**Antifungal Activity of Reworks Wormcast**

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## Background

The purpose this research is to investigate the effects of wormcast on several major turfgrass fungal pathogens as well as examining the microbial populations and antifungal volatile substances produced by wormcast. Forterra (formerly Reworks) has a vermiculture processing plant in Downsview, Ontario, which feeds vegetable and plant refuse in a proprietary system to produce wormcast. Wormcast was collected at the Downsview site on 24 January 2007, and stored at 4C, with the first tests started within one week of collection. Another set of wormcast was obtained in fall 2006 (manufactured Oct 2006). The old wormcast had been stored at room temperature before the start of these experiments.

## TEST 1: Slurry Inhibition and EC50 calculation

The purpose of this slurry test was used to look for inhibition of mycelial growth in amended agar plates from microorganisms which survive a short exposure to 60C molten agar or from non-labile inhibitory compounds previously produced in the wormcasts. Water content and dry weights were obtained by drying at 84C for 48 hr (Table 1).

Table 1. Water content of wormcast (dried at 84C for 48 hr) assessed on 2007/03/01.

| Wormcast | Wet weight (g) | Dry weight (g) | Moisture content (%) |
|---|---|---|---|
| 0701 (fresh) | 10 | 5.92 | 40.8 |
| 0610 (old) | 10 | 6.02 | 39.8 |

moisture content % = (wet weight - dry weight)/wet weight

A slurry was made from the wormcasts (0610 and 0701) by adding 200 g of each wormcast to 600 ml of cooled autoclaved water and mixing for 30 min. Different amounts of slurry (0.1 ml, 1 ml or 10 ml) were placed into 9-cm-diam petri plates, and then 15 ml molten potato dextrose agar (60C) was added to each 9-cm-diam petri plate, with four replicate plates per concentration for each isolate tested. These rates allowed for calculation of an EC50 value (Effective Concentration that reduces growth by 50%) using PROBIT analysis as implemented in the statistical program SAS. The slurry inhibition results are shown in Table 2. Note that hot agar may have killed off some propagules of microorganisms in the wormcast. On each plate, four 5-mm-diam inoculum plugs of each pathogen (*Sclerotinia homoeocarpa* SH30, *Rhizoctonia solani* RS100, *Colleotrichum graminicola* CG99410, *Microdochium nivale* MN96070, *Typhula incarnata* TN21 or *T. ishikariensis* TS94072) were placed on the surface of the slurry-amended agar, and incubated at 23C, or at 10C for the last two isolates. The colony diameters were recorded after 3 days (CG, MN, RS, SH) or 7 days (TN, TS) of incubation.

## Results

Abundant growth of the contaminant fungus *Rhizopus* was found on all plates involving old (0610) wormcast. The two higher rates of both wormcasts (old and fresh) showed significant inhibition of fungal growth for all six pathogens, except for MN96070 at 0.0156 g/ml of the fresh wormcast. At the lowest rate of 0.0017 g/ml wormcast, most tests showed significant inhibition except against MN96070 (old and fresh) or SH30 (fresh), where some stimulation of growth was observed. For TN21 and TS94072 (which cause grey snow mold), wormcast (old or fresh) significantly inhibited growth at all of the rates.

Table 2. Wormcast inhibition of growth of several turfgrass pathogens in 9-cm-diam glass plates containing potato dextrose agar amended with wormcast. The diameter of colonies growing from four inoculum plugs per plate were measured after incubation of 3 days at 23C (CG, MN, RS, SH) or 7 days at 10C (TN, TS). Inhibition was calculated as (diam of control - diam of treatment )/ diam of control, and each value is based on four replicate plates. EC50 values (g/ml) are also presented.

