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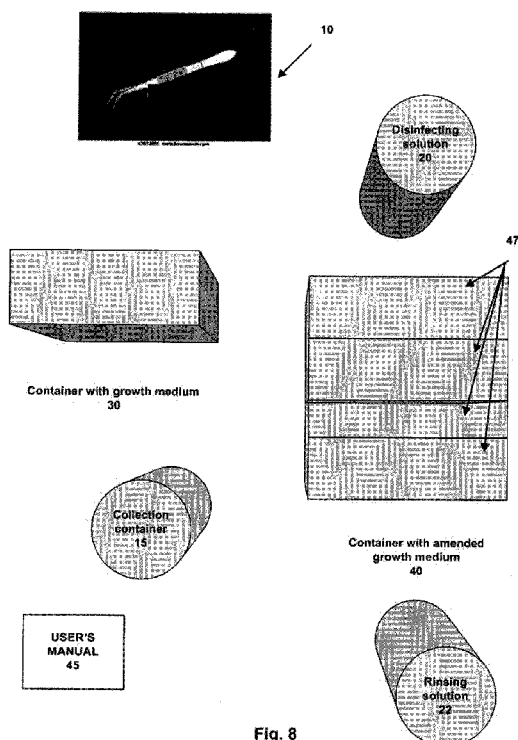


Fig. 8

(57) Abstract: A method for determining "fungicide resistance" in fungal pathogens on turf grasses includes collecting infected leaf blades, sterilizing, and placing leaf blades in a growth medium. A kit for determining "fungicide resistance" in fungal pathogens on turf grasses is also described.



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FIELD KIT AND METHOD FOR DETECTION OF FUNGICIDE RESISTANCE IN
FOLIAR TURFGRASS PATHOGENS

BACKGROUND

5 These teachings relate generally to Field detection of fungicide resistance in fungal pathogens on turf grass.

 Dollar spot, caused by the ascomycete fungus *Sclerotinia homoeocarpa*, is a major turfgrass disease that causes significant damage to turfgrass swards from May to October on North American
10 golf courses. *Sclerotinia homoeocarpa* has a wide host range of cool and warm season grasses, but is of most significance on annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* L. syn = *A. palustris* Huds), which constitute a large portion of finely managed turfgrass found on golf course
15 fairways, putting greens and tee boxes. Turfgrass infection symptoms begin with individual leaf blades, which exhibit water soaked lesions and progress to straw or bleach colored hourglass shaped lesions marked by a tan to reddish brown border. With the exception of coarse textured turfgrass leaves, lesions will
20 enlarge and extend across the entire leaf blade, and onto multiple plants, causing a blighted appearance. The coalescence of multiple leaf blades from multiple plants in an infection center results in small, sunken patches of blighted turf. This pitting damage is a trademark of *S. homoeocarpa* infection, and
25 severely reduces the aesthetic quality and playability of golf course turf swards.

 Cultural practices often do not provide adequate *S. homoeocarpa* control and multiple fungicide applications are made each year to maintain acceptable turf quality. Frequent
30 fungicide applications on golf courses has led to the selection of *S. homoeocarpa* isolates resistant to benzimidazole and dicarboximide, and insensitive to sterol demethylation inhibitor

(DMI) fungicide classes. Previous regional monitoring studies from across the United States have confirmed resistance and reduced sensitivity to the aforementioned fungicide classes. Putman et al. recently confirmed resistance and decreased *in vitro* sensitivity to *S. homoeocarpa* isolates collected from golf courses in the northeastern United States to the benzimidazole, dicarboximide and DMI classes. Confirmation of DMI insensitivity in the Northeast is of great concern because DMI fungicides are an important component of fungicide programs used to control *S. homoeocarpa* on golf course fairways and tee boxes. Golf course fairways and tee boxes consist of considerable acreage (approximately 20-40 acres per 18 holes), therefore broad-spectrum systemic fungicides such as the DMIs are the preferred control option, because they provide longer residual control than contact fungicides and allow turfgrass managers to reduce fungicide applications.

The development of fungicide resistance (benzimidazole and dicarboximide classes) or insensitivity (DMI class) has led to increased fungicide applications to obtain the desired level of dollar spot control. Each golf course has a different fungicide use history, therefore resistance and insensitivity to different classes may develop differently at each golf course. This leads to site-specific resistance profiles and the need to be accurately identified. Currently, fungicide resistance assays are preformed in diagnostic labs at Universities and by companies producing fungicides, but this process can be time consuming and expensive.

Field detection kits for fungicide resistance have been developed for pathogens on peaches. Those kits are complex in procedure and require a person having the knowledge of an agricultural extension agent in order to operate the kit correctly.

There is a need for a field detection kit for fungicide resistance in dollar spot and other diseases on turf grasses, where the kit can be used by golf course superintendents and can be used on-site.

5

BRIEF SUMMARY

One embodiment of the method of these teachings for determining fungicide resistance or insensitivity (hereinafter referred to as "fungicide resistance") in fungal pathogens on turf grasses includes collecting infected leaf blades, sterilizing, and placing leaf blades in a growth medium. After leaf blades are identified, collected and sterilized, they are then placed on differing growth mediums to determine the resistance profile. A control sample is created by placing an infected leaf blade on fungicide-free growth medium. Other disinfected samples are placed on a growth medium amended with a predetermined discriminatory dose of a predetermined fungicide, thereby creating a test sample. Fungicide resistance is determined after a predetermined interval of time by comparing growth of the pathogen in the test sample to growth on the control sample.

In one instance, the fungal diseases are caused by *Sclerotinia homoeocarpa* and the fungicide is a DMI class fungicide, such as or preferably propiconazole. Since cross-resistance has been confirmed among seven DMI active ingredients (fenarimol, metconazole, myclobutanil, propiconazole, tebuconazole, triadimefon and triticonazole), no loss of generality results from selecting only propiconazole.

In one embodiment, the kit for determining fungicide resistance for the fungal pathogen *Sclerotinia homoeocarpa* compares mycelium growth out of infected grass leaf tissue on non-amended media (the control sample) to growth on fungicide

amended media (the test sample) each containing one discriminatory concentration of a fungicide. In one exemplary embodiment, the fungicides are thiophanate-methyl (benzimidazole), vinclozolin (dicarboximide) and propiconazole (DMI). The kit includes but is not limited to: a collection container, a collection device for collecting infected leaf tissue, sterilization solutions, a container with the control media, a number of containers, each amended with one fungicide class. In one instance, the kit also includes a user manual.

For a better understanding of the present teachings, together with other and further needs thereof, reference is made to the accompanying drawings and detailed description and its scope will be pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the Joseph Troll Research Facility (JTRF) relative mycelium growth percentage (RMG%) on 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ propiconazole concentrations. *Sclerotinia homoeocarpa* isolates were sampled from untreated (initial and 7-DAT) and propiconazole (initial).

Figure 2 shows the Hartford Golf Club (HGC) relative mycelium growth percentage (RMG%) on 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ propiconazole concentrations. (A) Frequency distribution of isolates sampled from propiconazole treated (initial) and untreated (initial and 7-DAT) plots. (B) Frequency distribution of isolates sampled from propiconazole treated plots (7-DAT).

Figure 3 shows Hickory Ridge Country Club (HRCC) relative mycelium growth percentage (RMG%) on 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ propiconazole concentrations. (A) Frequency distribution of isolates sampled from propiconazole treated (initial) and untreated (initial and 7-DAT) plots. (B) Frequency

distribution of isolates sampled from propiconazole treated plots (7-DAT).

