ENGINEERING DISEASE RESISTANCE TO DOLLAR SPOT IN SEASHORE PASPALUM

by

KATHERINE ELLEN CATCHING

(Under the Direction of Paul L. Raymer and Wayne A. Parrott)

ABSTRACT

Seashore paspalum (*Paspalum vaginatum* Swartz.) is a warm-season turfgrass that is highly susceptible to dollar spot caused by *Clarireedia spp.* Embryogenic callus lines of seashore paspalum were transformed with a wheat germin gene for oxalate oxidase, *gf*-2.8. Transformed lines were then evaluated for gene presence, expression, and disease resistance. Analysis by PCR indicated that breeding lines 17-726 and 17-756 had higher transformation rates than SS13. A colorimetric oxalate oxidase enzyme assay measured differences between lines ability to convert oxalic acid to H_2O_2. Using qRT-PCR, it was determined that lines 1B-11, 1B-13, 1B-15, 1B-17, 1B-19, R2-2BA, 5A-10, 5A-3, 6A-2, 7A-53, and 4-CC overexpressed *gf-2.8*. The studies conducted are not able to conclusively determine if the overexpression of *gf-2.8* in seashore paspalum increases disease resistance. Further screening in the greenhouse as well as the field are recommended with transformed lines that showed upregulation of the gene through qRT-PCR.

INDEX WORDS: Seashore paspalum, dollar spot, turfgrass, *Paspalum vaginatum, Clarireedia spp.*

ENGINEERING DISEASE RESISTANCE TO DOLLAR SPOT IN SEASHORE PASPALUM

by

KATHERINE ELLEN CATCHING

B.S., HUMBOLDT STATE UNIVERSITY, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

ENGINEERING DISEASE RESISTANCE TO DOLLAR SPOT IN SEASHORE PASPALUM

by

KATHERINE ELLEN CATCHING

Major Professors:	Paul L. Raymer
	Wayne A. Parrott
Committee:	David Jespersen
	Alfredo D. Martinez-Espinoza

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
December 2020

DEDICATION

For my siblings; Adam, Sarah, and Leah, my incredibly supportive parents; Ben and Kris and to my amazing partner, Ray. Without all your love and support, I could not have done this. I love you guys all so much.

ACKNOWLEDGEMENTS

This research was supported and funded by The University of Georgia Institute of Plant Breeding, Genetics, and Genomics and the Department of Crop and Soil Sciences. I would like to extend my thanks and gratitude to my colleagues who have helped me through this whole process. This thesis is a result of the work of many individuals and it would not have been possible without the help of everyone involved.

I would like to especially thank my advisors, Dr. Paul Raymer and Dr. Wayne Parrot. Thank you for believing in me and pushing me to be the best I can be. I have gained so much from working with these amazing people and the knowledge I developed with their guidance is priceless.

I would also like to acknowledge my other committee members, Dr. David Jespersen and Dr. Alfredo Martinez-Espinoza. I greatly appreciate the help and support in developing experiments and interpreting results. This project would not be the same without their assistance.

Additionally, I would like to thank everyone at the Griffin and Athens campuses. Especially Mary Flynn and Deborah Franco, who have worked tirelessly to support my work and their guidance through the maze that is paperwork and forms was absolutely essential. Thanks also to Lewayne White, Dr. Zhenbang Chen, and Jihong Xue, all of whom have assisted with various lab work, acquiring materials, and teaching of protocols, I can not express how much I appreciate all your hard work and patience.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Seashore paspalum (*Paspalum vaginatum* Sw.) is a warm-season perennial turfgrass primarily used on golf greens and fairways (Brosnan & Deputy, 2008). It is an important species of turfgrass in that, once established, it is one of the most salt tolerant of turf species (Lee, et al., 2004). This is crucially important as brackish and effluent water are more commonly being used to irrigate golf courses, athletic fields, and other landscapes. Seashore paspalum is a durable grass species that forms a high-quality turf even under low light and water-logged conditions (Brosnan & Deputy, 2008). Additionally, it can withstand a soil pH range of 3.6 to 10.2 (Lee, et al., 2004).

While being affected by only a handful of pathogen species, by far the most common pathogen on *P. vaginatum* Sw. is a fungal disease commonly known as dollar spot. The causal agent of this disease is referenced in most literature as *Sclerotinia homoeocarpa* F.T. Benn. but was recently divided into four new species under the genus *Clarireedia* (Salgado-Salazar, 2018). Two species, *C. jacksonii* and *C. monteithiana*, have been identified in the Georgia (Sapkota et al., 2020) and *C. monteithiana* has been found on warm-season turfgrasses. For golf course managers, dollar spot is the most expensive pathogen to control (Vargas, 1994). In this regard, enhancement of host resistance to dollar spot would be economically beneficial.

