OTRF Project No.

OTRF Funded Research Project

☐ Interim Report ■ Final Report

Title	Variation in responsiveness of Agrostis cultivars to defense activators, and correlation with defense response genes.
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Graduate Student/Research Associates and Affiliation	Ms. Weihong Gao, Research Assistant, University of Guelph
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Executive Summary

There is a A new group of disease control compounds known as defense activators or resistance activators. These compounds cause plants to show greater resistance to diseases, and do not have direct inhibitory effects on fungi as is found with conventional fungicides. However, the disease suppression afforded by these compounds is rarely as potent as that of conventional fungicides, and there are also reports of variation in results when using such compounds in the field. Because these compounds work by activating the natural defense mechanisms in a plant, the particular genetic make-up of a plant (i.e. cultivar) may have a major impact on their effectiveness, and the purpose of this work was to look at this source of variation in responsiveness to several commercially available defense activators. In some preliminary work, we have found differences between cultivars of *Agrostis* species in their ability to show disease resistance induction by some of these compounds. In this study, we conducted more extensive experiments in the lab and the field and found that responsiveness to disease resistance activation does vary by cultivar with some showing very high stimulation (disease reduced to half the untreated level), while others show no effect or even some increase in disease. Field test results from summer 2012 showed that one defense activator treatment actually increased disease over the inoculated control (creeping bentgrass L93) while Penncross, a commonly used creeping bentgrass cultivars, and a colonial bentgrass cultivar showed reductions in dollar spot disease.

The molecular mode of action and genes associates with this resistance activation were also investigated by a new method called genome-wide Next Generation Sequencing, and preliminary analysis of the results has been completed showing that thousands of genes may be affected by such chemicals.

How these results benefit target groups is that there is now a better understanding of how such activators work, and some insight into their limitations. We have produced a list of cultivars that show high responsiveness, and this can be immediately be put to use if turf managers or sod growers are seeding or overseeding, and intend to use commercially available resistance activator compounds to replace or supplement their synthetic fungicide use.

The next steps for this project are to continue analysis of the many gigabytes of Next Generation Sequencing data, and to further test these products in the field. Since most of the defense activators in

this study are commercially available and registered for use in Canada (Civitas, Harmonizer and Actigard), the technology can be adopted immediately if turf managers choose particular responsive varieties to grow, and select particular resistance activators for use. In conventional fungicide tests, efficacy is more dependent on the toxicity of the product toward fungi directly and the environment under which it is being used. For defense activators, it seems that the condition or inherent genetics of the host plant also play very critical roles that need further investigation.

Background

Defense activators such as Actigard, Civitas, and other newly developed compounds are known to decrease severity of turfgrass diseases in *Agrostis* species (1,2) . The mode of action for most of these activators is SAR (systemic acquired resistance), mediated by the salicylic acid pathway, or ISR (induced systemic resistance), mediated by jasmonic acid or ethylene related pathways. However, some defense activators work via pathways that are not fully characterized. Because these chemicals work through plant defense gene expression of the host, the plant genotype (cultivar) can have a major impact on their effectiveness. The effect of plant genotype on defense gene expression has not been clearly elucidated in *Agrostis* species or other turfgrass species. In some preliminary work, we have found differences between cultivars of *Agrostis* species in their ability for disease resistance induction by some of these compounds. We would like to verify this further in more extensive experiments using newly developed technologies.

References:

(1) Cortes-Barco AM, Hsiang T, Goodwin PH. 2010. Induced systemic resistance against three foliar diseases of *Agrostis stolonifera* by (2R,3R)-Butanediol or an isoparaffin mixture. Annals of Applied Biology 157:179-189.

(2) Hsiang T, Goodwin PH, Cortes-Barco AM. 2011. Plant defense activators and control of turfgrass diseases. Outlooks on Pest Management 22:160-164.