Figure 4 shows Shuttle Meadow Country Club (SMCC) relative mycelium growth percentage (RMG%) on 0.1, 0.3, 0.5 and 1.0 μg a.i. ml^{-1} propiconazole concentrations. (A) Frequency distribution of isolates sampled from propiconazole treated (initial) and untreated (initial and 7-DAT) plots. (B) Frequency distribution of isolates sampled from propiconazole treated plots (7-DAT);

Figure 5 shows Wintonbury Hills Golf Club (WBGC) relative mycelium growth percentage (RMG%) on 0.1, 0.3, 0.5 and 1.0 μg a.i. ml^{-1} propiconazole concentrations. (A) Frequency distribution of isolates sampled from propiconazole treated (initial) and untreated (initial and 7-DAT) plots. (B) Frequency distribution of isolates sampled from propiconazole treated plots (7-DAT);

Figure 6 shows aerial mycelium growing from 5-mm agar plug placed on PDA amended with 0.5 μg a.i. ml^{-1} of propiconazole. Aerial mycelium displays vertical growth and does not exhibit prostrate growth on fungicide amended media surface;

Figure 7 shows *in vitro* mycelial growth of field resistant (HRCC) and field sensitive (JTRF) isolate of *Sclerotinia homoeocarpa* on PDA media amended with propiconazole at 0, 0.1, 0.3, 0.5 and 1.0 μg a.i. ml^{-1} . Mycelium growth observed on the 0.3 and 0.5 μg a.i. ml^{-1} concentrations was commonly associated with aerial mycelium that did not make contact with propiconazole-amended media; and

Figure 8 is a graphical schematic representation of one embodiment of the kit of these teachings.

DETAILED DESCRIPTION

The following detailed description presents the currently contemplated modes of carrying out the present teachings. The description is not to be taken in a limiting sense, but is made
5 merely for the purpose of illustrating the general principles of the present teachings.

The present teachings will be more completely understood through the following description, which should be read in conjunction with the drawings. In this description, like
10 numbers refer to similar elements within various embodiments of the present disclosure. Within this description, the claims will be explained with respect to embodiments. The skilled artisan will readily appreciate that the methods, apparatus and systems described herein are merely exemplary and that
15 variations can be made without departing from the spirit and scope of the disclosure.

As used herein, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

20 Except where otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about."

A "discriminatory concentration," as used herein, is a
25 concentration of fungicide that suppresses mycelium growth between 99.1 % and 100.0 % in isolates from a baseline facility that has had minimal fungicide exposure while allowing mycelial growth in isolates with fungicide resistance from a location with prior exposure. The discriminatory concentration is a
30 concentration that represents a threshold for determining when fungicide resistance detected in vitro indicates a practical resistance in vivo.

The discriminatory concentration should be distinguished from a concentration that produces at least 40% RMG (Percent relative mycelial growth) in isolates from fungicide resistant locations, which is a concentration that represents a threshold
5 for determining when fungicide resistance detected in vitro indicates a practical resistance in vivo.

The discriminatory concentration should be distinguished from a concentration that produces a 50% reduction in RMG (Percent relative mycelial growth) in isolates from a facility
10 that has had minimal fungicide exposure (often referred to as DG_{50}) or a concentration that produces at least an 80% reduction in RMG in isolates from a facility that has had minimal fungicide exposure.

One embodiment of the method of these teachings for
15 determining fungicide resistance in fungal pathogens on turf grasses includes collecting infected leaf blades, sterilizing, and placing leaf blades in a growth medium. After leaf blades are identified, collected and sterilized, they are then placed on differing growth mediums to determine the resistance profile. A
20 control sample is created by placing an infected leaf blade on fungicide-free growth medium. Other disinfected samples are placed on a growth medium amended with a predetermined discriminatory dose of a predetermined fungicide, thereby creating a test sample. Fungicide resistance is determined after
25 a predetermined interval of time by comparing growth of the pathogen in the test sample to growth on the control sample.

In one exemplary embodiment, a discriminatory concentration of each fungicide class (benzimidazole, dicarboximide, and demethylation inhibitor) to be tested was developed.

30 In one embodiment, the kit for determining fungicide resistance in the fungal pathogen *Sclerotinia homoeocarpa*, compares mycelium growth out of infected grass leaf tissue on

non-amended media (the control sample) to growth on fungicide amended media (the test sample), each containing one discriminatory concentration of a fungicide. In one exemplary embodiment, the fungicides are thiophanate-methyl (benzimidazole), vinclozolin (dicarboximide) and propiconazole (DMI). The kit includes but is not limited to: a collection container, a collection device (also referred to as a selection device) for collecting infected leaf tissue, sterilization solutions, a container with the control media, a number of containers, each amended with one fungicide class. In one instance, the kit also includes a user manual.

Figure 8 shows one embodiment of the kit of these teachings. Referring to Figure 8, in the embodiment shown therein, the kit includes a collection device 10 (tweezers in the embodiment shown), a collection container 15, a container having disinfectant solution 20, a container having a rinsing solution 22, a container with growth medium (the control media) 30, a container with amended growth media 40 and a user's manual 45. In the embodiment shown the container with amended growth media has a number of sub-containers 47. In one embodiment, at least some of the sub-containers can each be amended with one fungicide class. In another embodiment, the container with growth media 30 can be one or more of the sub-containers in the container 40, where those one or more sub-containers are not amended but contain growth medium used for the control media.

The steps of the method of these teachings, such as sample collection, disinfection, and growth on an artificial medium have been optimized here for efficiency. In one instance, the method is as follows: a number of symptomatic leaf blades (12 in one instance, but not a limitation of these teachings) are individually sampled with a collection device (tweezers in one instance, but not limitation of these teachings) from dollar spot

infection centers and stored in a storage container (in one instance, a 1.5 ml micro centrifuge tube, not a limitation of these teachings) until isolation (within 24 hours). Full strength acidified potato dextrose agar (PDA) (acidified potato dextrose agar is referred to as APDA) (for example, from Difco Laboratories, Detroit, MI) is prepared, in one instance, by sterilizing PDA for 45 minutes at 121°C in an autoclave (Tuttnauer 3850 M, Hauppauge, NY) and adding 1 ml of 85% lactic acid (Fisher Scientific, Fair Lawn, NJ) per 1 liter of full strength PDA (other sterilization techniques are within the scope of these teachings) and is poured into 12 well (4x3 well) polystyrene cell culture plates (for example, from Corning Incorporated, Corning, NY) (other culture containers are within the scope of these teachings), is allowed to solidify, and is stored at 2°C until a usage. Each row of 3 cells in the culture plates will hold a different discriminatory concentration of fungicide (control, in one instance, benzimidazole, dicarboximide, and demethylation inhibitor). Full strength APDA was selected because it allows for quick growth of mycelium out of infected leaf tissue and enables the test to be completed within 48-72 hours. In one instance, after sample collection, individual leaf blades are surface sterilized for 1 min in 3% sodium hypochlorite solution, air-dried on sterile filter paper and then each placed in all wells of the amended culture plates. Culture plates are then stored at 25°C (room temperature) for 2 to 3 days before assessing fungicide resistance based on mycelium growth on fungicide amended plates and the control amended plate. No growth or growth of a characterized contaminant indicates a fungicide sensitive result. A detailed user manual is prepared with the kit including images of common contaminants and how to assess results with recommendations for fungicide resistance management. It should be noted that the above described exemplary

embodiment is presented herein to elucidate these teachings, but these teachings are not limited only to the above described exemplary embodiment.