Methods used to control dollar spot typically include regularly applying nitrogen fertilizers, contact and systemic fungicides, and manual removal of dew (Vargas, 1994). These practices can be costly and labor intensive, which only adds to the expense of managing seashore

paspalum. These methods are also not completely effective as the pathogen can remain dormant within infected plant tissue and is easily distributed by equipment, people, wind, and water (Smiley et al, 2005). The disease does not produce spores, but can easily spread through mycelium and the movement of infected tissue.

There have also been reported cases of fungicide resistance in dollar spot. The most effective fungicides for control of dollar spot are DMIs, dicarboximides (Vargas, 1994) and succinate dehydrogenase inhibitors (SDHI) (Popko et al., 2018) and it is possible that their overuse has led to resistance in dollar spot (Vargas, 1994). Continual applications of nitrogen fertilizers and fungicides are not an economically or environmentally viable long-term solution. A better solution for the management of dollar spot is needed if the use of *P. vaginatum* as turf species is to continue to spread and grow. Improved host resistance to dollar spot should be the next course of action to ensure that seashore paspalum remains an environmentally viable turfgrass.

Seashore Paspalum

Paspalum vaginatum can be found growing in coastal areas and marshes. It is a warm-season turf species that does well in areas with high salt levels (Lonard, et al., 2015). Most commonly it is used for golf courses and other athletic fields. Due to its evolution in coastal and other high saline areas, seashore paspalum is not affected by as many pests or diseases as other turf species (Carrow and Duncan, 1999). Dollar spot is the exception to this. Previous research indicated that high levels of resistance to dollar spot are not present within the current germplasm of cultivated seashore paspalum lines (Steketee et al., 2017). Since sources of host plant resistance to dollar spot are limited within the existing germplasm, major progress using a

conventional breeding approach is unlikely. One option for improving disease resistance in seashore paspalum is to use biolistic gene transformation to incorporate a foreign defense gene that could potentially enhance resistance to dollar spot.

Studies have been conducted demonstrating that seashore paspalum can be transformed with *Agrobacterium tumefaciens* (Kim, 2009). Biolistic gene transformation using "pest-free" construct could be used in order to reduce regulatory constraints should a product of this research prove to have commercial potential. There are currently no reports of successful transformation in seashore paspalum using biolistics. Other grass species, such as switchgrass and bahiagrass, can be transformed using biolistic gene transformation (Altpeter and James, 2005, King, et al., 2014). A reasonable assumption was to presume that seashore paspalum can be transformed using biolistics as well.

Dollar Spot

The fungal pathogen that causes dollar spot has recently been renamed into four different species, two of which are currently in the United States. *Clarireedia jacksonii* has been found infecting C3 plants while *Clarireedia monteithiana* infects mainly C4 grasses including seashore paspalum (Sapkota et al, 2020). However, before this year, the dollar spot pathogen has been mainly referred to in the literature as *Sclerotinia homoeocarpa*. We will refer to *S. homoeocarpa* when literature uses this taxonomic name. This fungus is a non-sporulating species, and therefore the main sources of inoculum are mycelium and infected plant material. Symptoms of infection on individual leaves start with chlorotic lesions that develop a water-soaked appearance followed by a bleaching of the infected site (Smiley, et al.,1992). Common symptoms in golf turf are

small, sunken patches of bleached leaves. On higher mowed lawns, such as residential lawns, patches may be irregularly shaped and 2-15 cm in diameter (Smiley, et al., 1992). When actively growing, dollar spot mycelia can be visualized growing on and between leaf blades during heavy dews (Smiley, et al., 1992).

The presence of aerial mycelium is often used to diagnose dollar spot. High humidity in the leaf canopy leads to increased fungal growth and in turn increases infection as the aerial mycelium can penetrate the leaf surface and cause infection (Smiley, et al., 1992). The most favorable conditions for dollar spot are temperatures between 15 ºC and 32 ºC (Vargas, 1994) in combination with long periods of high humidity (Walsh, et al., 1999). Dollar spot also survives unfavorable conditions, like dry and cold periods, as mycelia and stromata on leaf surfaces (Smiley, et al., 1992).