Objectives

PRACTICAL OBJECTIVES: The practical objectives of this work were to assess whether *Agrostis* species and cultivars vary in their ability to benefit from defense activator applications. There is some previous work showing that reduction in disease severity differs between *Agrostis* cultivars when exposed to compounds such as Actigard or Civitas. The practical outcome of this was to identify species and cultivars with defense-activator responsiveness, and perhaps selectable gene expression markers that could be used by breeders to screen for this trait if this exists. These practical objectives have been achieved.

SCIENTIFIC OBJECTIVES: The use of transcriptome sequencing to examine changes in the expression of genes on genome-wide scale is just starting to become popular as the prices for Next Generation Sequencing become much less expensive and affordable. This research compared genome-wide changes in gene expression caused by defense activator exposure, and related this to cultivar differences in their response to defense activators. Among the thousand of genes that showed increased expression, so of them are thought to be related to plant disease resistance, and these need further study. In addition, changes in expression of many plant genes may give insights into other effects that defense activators may be having on the treated plants such as improved drought and heat tolerance as well as improved root growth. These scientific objectives require further study.

Methods & Results

Activity One: Test cultivars for their response to select resistance activators in lab and field (December

13, 2012 to June 30, 2013)

Methods: We compared cultivars of *Agrostis* species for variation in their ability to respond to defense activators to induce systemic acquired resistance (SAR) and ISR (induced systemic resistance) against turf pathogens (*Waitea circinata* var. *circinata* causing brown ring patch) or *Sclerotinia homoeocarpa* causing dollar spot). Responsiveness to defense activators is defined as the ability of the chemical to reduce the severity of disease in plants treated with defense activator and then inoculated compared to inoculated water-treated plants. This work has been done in the lab and in the field. In the lab in 2012, we tested the brown ring patch pathogen, *Waitea circinata* because we found it an easier model system than some other more common and severe turfgrass pathogens. However, other lab work in 2012 and 2013 was done with the dollar spot pathogen, and field work in 2012 was conducted with the dollar spot pathogen on a range of different cultivars.

2012 Lab results: Cultivars of *Agrostis stolonifera* and *A. capillaris* were screened in vitro (Figure 1) for their level of responsiveness to the ISR activator, 2R, 3R-butanediol (BD), by measuring the reduction in disease symptoms caused by the fungal pathogen *Waitea circinata* var. *circinata* compared to a water control. Defense activation in cultivars 'SR7100' and 'SRP1GMC', as measured by reduced yellowing and mycelial coverage (Figure 2), was strongly responsive to BD. In contrast defense activation of cultivars 'Penn A4' and 'Providence' was weakly responsive, and BD treatment even led to increased yellowing and mycelial coverage.

Figure 1: Turfgrass cultivars were screened inside 15 ml glass vials with grass grown on a sand base. After one to two weeks of growth from seed, defense activators were applied. After another week, grass was inoculated with ground-up grain inoculum of the brown ring patch fungus, *Waitea circinata*.