In the fungal pathogen *Sclerotinia homoeocarpa* a discriminatory concentration for the benzimidazole, dicarboximide, and demethylation inhibitor (DMI) has been determined. For the benzimidazole fungicide class, a discriminatory concentration of thiophanate-methyl at about 1,000 $\mu\text{g a.i. ml}^{-1}$ is used. For the fungicide class dicarboximide, a discriminatory concentration of vinclozolin of about 5 $\mu\text{g a.i. ml}^{-1}$ is used. Cross-resistance has been confirmed for all seven DMI active ingredients (fenarimol, metconazole, myclobutanil, propiconazole, tebuconazole, triadimefon and triticonazole) and the assay is accurate for all DMIs when the test includes only propiconazole. In these teachings, the predetermined discriminatory dose of propiconazole is greater than 0.5 $\mu\text{g a.i. ml}^{-1}$ and not greater than 1.0 $\mu\text{g a.i. ml}^{-1}$; preferably about 1.0 $\mu\text{g a.i. ml}^{-1}$.

Further illustration of the determination of the predetermined discriminatory dose for the exemplary embodiment, propiconazole, is provided herein below.

Isolate Selection

Isolates from five locations (HGC, HRCC, JTRF, SMCC and WBGC) were utilized in this experiment. Field trials conducted in 2009 and 2010 determined that *S. homoeocarpa* populations from HGC, HRCC, SMCC and WBGC exhibited practical propiconazole field resistance and that propiconazole was highly effective for dollar spot control at JTRF. *Sclerotinia homoeocarpa* isolates were obtained from plots treated with 0.44 kg a.i. ha⁻¹ of propiconazole. From this point forward, propiconazole treated

plots will refer exclusively to plots treated with 0.44 kg a.i. ha⁻¹ propiconazole.

Isolates selected for this study were sampled from two different time points in 2009: initial (prior to treatment) and 7 days after treatment (7-DAT). Isolates from the initial sample were collected prior to fungicide application from propiconazole-treated and untreated plots at all locations. Isolates from the 7-DAT-sample time were collected from propiconazole-treated and untreated plots at HGC, HRCC, SMCC and WBGC.

Isolate collection 7-DAT did not occur at JTRF because dollar spot infection centers were not present until 50-DAT on propiconazole-treated plots. This was the result of propiconazole being highly effective on a DMI sensitive population. Isolates were collected 7-DAT from dollar spot infection centers with active mycelia or newly infected lesions and up to ten infection centers per plot were sampled. The 7-DAT sampling was employed to determine the sensitivity of propiconazole-field-resistant isolates, since 0.44 kg a.i. ha⁻¹ of propiconazole is labeled to control dollar spot for 14 days. The 7-DAT sampling did not occur in the same spray interval at all locations, since disease pressure at each field trial location varied. HGC 7-DAT isolates were collected after the first application, HRCC and SMCC 7-DAT isolates were collected after the second application, and WBGC 7-DAT isolates were collected after the fourth application.

Fungal Isolation and In Vitro Fungicide Sensitivity Assay

Fungal isolation was conducted following procedures described by Jo et al. *Sclerotinia homoeocarpa* isolates from the summer of 2009 were stored at ambient temperature in dried pure cultures. Isolates from untreated and propiconazole-treated plots (initial and 7-DAT) were transferred to APDA and grown for 2-3 days before being transferred to PDA. Two to three days after

transfer to PDA, isolates were assayed on propiconazole-amended PDA to determine relative mycelium growth percentage (RMG%) at multiple discriminatory concentrations. Propiconazole amended PDA was prepared by performing serial dilutions of commercial grade propiconazole (Banner MAXX 13EC, Syngenta Crop Protection, Greensboro, NC) in sterile de-ionized water and added to PDA resulting, in final concentrations of 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$. One 5-mm agar plug was transferred from actively growing mycelium of pure *S. homoeocarpa* cultures to the center of each propiconazole-amended PDA (0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$) and non-amended PDA Petri plate. This step was replicated twice for each isolate. Forty-eight hours after transfer, three radial points approximately 120° apart on the circumference of actively growing *S. homoeocarpa* mycelial colonies were measured with digital calipers (Mahr 16EX, Göttingen, Germany). The average radial growth on propiconazole-amended PDA was divided by the average non-amended radial growth and multiplied by 100 to give RMG% for each Petri plate. Table 3.1 contains a list of the number of isolates that were assayed from at each location.

Table 3.1. Location, number of fungicide applications applied in 2009 field efficacy trial and number of *Sclerotinia homoeocarpa* isolates collected from golf courses in 2009.

Abbreviation	Location	No. of Applications ^w	No. of isolates			
			Initial ^x		7 DAT ^y	
			Unt.	PP	Unt.	PP
WBGC	Bloomfield, CT	4	14	36	34	19
JTRF ^z	South Deerfield, MA	4	32	37	40 ^z	35 ^z
SMCC	Kensington, CT	3	20	15	31	17
HGC	West Hartford, CT	3	31	29	39	16
HRCC	Hadley, MA	2	28	29	33	26

^w Total number of DMI applications made during the field trial.

^x Isolates collected during the initial sampling (Initial) had not been treated with fungicides prior to sampling. Untreated (Unt.) and propiconazole treated (PP) plots represent the type of fungicide treatment plots received after sample collection.

^y Isolates collected seven days after treatment (7 DAT) were either untreated (Unt.) or treated with 0.44 kg a.i. ha⁻¹ of propiconazole (PP). Isolates were only collected from *S. homoeocarpa* infection centers displaying active mycelium growth or newly infected lesions.

^z Isolates collected from JTRF were sampled 50 days after treatment. *Sclerotinia homoeocarpa* infection was not observed on until 50 days after treatment days within plots treated with 0.44 kg a.i. ha⁻¹ of propiconazole.

Qualitative Discriminatory Concentration Selection

JTRF is a DMI-sensitive population and propiconazole provided a high level of *S. homoeocarpa* control during 2 years of field-efficacy testing. Populations HGC, HRCC, SMCC and WBGC were determined to be field resistant to propiconazole during 2 years of field efficacy testing. HGC and HRCC both displayed bimodal population distributions (sensitive and resistant subpopulations) during the 2009 initial sample and propiconazole application selected the resistant subpopulation in propiconazole-treated plots at each location 7-DAT. Therefore, qualitative discriminatory concentration selection was focused on determining the concentration that suppressed growth of JTRF isolates and sensitive isolates from HGC and HRCC, while allowing field-resistant isolates from HGC, HRCC, SMCC and WBGC to exhibit growth. RMG% was calculated for all concentrations (0.1, 0.3, 0.5 and 1.0 µg a.i. ml⁻¹), and histograms were constructed to show isolate growth on the respective concentrations. Isolates sampled from untreated plots (initial and 7-DAT) were pooled with isolates from propiconazole plots initial sample in histograms since those isolates were not collected from plots that were not under direct selection pressure prior to sampling. Isolates

collected 7-DAT from propiconazole-treated plots were presented in separate histograms for HGC, HRCC, SMCC and WBGC.

