenic action.

In a review about nutrients influence in phytopathogens behavior, Zambolim and Ventura (1993) related each nutrient role in saprophytic and biotrophic phytopathogens. Some nutrients stimulated them and others inhibited, because each fungal specie has its own nutritional demand. Diseases caused by *Fusarium*, *Rhizoctonia* and *Aphanomyces* genus, for instance, may be stimulated by ammonium and reduced by nitrate, according to chemical structure of nitrogen.

In balanced conditions, some diseases can be controlled by nutritional management or crop rotation (Reis et al., 2011). However, pathogens like *S. sclerotiorum* and *S. rolfsii* form resistance structures (sclerodium) that stay in soil independent of its nutritional status. These ones may be controlled by suppressiveness induction over time. Our results demonstrate that high doses in little time inhibit these phytopathogens growth.

SWW appliance, however, may also not show suppressive effect. Durigon (2012) evaluated successive SWW introduction in corn crop leading to a populational increase in *Fusarium spp.* as time passed. For this reason, correct handling and dosage addition into soil must to be take into account, remembering that the cultivated crop is an essential parameter (Silveira et al., 2011).

Diseases are exceptions in soil so its presence indicates ecological imbalance. Pig farming waste submitted to anaerobic biodigestion are composed by nutrients and microorganisms with beneficial action in soil since whereas well handled. Other waste types, as sewage, household waste, agroindustrial residues can also suppress

phytopathogens in soil, especially fungi. Process as vermicomposting, composting, anaerobic digestion favors antagonistic action of microorganisms present in soil against phytopathogens, beyond chemical factors already pointed (Singh et al., 2013).

Santos et al. (2014) evaluated current systems of SWW treatment in Brazil. Besides their results show satisfactory reduction of pollute load in SWW effluents, there is no study pointing the costs of each system. Our study provides valuable information on *in vitro* SWW potential as reducer of *S. rolf sii* and *S. sclerotiorum* development and encourage further trials in greenhouses, once SWW showed potentiality to control *S. rolf sii* and *S. sclerotiorum*, so it would optimize treatment systems costs.

## 5. Conclusion

We accepted the hypothesis because swine wastewater inhibited mycelial growth of *Sclerotinia sclerotiorum* and *Sclerotium rolf sii* under *in vitro* conditions. The present study contributed to the understanding of sustainable propagation of residues from pig farming to the preventive handling of soiborn phytopathogens.

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