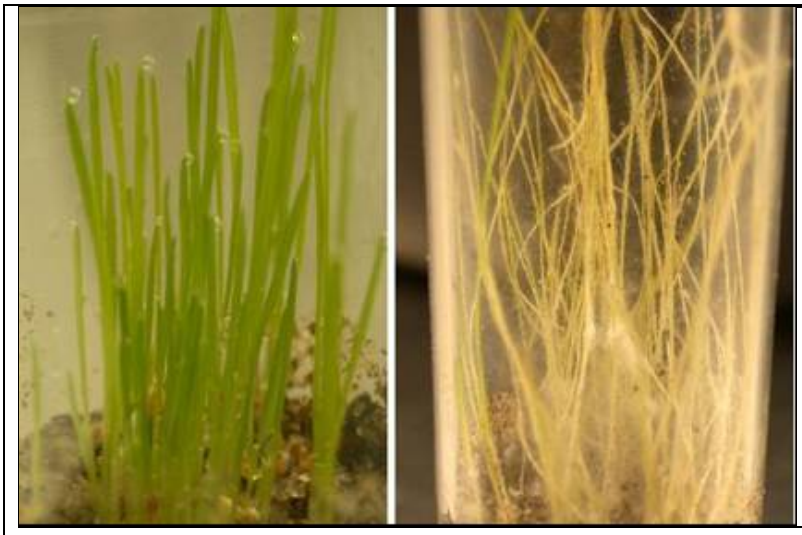

Figure 2: Turfgrass cultivars were screened inside 15 ml glass vials with grass grown on a sand base. The left panel shows untreated grass, while the right panel shows disease, extensive yellowing and the presence of sclerotia of *Waitea circinata* at one week after inoculation.

2012 Field results: Cultivars of turfgrass species were screened in the field in summer 2012 for their responsiveness to the SAR activator BTH (benthiadazole, the active component in the Syngenta product Actigard used at 7 mg / m^2), the ISR activator Civitas alone (produced by Petro Canada and used at 24 ml / m^2 at greens height and double at lawn height) and the combination of Civitas and the pigment, Harmonizer (produced by Petro Canada, at same rate for Civitas and 1/16 of that for Harmonizer). These treatments were applied on a three week interval from early July to late August, and the plots were rated weekly for dollar spot (the wheat grain inoculum had been applied to the plots in early July at 5 g / m^2). Plots of these grasses were available in the display garden near the GTI building or on the research greens and ranges. A total of 15 species/cultivars were tested in these experiments, listed in Table 1 below:

Table 1: The cultivars tested at GTI with resistance activators and their specific locations. Greens refers to a low height of cut ranging from greens height to fairway height. Lawn refers to a high height of cut of around 5 cm.

Species	**Location**	**Height**
Creeping bentgrass, *Agrostis stolonifera* 'Penncross'	Pathology green	greens
Creeping bentgrass, *Agrostis stolonifera* 'A4'	Upper sand green (west half)	greens
Creeping bentgrass, *Agrostis stolonifera* 'SR7200'	Range 3 (30 m from west end)	greens
Creeping bentgrass, *Agrostis stolonifera* 'L93'	Upper sand green (west half)	greens
Creeping bentgrass, *Agrostis stolonifera* 'Cobra'	California green	greens
Creeping bentgrass, *Agrostis stolonifera* 'MacKenzie'	Upper sand green (west half)	greens
Perennial ryegrass, *Lolium perenne*	Display garden	lawn
Tall Fescue, *Lolium arundinaceum*	Display garden	lawn
Red Fescue, *Festuca rubra*	Display garden	lawn
Rough bluegrass, *Poa trivialis*	Display garden	lawn
Kentucky bluegrass, *Poa pratensis*	around Pathology green	lawn
Annual bluegrass, *Poa annua*	Display garden greens	greens
Velvet bentgrass, *Agrostis canina*	Display garden greens	greens
Colonial bentgrass, *Agrostis capillaris*	Display garden greens	greens
Redtop, *Agrostis gigantea*	Display garden	lawn

Throughout most of the trial, there were few significant differences between treatments. The lawn height grasses never showed noticeable dollar spot development. On the greens height grasses, one of the issues was that dollar spot came on slowly, but then dramatically increased in late July and seemed to

overwhelm the defenses of the grasses, except for creeping bentgrass cultivar 'McKenzie' which showed the slowest dollar spot development. The disease data for the cultivars by the end of the trial in late September are shown in Table 2, and Figure 3 gives an example of the plots.

Table 2: Effects of defense activator treatment compared to the water control on various bentgrass cultivars plus *Poa annua*, with the the number of spots or the area affected per 0.5 m by 0.5 m plot. Shaded boxes show significantly decreased disease compared to the inoculated control. Numbers in bold show significant enhancement of disease.