Results

5 *Qualitative Discriminatory Concentration Evaluation*

JTRF RMG% ranged from 9.7-48.4% ($0.1 \mu\text{g a.i. ml}^{-1}$), 0.0-23.6% ($0.3 \mu\text{g a.i. ml}^{-1}$) and 0.0-18.6% ($0.5 \mu\text{g a.i. ml}^{-1}$). Complete suppression of all isolates was observed at $1.0 \mu\text{g a.i. ml}^{-1}$ (Figure 1). Isolates sampled 7-DAT from
10 propiconazole-treated plots at HGC, HRCC, SMCC and WBGC were previously determined to be field resistant, and RMG range data will be presented separately. RMG% ranges observed from HGC were: 62.3-91.8%, 52.8-87.8%, 46.5-71.4% and 38.2-77.7% for 0.1, 0.3, 0.5 and $1.0 \mu\text{g a.i. ml}^{-1}$ of propiconazole,
15 respectively. RMG% ranges observed from HRCC were: 66.5-97 %, 54.8-78.5%, 39.9-70.4% and 26.3-58.0% for 0.1, 0.3, 0.5 and $1.0 \mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. RMG% ranges observed for SMCC were: 46.5-63.0%, 32.3-57.7%, 31.4-59.8% and 6.4-41.5% for 0.1, 0.3, 0.5 and $1.0 \mu\text{g a.i. ml}^{-1}$ of
20 propiconazole, respectively. RMG% ranges observed for WBGC were: 59.3-89.8%, 44.5-79.8%, 35.4-69.0% and 23.2-55.9% for 0.1, 0.3, 0.5 and $1.0 \mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. Figures 2-B, 3-B, 4-B and 5-B show complete population distributions for HGC, HRCC, SMCC and WBGC. Field-
25 resistant isolates from HGC, HRCC, SMCC and WBGC exhibited 100% growth on 0.3 and $0.5 \mu\text{g a.i. ml}^{-1}$ of propiconazole and 98.7 % of isolates exhibited growth on $1.0 \mu\text{g a.i. ml}^{-1}$ of propiconazole.

Isolates from untreated plots (initial and 7-DAT) and
30 propiconazole plots initial sample were pooled for RMG% range description. RMG% ranges observed from HGC were: 17.4-95.5 %, 0.0-85.4 %, 0.0-82.7 % and 0.0-62.0% for 0.1, 0.3, 0.5 and 1.0

$\mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. RMG% ranges observed from HRCC were: 4.4-99.6%, 0.0-98.5%, 0.0-83.4% and 0.0-62.4% for 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. HGC and HRCC displayed bimodal population distributions in absence of propiconazole selection pressure; whereas propiconazole plots 7-DAT displayed solely unimodal populations distributions (Figures 2 and 3). RMG% ranges observed from SMCC were: 29.9-76.1 %, 9.7-70.6 %, 0.0-56.1 % and 0.0-48.1 % for 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. RMG% ranges observed from WBGC were: 61.2-97.1%, 40.6-80.2%, 32.1-71.4% and 6.9-59.8% for 0.1, 0.3, 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ of propiconazole, respectively. Figures 2-A, 3-A, 4-A and 5-A show complete population distributions for HGC, HRCC, SMCC and WBGC.

Selection of a Qualitative Discriminatory Concentration

Practical field-resistance analysis with a qualitative discriminatory concentration analyzes population distributions based on the presence or absence of mycelium growth. The 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ concentrations of propiconazole completely suppressed mycelium growth of 99.1 % and 100.0 % of isolates from JTRF, respectively. The 1.0 $\mu\text{g a.i. ml}^{-1}$ concentration of propiconazole was also more effective at reducing aerial mycelia. Aerial mycelia resulted from mycelium growing vertically from the agar plug placed on propiconazole-amended media (Figure 6). Aerial mycelia was not quantified, however Figure 7 shows the presence of aerial mycelium in a greater abundance on PDA amended with 0.5 $\mu\text{g a.i. ml}^{-1}$ than PDA amended with 1.0 $\mu\text{g a.i. ml}^{-1}$ of propiconazole. This trend in aerial mycelium was observed in general among isolates from all locations.

Field-resistant isolates sampled from propiconazole-treated plots at HGC, HRCC, SMCC and WBGC 7-DAT were analyzed to determine the appropriate qualitative discriminatory concentration. Population distributions show that field-resistant isolates exhibited growth on all discriminatory concentrations tested and both 0.5 and 1.0 $\mu\text{g a.i. ml}^{-1}$ would be suitable for qualitative assessment of field resistant isolates. Due to reduced aerial mycelium growth on PDA amended with 1.0 $\mu\text{g a.i. ml}^{-1}$ of propiconazole, this concentration was determined to be more effective for qualitative *in vitro* detection of propiconazole-field-resistant isolates (Figure 7).

Although the above results relate to propiconazole, it should be noted that cross-resistance has been confirmed among seven DMI active ingredients (fenarimol, metconazole, myclobutanil, propiconazole, tebuconazole, triadimefon and triticonazole).

The present results apply to the benzimidazole and dicarboximide fungicide classes that are used to control *S. homoeocarpa*. The methods presented can also apply to other turfgrass pathogens that have developed fungicide resistance. The exemplary embodiment above illustrates the methods used for providing a kit to determine fungicide resistance in several fungal pathogens of turfgrasses. In one embodiment, the methods determined a discriminatory concentration, selected a growth medium based on pathogen growth in a predetermined time and the predetermined time enabled use of the kit on site.

It should be noted that the DMI (demethylation inhibitor) class of fungicides represent the most difficult class in terms of determining fungicide resistance.

It should also be noted that the present teachings apply to the other two of the three fungicide classes (benzimidazole, dicarboximide, and demethylation inhibitor) applied to *S.*

homoeocarpa and that the present teachings can also apply to other turfgrass pathogens that have developed fungicide resistance.

Although these teachings are not limited to the above exemplary embodiment, the above exemplary embodiment illustrates the method of these teachings for providing a kit for determining fungicide resistance in fungal diseases on turfgrasses. In one embodiment, the method of these teachings for providing a kit for determining fungicide resistance in fungal diseases on turfgrasses includes determining a discriminatory concentration, as described herein, selecting a growth medium based on growth of the fungal disease in at most a predetermined time, the predetermined time enabling use of the kit on site, and based on ease of use on site.

For the purposes of describing and defining the present teachings, it is noted that the term "substantially" is utilized herein to represent the inherent degree of uncertainty that may be attributed to any quantitative comparison, value, measurement, or other representation. The term "substantially" is also utilized herein to represent the degree by which a quantitative representation may vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

Although these teachings have been described with respect to various embodiments, it should be realized these teachings are also capable of a wide variety of further and other embodiments within the spirit and scope of the appended claims.

What is claimed is:

CLAIMS

1. A method for determining fungicide resistance in a fungal disease on turf grasses, the method comprising:
 - 5 collecting samples of leaf blades;
 - disinfecting the samples, after collection, thereby obtaining disinfected samples;
 - 10 rinsing the disinfected samples;
 - placing some of the disinfected samples in a growth medium, thereby forming a control sample;
 - 15 placing others of the disinfected samples in a growth medium amended with a predetermined discriminatory dose of a predetermined fungicide, thereby forming a test sample; and
 - 20 comparing the test sample and the control sample after a predetermined interval of time;
 - fungicide resistance being determined by comparing the test sample on the control sample.
- 25 2. The method of claim 1 wherein the fungal disease is *Sclerotinia homoeocarpa*; wherein the predetermined fungicide is propiconazole; and wherein a predetermined discriminatory dose of propiconazole is greater than 0.5 $\mu\text{g a.i. ml}^{-1}$ and not greater than 1.0 $\mu\text{g a.i. ml}^{-1}$; whereby resistance to demethylation
30 inhibitor (DMI) fungicide is determined.

3. The method of claim 2 wherein the predetermined discriminatory dose of propiconazole is about $1.0 \mu\text{g a.i. ml}^{-1}$.