| Wormcast treatment | Wormcast (g) | Total medium (ml) | Final wormcast amount (g/ml) | Colony growth inhibition by fungal strain (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CG99410 | MN96070 | RS100 | SH30 | TN21 | TS94072 |
| 0701 (fresh) | 0.025 | 15.1 | 0.0017 | 40.8 | 10.6 | 1.5 | 1.8 | 15.2 | 40.5 |
| 0701 (fresh) | 0.25 | 16 | 0.0156 | 40.8 | 5.9 | 11.7 | 51.2 | 47.8 | 42.9 |
| 0701 (fresh) | 2.5 | 25 | 0.1 | 59.2 | 76.5 | 92.7 | 87.8 | 56.6 | 52.4 |
| 0610 (old) | 0.025 | 15.1 | 0.0017 | 44.9 | 5.9 | 11.3 | 45.1 | 34.8 | 42.9 |
| 0610 (old) | 0.25 | 16 | 0.0156 | 38.8 | 20.0 | 33.6 | 55.5 | 37.0 | 42.9 |
| 0610 (old) | 2.5 | 25 | 0.1 | 57.1 | 60.0 | 85.4 | 79.9 | 52.2 | 47.6 |
| LSD (p= 0.05) [a] | | | | 9.9 | 19.8 | 12.8 | 29.1 | 5.7 | 4.4 |
| 0701 EC50 (g/ml) [b] | | | | 0.028 | 0.046 | 0.032 | 0.009 | 0.038 | 0.073 |
| 0610 EC50 (g/ml) | | | | 0.041 | 0.066 | 0.021 | 0.004 | 0.111 | 1.895 |

[a] LSD refers to the least significant difference between inhibition values within each column, and any means that differ by this amount are significantly different. Also any means that are more than this value show significant inhibition of growth while any means that are less than the negative of this value show significant stimulation of growth.

[b] EC50 (gram wormcast/ml media) refers to the amount of wormcast per ml agar medium that reduces fungal growth by 50%.

**Discussion**

At the highest rate of 0.1 g of wormcast per ml of growth medium, both old and fresh wormcast significantly decreased growth of all six turfgrass pathogens on agar plates. The major difference between old and fresh wormcasts was that the former was commonly colonized by saprophytic *Rhizopus* fungi. At the lowest rate, both types of wormcast showed stimulation of growth of MN96070 in this test. At all rates, both wormcasts significantly inhibited growth of the grey snow mold pathogens (*T. incarnata* and *T. ishikariensis*), although these were both incubated at 4C for 7 days which may have allowed time for more inhibitory materials to diffuse from the wormcasts.

**TEST 2: Center Ring Inhibition**

The purpose of this test was to assess inhibition of fungal growth by various amounts of wormcast (old & fresh) solution placed within small rings set on agar. Ten grams of each wormcast were added to 30 ml autoclaved water, and shaken vigorously for 30 s. This suspension was kept for 30 min before usage, allowing the solids to settle. An autoclaved metal ring (1.5 cm diameter, 0.8 cm width) was seated firmly in the center of a 9-cm-diam petri plate containing 15 ml of PDA. Wormcast suspension was then applied in amounts of 0.1, 0.5 or 1 ml into the autoclaved metal ring. Four 5-mm-diam inoculum plugs of each of the different turfgrass pathogens (*Sclerotinia homoeocarpa* SH30, *Rhizoctonia solani* RS100, *Colleotrichum graminicola* CG99410, *Microdochium nivale* MN96070, *Typhula incarnata* TN21 or *T. ishikariensis* TS94072) were placed at 1.5 cm radial distance away from the ring. This was replicated 3 times for each isolate by treatment combination. The plates were sealed with parafilm and incubated at 23C (SH, RS, CG, MN) or 10C (TN and TS). The proximal (growing toward ring) and distal radii for each of the inoculum plugs were measured after 3 days (SH, RS, CG, MN) or 7 days (TN and

TS), and any signs of microbial growth from the wormcast solutions were also noted. Plug inhibition results for solutions made from old and fresh wormcast are shown in Table 3 for fungal growth after 3 or 7 days. For fresh wormcast, fungal growth after 7 or 11 days is also shown in Table 4, but not for old wormcast since these plates were overgrown by *Rhizopus*.

Table 3. Inhibition of growth of fungal isolates SH30, RS100, MN96070, CG99410, TN21 and TS94072 by 25% (w/w) wormcast solutions (0.1, 0.5 or 1.0 ml) which were placed in 1.5-cm-diam rings in the center of 9-cm-diam petri plates containing 15 ml PDA. The hyphal growth from the inoculum plugs toward the ring (1.5 cm distance, proximal radius) was measured after 3 d at 23C (CG9940, MN96070, SH30 and RS100) or 7 d at 10C (TN21 and TS94072).