Cultivar	Water	BTH	Civitas	Civitas&Harmonizer	LSD (p=0.05)
Penncross (area)	19.8	6.5	5.5	8.3	10.1
Penncross (spots)	21.3	3.3	8.0	9.0	13.2
L93 (spots)	14.3	12.8	14.5	**22.0**	7.3
McKenzie (spots)	6.3	18.3	4.8	4.5	20.8
Cobra (area)	55.0	28.8	57.5	41.3	25.4
SR-7200 (spots)	5.3	4.8	3.5	5.8	6.4
Poa annua (spots)	3.8	4.5	3.8	5.0	3.7
Velvet bent (spots)	2.3	2.3	2.0	1.3	1.8
Colonial bent (spots)	7.8	5.5	4.0	3.0	3.7

BTH	Civitas
Water	Civitas & Harmonizer

Figure 3: An example of field plots treated with resistance activators three times between July & August. The boxes above show the arrangement of treatments. The plots were inoculated with the dollar spot fungus in early July, and this picture was taken at the end of September.

2013 Lab results: Cultivars of *Agrostis stolonifera* and *A. capillaris* were screened in the laboratory for their level of responsiveness to the ISR activators, 2R, 3R-butanediol (BD) and Civitas+ Harmonizer, which is produced by Petro-Canada, and the SAR activator, Actigard, which is produced by Syngenta. Induced resistance was measured as the reduction in disease symptoms caused by the fungal pathogens *Waitea circinata* var. *circinata* (causing brown ring patch) or *Sclerotinia homoeocarpa* (causing dollar spot) compared to a water control. As measured by reduced yellowing and mycelial coverage, disease severity in the cultivars 'SR7100' and 'SRP1GMC' was strongly responsive to BD, and disease severity in cultivars 'PennA4' and 'Brighton' was strongly responsive to Actigard. In contrast, cultivars 'PennA4' and 'Providence' were weakly responsive to BD, and cultivars 'SRPBLTR3' and 'SRX1WM' were weakly responsive to Actigard. For Civitas+Harmonizer treatments, cultivars '007', 'SR1150' and 'Cato' were strongly responsive, while cultivars 'Alpha', 'Tyee' and 'Providence' were weakly responsive as measured by reduced yellowing (Table 3). For weakly responsive cultivars, BD, Civitas+Harmonizer or Actigard treatment even led to increased yellowing and mycelial coverage.

Table 3: Percent yellowing in bentgrass cultivars grown in vials or jars at 12 days after inoculation with the dollar spot pathogen, *Sclerotinia homoeocarpa,* and measured 19 days after treatment.

Cultivar	Water	Civitas	Civitas & Harmonizer	Harmonizer
SR1150	43	32.5	21	15.5
007	75	44	26	19.5
Cato	76.5	27.5	42.5	34
Providence	81	72	60	47.5
Tyee	84.5	63.5	63.5	48
Alpha	76	78	67.5	45

Activity Two: Test highest and lowest responding cultivars with Next Generation sequencing tools to find differences, July 1, 2013 to March 15, 2014

Methods: Samples showing high and low response to Actigard or Harmonizer were prepared for RNA extraction using conventional methods. These samples were sent to the sequencing company (Genome Quebec) for Next Generation Sequencing with Illumina-Solexa HiSeq2000, using multiplexed libraries in 100 base-pair paired-end sequencing. The sequencing data were produced after 4 months in summer 2013. Considerable effort has been made to thoroughly analyze the data, but this technology is so new that standard protocols for analysis have not been universally accepted. The analysis is ongoing as new methods are evolving.

Results: Bioinformatic analysis has identified numerous potential candidate genes related to plant defenses activated by Harmonizer (Table 4) or by BTH (Table 5). This data will continue to be analyzed and future reports and publications produced and shared with OTRF.

Table 4: Twenty genes obtained by Next Generation Sequencing and selected for their annotations as being potentially related to disease, out of a pool of 1000 genes showing greater than two-fold overexpression (compared to water) in *Agrostis stolonifera* 'Penncross' treated with Harmonizer.