4. The method of claim 2 further comprising:

5 placing some others of the disinfected samples in a growth medium amended with a predetermined discriminatory dose of another predetermined fungicide, thereby forming another test sample; and

10 comparing the another test sample and the control sample after the predetermined interval of time;

15 resistance to the another predetermined fungicide being determined by comparing the another test sample to the control sample;

wherein the another predetermined fungicide is one of benzimidazole fungicide or dicarboximide fungicide.

20 5. The method of claim 4 wherein the another predetermined fungicide is the benzimidazole fungicide; and wherein the benzimidazole fungicide is thiophanate-methyl and a predetermined discriminatory dose of thiophanate-methyl is about $1,000 \mu\text{g a.i. ml}^{-1}$.

25 6. The method of claim 5 wherein the predetermined discriminatory dose of propiconazole is about $1.0 \mu\text{g a.i. ml}^{-1}$.

30 7. The method of claim 4 wherein the another predetermined fungicide is the dicarboximide fungicide; and wherein the dicarboximide fungicide is vinclozolin and predetermined discriminatory dose of vinclozolin is about $5 \mu\text{g a.i. ml}^{-1}$.

8. The method of claim 7 wherein the predetermined discriminatory dose of propiconazole is about $1.0 \mu\text{g a.i. ml}^{-1}$.

5 9. A kit for determining fungicide resistance in diseases caused by *Sclerotinia homoeocarpa* on turfgrasses, the kit comprising:
a first container having a disinfecting solution;
a second container having a growth medium; and
10 a third container having growth medium amended with a predetermined discriminatory dose of one or more predetermined fungicides.

10. The kit of claim 9 wherein the growth medium is acidified
15 potato dextrose agar (APDA).

11. The kit of claim 9 wherein the one or more fungicides comprises three fungicides; wherein the third container comprises at least three sub-containers; wherein at least one sub-container
20 has a growth medium amended with a predetermined discriminatory dose of benzimidazole fungicide, at least another sub-container has a growth medium amended with a predetermined discriminatory dose of dicarboximide fungicide, and at least yet another sub-container has a growth medium amended with a predetermined
25 discriminatory dose of at least one active ingredient of demethylation inhibitor (DMI).

12. The kit of claim 11 wherein the at least one active ingredient of DMI is propiconazole; whereby resistance to
30 demethylation inhibitor (DMI) fungicide is determined.

13. The kit of claim 12 wherein the benzimidazole fungicide is thiophanate-methyl and a predetermined discriminatory dose of thiophanate-methyl is about 1,000 $\mu\text{g a.i. ml}^{-1}$; wherein the dicarboximide fungicide is vinclozolin and a predetermined discriminatory dose of vinclozolin is about 5 $\mu\text{g a.i. ml}^{-1}$; and wherein a predetermined discriminatory dose of propiconazole is greater than 0.5 $\mu\text{g a.i. ml}^{-1}$ and not greater than 1.0 $\mu\text{g a.i. ml}^{-1}$.

14. The kit of claim 13 wherein the predetermined discriminatory dose of propiconazole is about 1.0 $\mu\text{g a.i. ml}^{-1}$.

15. The kit of claim 9 wherein the second container and third container are one container, said one container comprising at least four sub-containers; wherein the one or more fungicides comprises three fungicides; wherein at least one sub-container has a growth medium amended with a predetermined discriminatory dose of benzimidazole fungicide, at least another sub-container has a growth medium amended with a predetermined discriminatory dose of dicarboximide fungicide, at least yet another sub-container has a growth medium amended with a predetermined discriminatory dose of at least one active ingredient of demethylation inhibitor (DMI), and at least a further sub-container has the growth medium.

16. The kit of claim 15 wherein the at least one active ingredient of DMI is propiconazole; whereby resistance to demethylation inhibitor (DMI) fungicide is determined.

17. The kit of claim 16 wherein the benzimidazole fungicide is thiophanate-methyl and a predetermined discriminatory dose of thiophanate-methyl is about 1,000 $\mu\text{g a.i. ml}^{-1}$; wherein the

dicarboximide fungicide is vinclozolin and predetermined discriminatory dose of is about $5 \mu\text{g a.i. ml}^{-1}$; and wherein a predetermined discriminatory dose of propiconazole is greater than $0.5 \mu\text{g a.i. ml}^{-1}$ and not greater than $1.0 \mu\text{g a.i. ml}^{-1}$.

5

18. The kit of claim 17 wherein the predetermined discriminatory dose of propiconazole is about $1.0 \mu\text{g a.i. ml}^{-1}$.