| Wormcast treatment | Solution (ml) | Proximal radial growth inhibition by fungal strain (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | CG99410 | MN96070 | RS100 | SH30 | TN21 | TS94072 |
| 0701 (fresh) | 0.1 | 34.1 | 2.7 | 67.2 | 50.0 | 13.8 | 24.6 |
| 0701 (fresh) | 0.5 | 31.7 | 21.6 | 64.1 | 46.2 | 7.8 | 28.6 |
| 0701 (fresh) | 1 | 36.6 | 8.1 | 65.6 | 26.9 | 13.8 | 12.7 |
| 0610 (old) | 0.1 | 4.9 | 35.1 | 0.8 | 1.3 | 13.8 | 16.7 |
| 0610 (old) | 0.5 | 4.9 | 13.5 | 0.8 | 1.3 | 7.8 | 32.5 |
| 0610 (old) | 1 | 4.9 | 24.3 | 0.8 | 1.3 | 13.8 | 8.7 |
| LSD (p=0.05) | | 10.8 | 15.0 | 9.3 | 16.3 | 25.3 | 21.5 |

**Results**

Table 3 shows that fresh and old wormcasts different significantly in their inhibition of the various fungal pathogens after 3 days at 23C or 7 days at 10C. Old wormcast at all three concentrations stimulated growth of CG99410 and SH30 after 3 days of growth, whereas the fresh wormcast significantly inhibited these pathogens.

Table 4. Inhibition of growth of fungal isolates SH30, RS100, MN96070, CG99410, TN21 and TS94072 by 25% (w/w) wormcast solutions (0.1, 0.5 or 1.0 ml) which were placed in 1.5-cm-diam rings in the center of 9-cm-diam petri plates containing 15 ml PDA. The hyphal growth from the inoculum plugs surrounding the ring (1.5 cm distance) were measured after 7 d at 23C (CG9940, MN96070, SH30 and RS100) or 11 d at 10C (TN21 and TS94072).

| Wormcast treatment | Solution (ml) | Radial growth inhibition by fungal strain (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | CG99410 | MN96070 | RS100 | SH30 | TN21 | TS94072 |
| 0701 (fresh) | 0.1 | 50.0 | 44.4 | 70.8 | 59.3 | 56.5 | 61.9 |
| 0701 (fresh) | 0.5 | 46.7 | 33.3 | 66.4 | 56.0 | 39.1 | 55.5 |
| 0701 (fresh) | 1 | 43.3 | 42.9 | 63.5 | 48.4 | 56.5 | 49.2 |
| LSD (p=0.05) | | 19.5 | 19.1 | 4.4 | 15.1 | 11.4 | 15.5 |

After 7 at 23C or 11 days at 10C incubation, fresh wormcast significantly inhibited growth of all pathogens tested. Old wormcast at all rates showed significant suppression of growth of MN96070, RS100 and TS94072, while it stimulated growth of CG99410 and SH30. At 0.5 ml, it stimulated growth of TN21, but at 0.1 and 1 ml, it inhibited growth. After 7 or 11 days, Rhizopus was full of plates, it was hard to estimate the mycelial growth.

## Discussion

The center ring inhibition test allows for diffusion of water soluble possibly inhibitory (or stimulatory) substance from the solution placed in a center ring. The results from Table 3 & 4 demonstrate that the solution from old and fresh wormcasts contain both inhibitory and stimulatory substances depending on pathogen.

**TEST 3: Assessment of microbial populations**

The purpose of this test was to assess the presence of microorganisms in the wormcasts using standard dilution plate techniques to count the total number of propagules of bacteria, fungi and yeasts. Three different media were used: PDA, PDA amended with antibiotics (streptomycin 100 mg/L, tetracycline 50 mg/L), and PDA amended with the fungicide benomyl (2 µg/ml). To obtain PDA amended with benomyl at 2 µg/ml, 10 ml of 200 µg/ml benomyl solution were added to 990 ml of molten PDA (60C). Fresh wormcast or old wormcast (10 g) was added to 30 ml of cooled autoclaved water added to make a 25% (w/w) suspension. A dilution series was made from wormcast (fresh and old), and plated onto three different media. Wormcast dilutions were initially 1:1, 1:10 and 1:100, but the highest dilution still yielded over 250 colonies per plate, so a new dilution series was made with dilutions of 1:1000, 1:10000, and 1:100000. There were three replications for each dilution. For dilution plates, 50 µl of each solution were spread over the different media with three replicates. The plates were incubated at 23C for 3 or 4 days, and the colonies were checked daily. After 3 or 4 days, the colonies of each different type of microbial group was counted using the following criteria:

- Bacteria: slimy smaller colonies, with cells up to 5 um long
- Yeast: creamy white or yellow colonies, with rodshaped cells up to 8 um long.
- Actinomycetes: hardlooking colonies, with an earthy smell, fuzzy with irregular edges
- Filamentous Fungi: fluffy or powdery large colonies of various colors

On PDA amended with antibiotics, colonies of all different types of microorganisms were found, but generally only bacteria were present on benomyl-amended PDA. Seven types of bacteria were seen on dilution plates: two white types, a yellow, a red, a pink an orange and a grey. The red one grew on PDA plates amended with antibiotics. These counts were converted to CFU per gram dried soil, and the results are shown in Table 5.