Gene	Expression
Cell wall-associated hydrolase	2.0x
PAL-4	2.0x
PAL-1	2.1x
PR class i	2.1x
WRKY-6	2.1x
Bacterial-induced peroxidase	2.2x
Harpin-induced protein	2.2x
Ethylene responsive protein	2.3x
Hypersensitivity related protein	2.5x
Disease resistance protein	2.8x
PR-5	2.8x
Chitinase	3.0x
Jasmonate-induced protein	3.2x
Lipoxygenase	3.3x
Nematode-resistance protein	3.5x
Endo-beta glucanase	3.6x
Rapidly elicited protein	4.1x
4-coumarate coenzyme A ligase	4.5x

PR-10	4.5x
PR-1 (basic)	20x

Table 5: Seventeen genes obtained by Next Generation Sequencing and selected for their annotations as being potentially related to disease, out of a pool of 1000 genes showing two-fold or more overexpression (compared to water) in *Agrostis stolonifera* 'Penn A4' treated with BTH at 7 days after treatment.

Node[1]	Coverage[2]	Assigned function[3]	Contig (bp)[4]	E-value[5]	Match (bp)[6]	BTH/water[7]
410106	2.6	Agmatine coumaroyltransferase	1753	0	562/693	6.82
162219	32.5	Aspartic proteinase	548	1.00E-124	408/510	3.67
184562	2.1	Beta glucanase	309	2.00E-65	185/219	2.23
199733	3.3	Blight resistance	284	1.00E-29	139/178	2.13
237688	16.7	Disease resistance NB-LRR	1585	3.00E-112	397/511	2.46
8046	14.2	Disease resistance protein	903	1.00E-95	413/550	2.62
375501	4.9	Ferrulate-5-hydroxylase	612	1.00E-47	134/150	17.27
166620	24.1	Glutathionine transferase	520	2.00E-152	428/512	12.72
11420	13.7	Harpin-inducing protein	974	5.00E-88	318/407	2.18
174361	14.1	HR-induced protein	1258	0	646/838	3.98
209575	5.6	Lipoxygenase	2821	0	1017/1189	2.99
300352	4.6	NPR1-interacting protein	616	4.00E-66	234/288	6.63
416123	1.4	Polyphenol oxidase	1080	8.00E-30	144/192	5.47
231978	8.2	PR-5	173	1.00E-23	107/135	2.46
145358	3.5	Resistance protein RGA2	419	3.00E-53	248/336	2.44
178630	6	Serine threonine kinase	161	3.00E-62	150/159	1.96
401659	8.2	WRKY transcription factor	652	5.00E-78	294/375	8.32

[1] Number assigned to unique contigs as they are assembled.
[2] Average number of reads matching each node across the length of the node.
[3] Gene ontology assigned to a contig from BLAST2GO analysis.
[4] Length of assembled contig.
[5] Minimum e-value of BLASTN search result when using the contig as query.
[6] Proportion of bp matching between the contig and its highest scoring BLASTN result.
[7] Fold overexpression of the contig in the BTH-treated sample versus water control.

Conclusions [Final Report]	
This is very promising research on the use of an environmentally friendly class of products (disease resistance activators that do not directly kill pathogens) for controlling diseases in the field, and shows that particular turfgrass cultivars react differently to these activators. This is very cutting edge research analogous to the development of personalized human medicine when treatments are targeted for specific individual based on their genetic make-up. We have found that certain activators work much	

better with certain plant lineages (i.e. cultivars), and are still working at uncovering the genetic basis of such activation through Next Generation Sequencing technology.

Out of over a dozen creeping bentgrass cultivars tested, here are results of high responding (disease reduced=less yellowing from disease treatment) and low responding (disease slightly or not significantly reduced and sometimes increased). The BD and BTH tests were done against brown ring patch caused by*Waitea circinata* while the test with Civitas and Harmonizer were done against dollar spot caused by *Sclerotinia homoeocarpa*.