19. The kit of claim 9 further comprising a collection device.

10

20. The kit of claim 9 further comprising a collection container.

21. The kit of claim 9 further comprising a container having a rinsing solution.

15

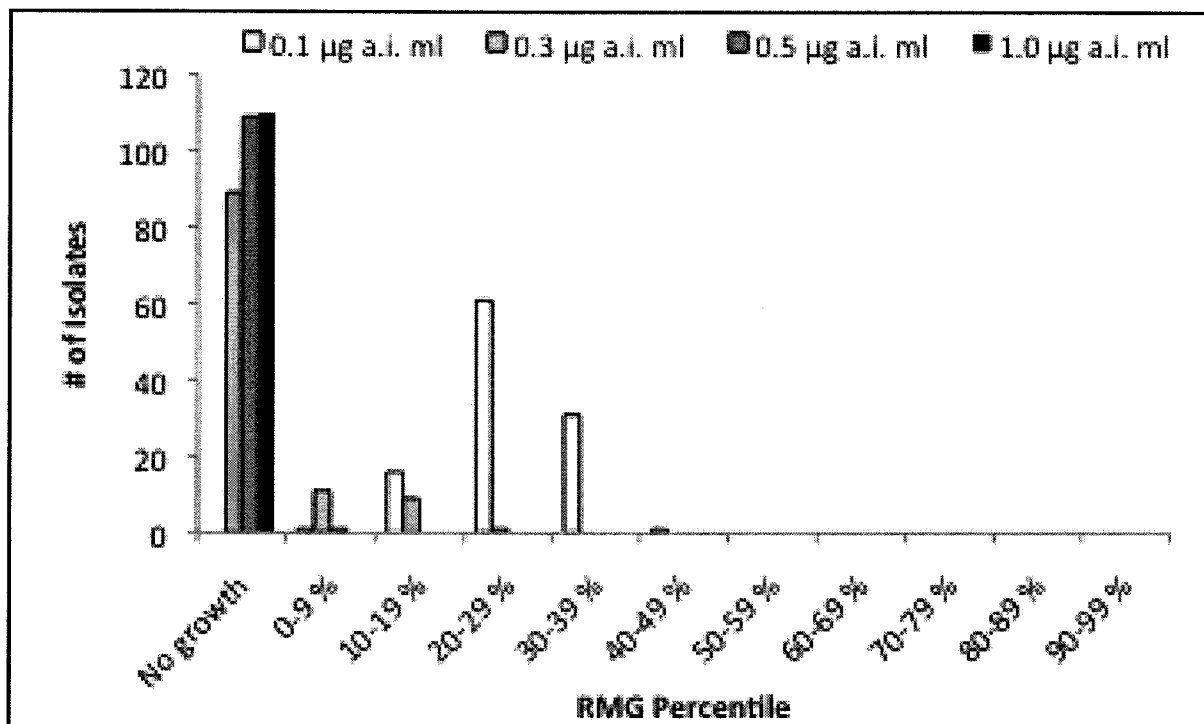


Fig. 1

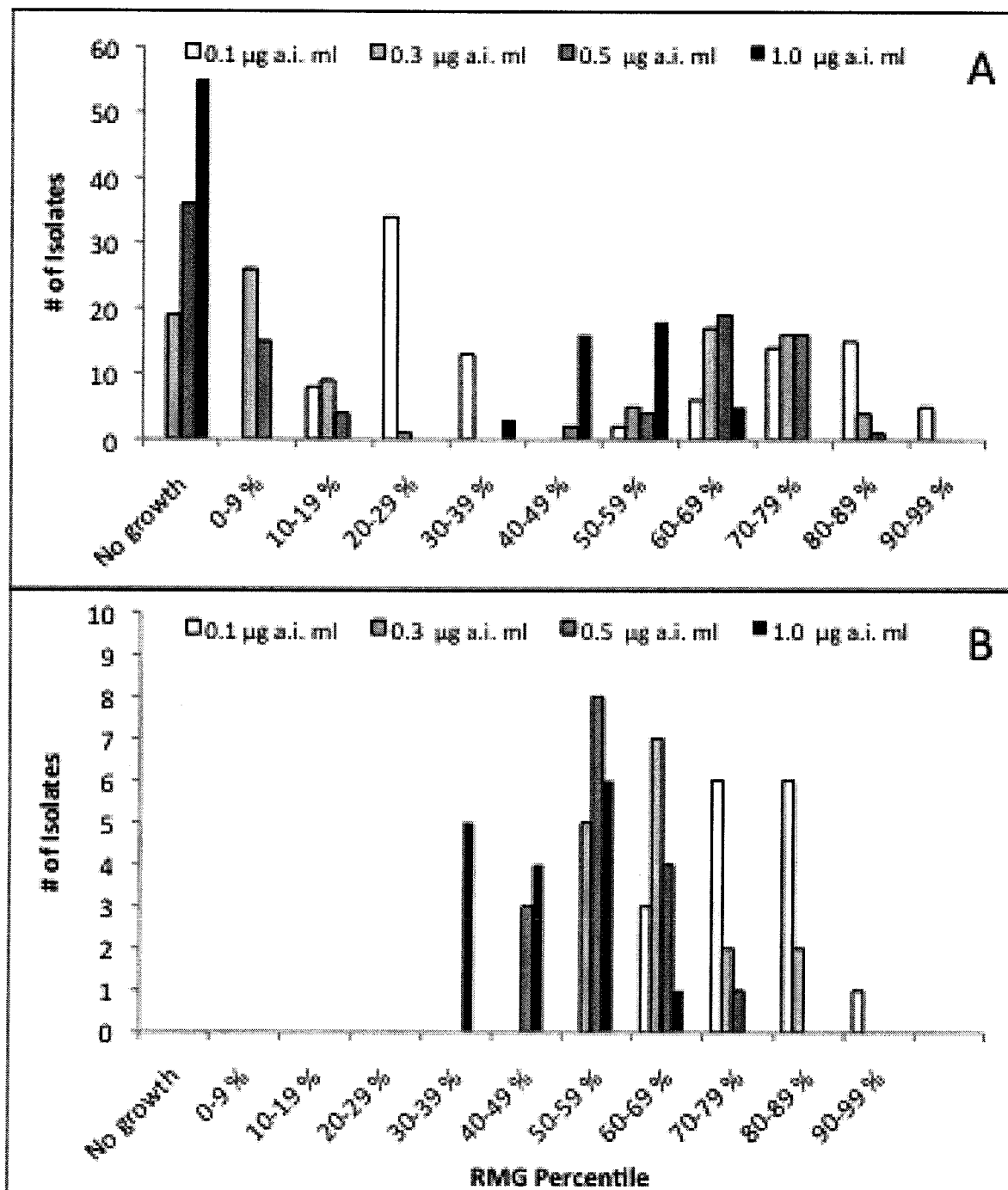


Fig. 2.

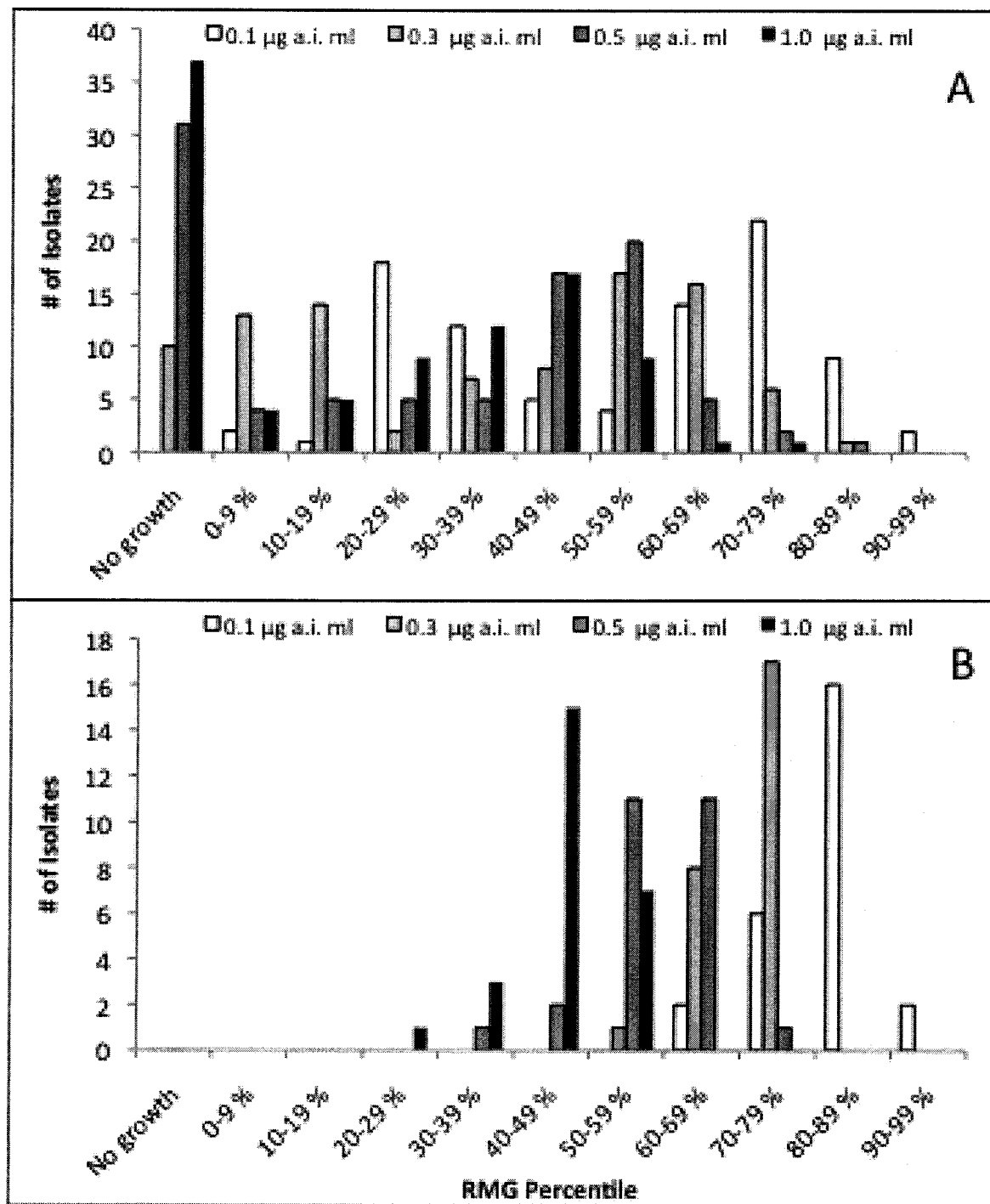


Fig. 3.