Table 5. The number of bacteria, fungi and yeasts colonies in CFU per gram dried soil (see Table 1 for dry weights) assessed on different media using standard dilution plating techniques, with three replications.

| Treatments | No. of CFU per gram dried soil | | | |
|---|---|---|---|---|
| | Bacteria | Filamentous Fungi | Actinomycetes | Yeast |
| 0701 (fresh) | 7. 94∀0.08 □ $10^5$ | 1.46∀0.10 □ $10^3$ | 2.53∀0.01 □$10^2$ | 0.23∀0.05 |
| 0610 (old) | 8.75 ∀0.46 □ $10^4$ | 1.88∀0.10 □ $10^3$ | 0 | 0.39∀0.10 |

∀ indicates standard deviation.

## Results

Both old and new wormcasts contained bacterial propagules as the major type of microorganism present at over half a million per gram dried soil, although the fresh wormcast contained significantly more propagules. The second highest counts were for filamentous fungi which ranged from 1 to 2 thousand propagules per gram of dried soil. Some actinomycetes were also observed, but only in the fresh wormcast. A few yeast colonies were observed in both types of wormcasts, but at very low levels (less than one per gram dried soil).

## Discussion

Fresh wormcast differed significantly from old wormcast in the total number of bacterial propagules by a full order of magnitude, and also had more filamentous fungi and actinomycetes.

**TEST 4: Production of inhibitory volatile substances by wormcasts**

Fresh or old wormcast (2 g) was placed onto PDA plate lids. Autoclaved water (2 ml) was added and mixed with the wormcast. Agar plugs (5 mm diam) of several pathogens (CG99410, MN96070, RS100, SH30, TN21 and TS94072) were placed on the agar surface. The bottom halves of the PDA plates with the medium and fungal plugs were inverted and placed over the plate lids which had wormcast solutions. The plates were wrapped with parafilm and incubated in this inverted fashion at 23C (room temperature), with four replicate plates per isolate by wormcast combination. Mycelial growth from the agar plugs was measured after 10 days. Inhibition was calculated as (diam of control - diam of treatment/ diam of control). The results are presented in Table 6.

Table 6. Production of inhibitory volatile substances by wormcasts, as measured in petri plates containing 2 g wormcast plus 2 ml water on the inverted lid, and with fungal agar plugs on the plate agar surface. Plates were sealed with parafilm and incubated in this inverted fashion at 23C (SH30, RS100, MN96070 and CG99410) and 10 C (TN21 and TS94072). Colony growth was measured after 10 days with four replicates per treatment.

| Wormcast | Radial growth inhibition by fungal strain (%) | | | | | |
|---|---|---|---|---|---|---|
| | CG99410 | MN96070 | RS100 | SH30 | TN21 | TS94072 |
| 0701 (fresh) | 7.5 | 42.4 | 39.2 | 40.5 | 5.2 | 12.7 |
| 0610 (old) | 7.5 | 39.5 | 19.6 | 26.7 | 12.5 | 29.1 |
| LSD (p=0.05) [a] | 74.6 | 21.6 | 19.1 | 24.7 | 21.4 | 18.3 |

[a] LSD refers to the least significant difference between inhibition values within each column, and any means that differ by this amount are significantly different. Also any means that are more than this value show significant inhibition of growth while any means that are less than the negative of this value show significant stimulation of growth.

## Results

Fresh and old wormcasts produced volatile substance(s) which significantly inhibited fungal growth of MN96070, RS100, and SH30.

## Overall Conclusions

Fresh (week old) and old (3 month old) wormcast showed inhibitory activity to turfgrass pathogens to some extent with various methods. The wormcasts produce some volatile substances which can significantly inhibit some pathogens. The results demonstrate that the solution from wormcasts contain both inhibitory and stimulatory substances. After wormcast is stored at room temperature for three months, abundant growth of the saprophytic fungus *Rhizopus* can be found, as well as a reduction in bacterial and fungal counts.

## Acknowledgments

This work was supported by Reworks (now called Forterra).