ISR Activators (BD and Civitas) reduced disease in SR7100 and SRP1GMC

ISR Activators did not reduce or slightly reduced disease in PennA4 and Providence

SAR Activators (BTH, Actigard) reduced disease in PennA4 and Brighton

SAR Activators did not or slightly reduced disease in SRPBLTR3 and SRX1WM

Civitas+Harmonizer reduced disease in 007, SR1150, and Cato

Civitas+Harmonizer did not reduce or slightly reduced disease in Alpha, Tyee and Providence

The lab results served to demonstrate that there are quantifiable differences in responsiveness of different cultivars to different defense activators under highly controlled environments, and that different cultivars may respond quite differently to different defense activators (e.g. PennA4. This information is important since it demonstrates that how well a product works may also depend on the particular subject (i.e. cultivar) being treated, rather than the pure efficacy of the compound itself. In conventional fungicide tests, efficacy is more dependent on the toxicity of the product toward fungal pathogens directly, and the environment under which it is being used. For choosing when and which defense activators to apply is also dependent on choice of cultivar. Perhaps some way to encourage responsiveness to defense activators can be found through conventional cultural management.

The field results further enforce the idea that defense activators can show effects that vary by cultivar responsiveness. In one case, a treatment was actually found to increase disease over the inoculated control (L93 treated with Civitas&Harmonizer) while Penncross and colonial bentgrass showed responsiveness to different treatments with a reduction in disease.

The next steps for this project are to continue analysis of the many gigabytes of Next Generation Sequencing data, and to further test these products in the field. Since most of the defense activators in this study are commercially available and registered for use in Canada (Civitas, Harmonizer and Actigard), the technology can be adopted immediately if turf managers choose particular responsive varieties to grow, and select particular resistance activators for use. In conventional fungicide tests, efficacy is more dependent on the toxicity of the product toward fungi directly and the environment under which it is being used. For defense activators, it seems that the condition or inherent genetics of the host plant also play very critical roles that need further investigation. We believe that changes in expression of many plant genes may give insights into other effects that defense activators may be having on the treated plants such as improved drought and heat tolerance as well as improved root growth. These scientific objectives require further study.

Graduate Student	Provide a brief update of the status of any graduate student involved on project.
We used the services of a research assistant for the hands-on work, but the under constant supervision. The work was considered too exploratory for a graduate student thesis (i.e. many	

techniques had to be worked out and the next generation sequencing component was too difficult for a typical M.Sc. student to master in the time frame provided).

Project Communication	List all industry and academic presentations and submitted publications

Hsiang T. 2014. Conventional fungicides vs. resistance activators. Canadian International Turfgrass Conference and Trade Show, Vancouver, B.C., February 18, 2014. (Invited speaker, Industry)

Hsiang T, Goodwin P, Cortes-Barco A and Nash B. 2013. Activating disease resistance in turfgrasses against fungal pathogens. pp. 331-342 in: Imai R, Yoshida M, Matsumoto N (eds). Plant and Microbe Adaptations to Cold in a Changing World. Springer, Berlin. (Refereed Book Chapter)

Hsiang T, Goodwin P, Cortes-Barco A, Nash B. 2013. Activated resistance against turfgrass diseases. PetroCanada talk series: Michigan State University Hancock Research Center (July 31), Rutgers University Turfgrass Research Center (August 6), South Carolina Pee Dee Research Center (Aug,12, , 2013).

Tung J, Goodwin PH, Hsiang T. 2013. Genotype-based variation in induced systemic resistance activated by the volatile organic compound, (2R,3R)-butanediol, in Agrostis species. International Turfgrass Research Conference, Beijing, China. July 15-18, 2013. (Research)

Tung J, Goodwin PH, Hsiang T. 2013. Genotype-based variation in induced systemic resistance activated by the volatile organic compound, (2R,3R)-butanediol, in *Agrostis* species. International Turfgrass Research Journal 12:81-89.

Project Funding Provided By	

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