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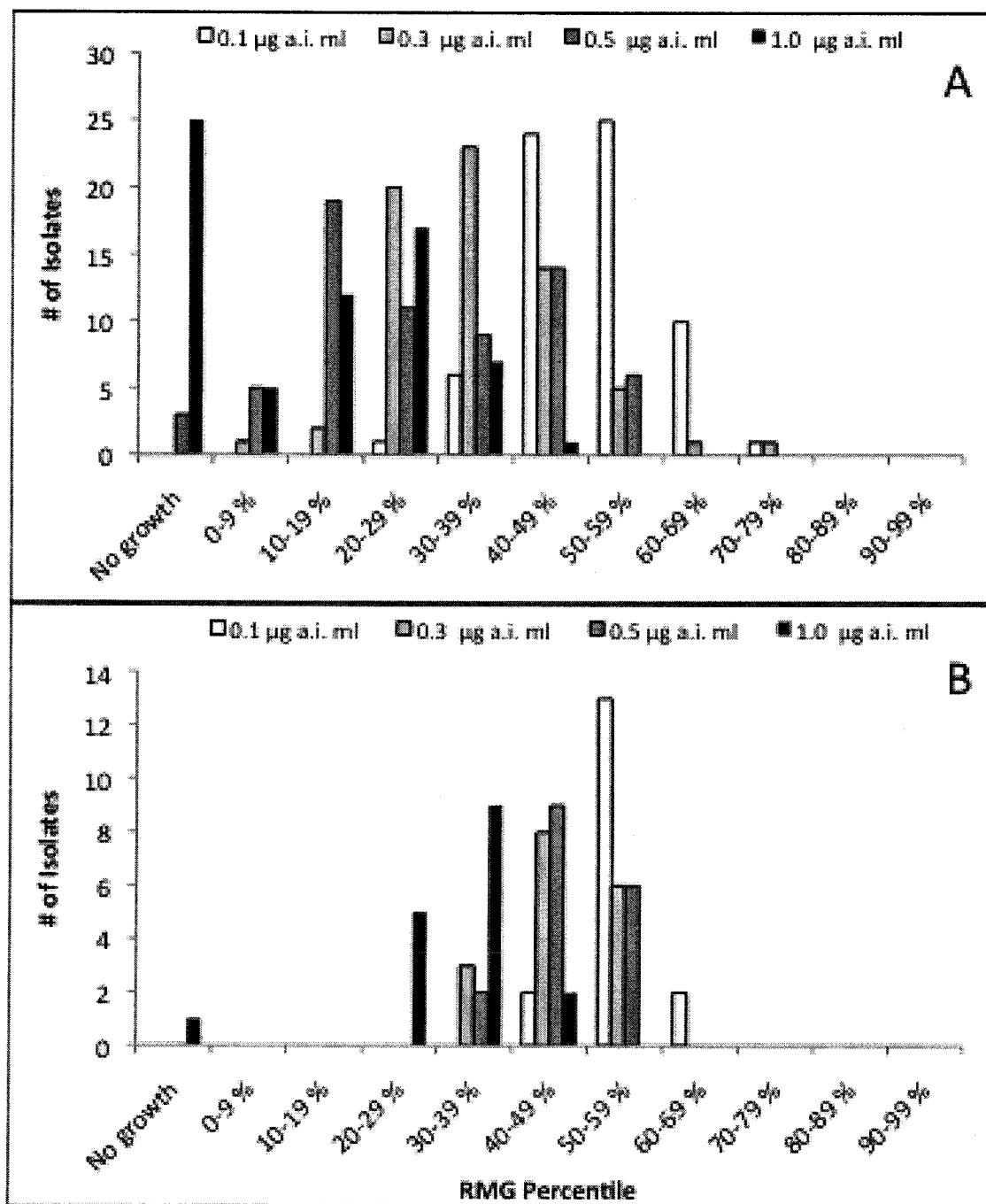


Fig. 4

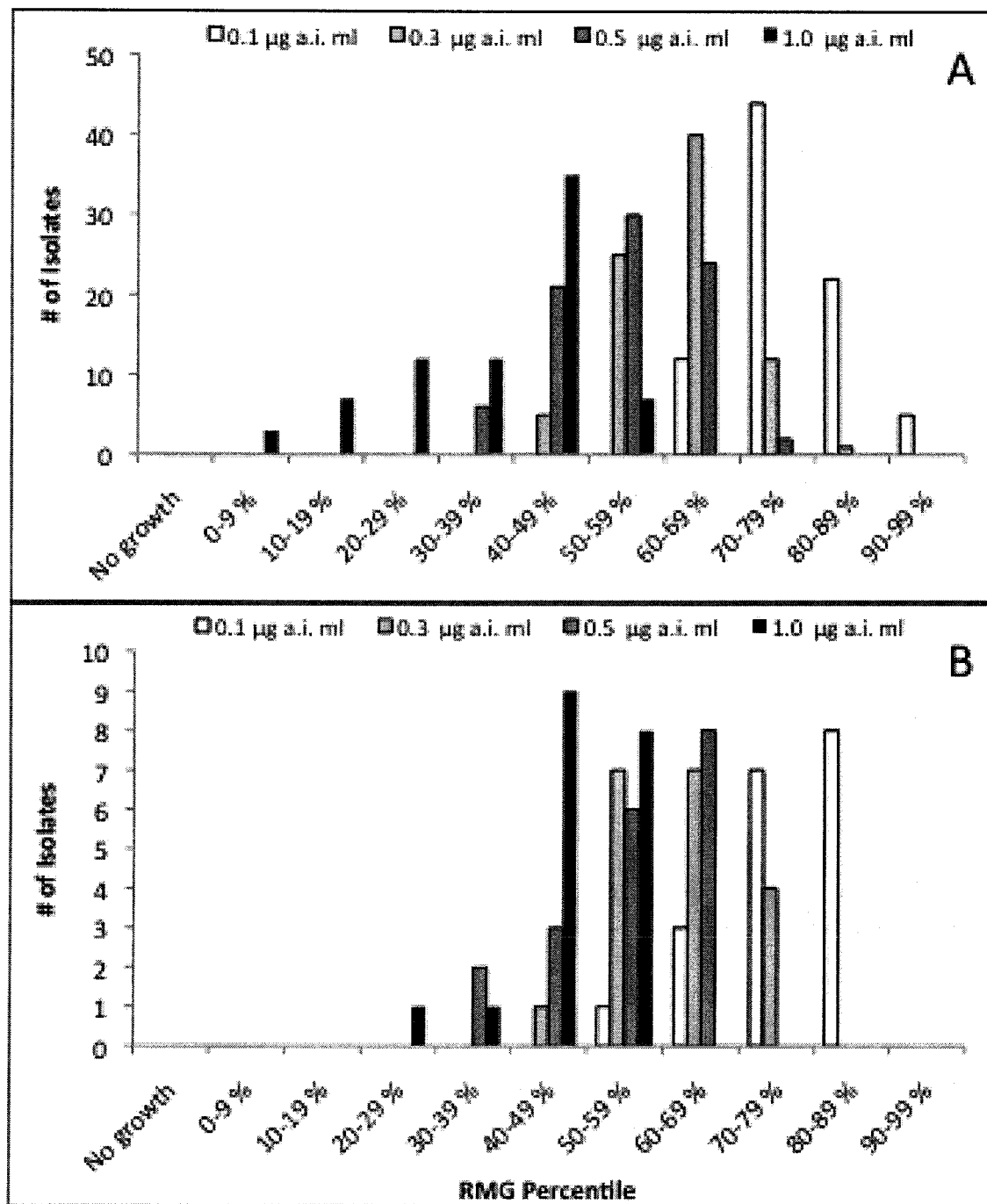


Fig. 5

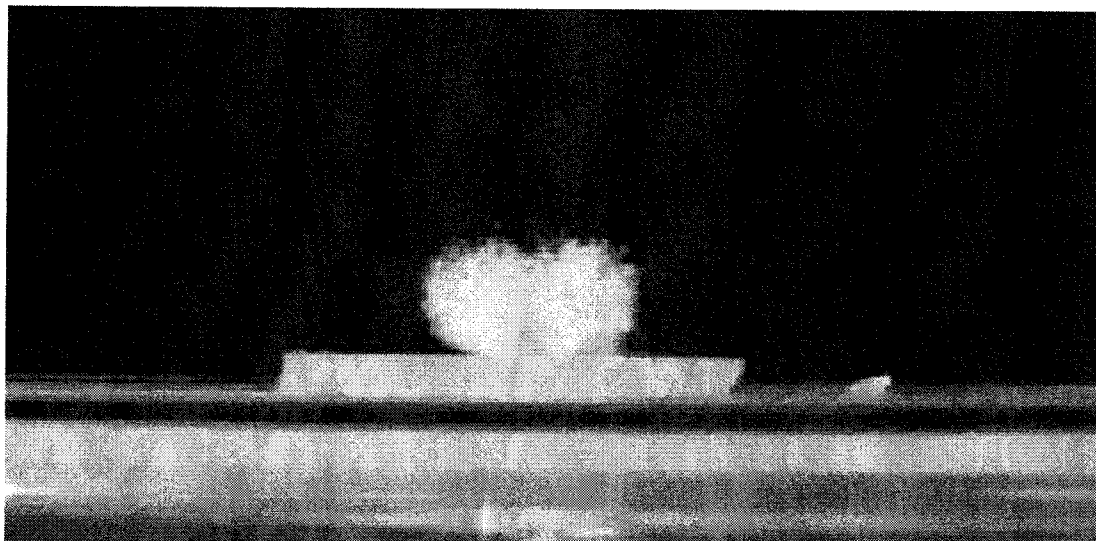


Fig. 6

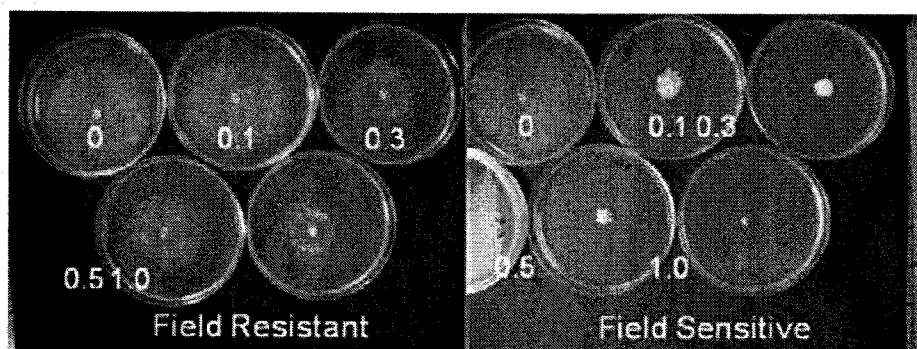


Fig. 7

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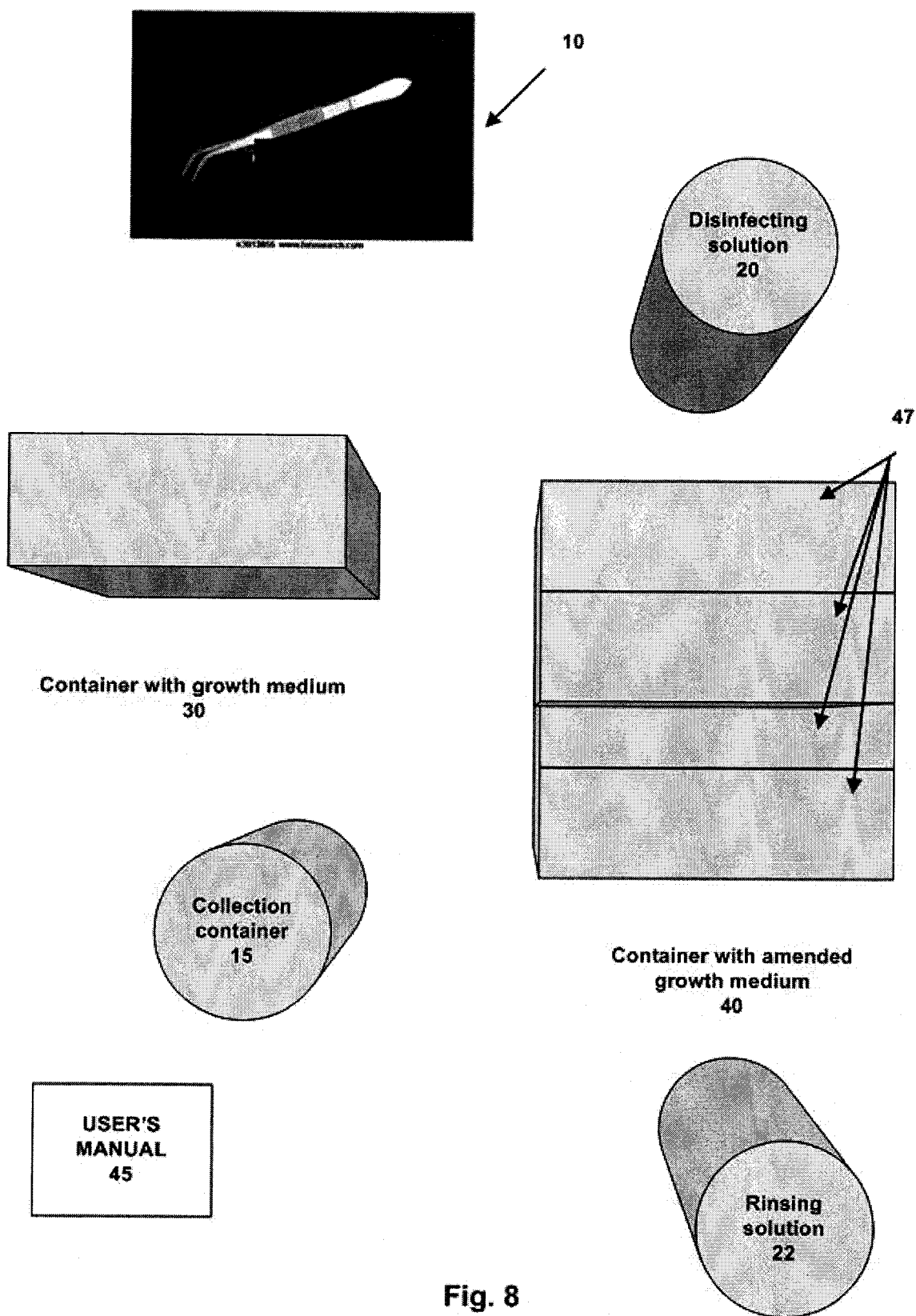


Fig. 8