Current Methods of Control

Nearly all known warm- and cool-season turfgrasses are susceptible to dollar spot (Burpee, 1997). Methods to control dollar spot include cultural, biological, and chemical (Vargas, 1994). The most effective cultural methods are maintaining high nitrogen levels, high soil moisture, and reducing the presence and duration of foliar moisture (Smiley, et al., 1992, Vargas, 1994). Frequently applying low rates of nitrogen and watering early in the day help to maintain vigorous growth and reduce dollar spot's ability to colonize leaf tissue. Foliar moisture from guttation water is rich in carbohydrates and amino acids and provides nutrients for the fungus to use, leading to increased fungal growth. Therefore, removing this water when possible can make the disease more manageable.

Biological control methods are not as commonly used as chemical controls, but applications of *Pseudomonas aureofaciens* has been shown to manage dollar spot. Applying concentrations of *P. aureofaciens* Tx-1 at 5.8 x 10^8 colony forming units (CFU) · mL^{-1}, was shown to reduce dollar spot when disease pressure is low (Hardebeck et al., 2004), but is not a practical method of disease suppression when disease pressure is high.

Chemical controls are by far the most common method of controlling dollar spot. Both contact and systemic fungicides are effective controls and can provide broad suppression of the disease. Systemic fungicides are effective, but increase the probability of resistant strains developing. Resistance has been identified in dollar spot to benzimidazoles, demethylation-inhibitors, and dicarboximides (Jo, et al., 2006). Dollar spot resistance to thiophanate has been known for many years now (Vargas, 1994). Because of the increased potential for fungicide resistance found in dollar spot strains, the need for genetic resistance has never been more important. There are currently no cultivars of seashore paspalum that have complete resistance to dollar spot (Steketee, et al., 2017).

Invasive Mechanism of Clarireedia monteithiana

The life cycle of dollar spot is characterized by the lack of spore production. It spreads by mycelium and stroma located both within plant tissues and on external surfaces (Smiley et al., 2005). Recent studies suggest that grass seeds may also be a source of inoculum (Rioux et al., 2014). The fungus survives plant dormancy within infected plant tissues and is spread through the movement of those tissues, either by human or animal traffic. When favorable conditions return, the dormant mycelia or stromae recolonize the tissue they are in (Smiley et al., 2005).

Mycelium may also survive in the leaf canopy when the humidity and temperature levels are favorable.

The method by which dollar spot infects plants is multifaceted and involves many molecular compounds. The compound that has been the focus of the most research is oxalic acid and is produced by many plant pathogens (Dutton and Evans, 1996). There is abundant evidence suggesting that oxalic acid is an important indicator of pathogen virulence, as infected plant tissues contain high levels of oxalic acid (Dutton and Evans, 1996). Plant lines that show increased fungal resistance also have plasma membranes that are more resistant to oxalic acid compared to those of susceptible lines (Dutton and Evans, 1996). The role that oxalic acid plays in plant pathogenicity was studied using fungal mutants that could not produce oxalic acid and comparing their virulence against that of wild-type lines. In Godoy et al. (1990), the mutant lines of *Sclerotinia sclertiorum* deficient in oxalic acid were nonpathogenic and could not replicate the same symptoms as the wild-type lines.

Optimization of Polygalacturonase Activity

Oxalic acid's role in pathogen virulence starts as a decrease in the pH, which favors fungal extracellular enzymes such as polygalacturonase (Bateman & Beer, 1965). Polygalacturonase is one of a class of pectolytic enzymes that a pathogen produces to destroy cells (Agrios, 2005). Oxalic acid increases the concentration of H^+ ions that depolarizes the cell membrane, inhibits the formation of lignin and pectin crosslinking, and together with the removal of calcium, leads to the disruption of the plasma membrane and eventual tissue maceration (Dutton and Evans, 1996; Lane, 1994). Bateman and Beer (1965) investigated the

influence of polygalacturonase and oxalic acid independently and as a combined mixture. Their findings proposed that lowering the pH of the host tissue optimizes polygalacturonase activity (Bateman and Beer, 1965). Later studies confirmed that the acidification associated with oxalic acid accumulation allows for increased polygalacturonase activity (Punja, et al., 1985).

Induction of Host Oxidative Burst

One of the earliest, observable forms of plant pathogen defenses is the oxidative burst, which is a rapid production of reactive oxygen species (ROS) (Wojtaszek, 1997). The oxidative burst response can be triggered by bacteria, fungi, viruses as well as cultured cells exposed to pathogen fragments and mechanical stress (Wojtaszek, 1997). It typically occurs at the plant cell surface and involves pre-exisiting components, not downstream biosynthetic cellular machinery (Wojtaszek, 1997). The oxidative burst is typically a primary response to pathogen attack and plays a role in secondary defense responses, including the hypersensitive response (HR) (Wojtaszek, 1997). The primary forms of ROS produced by oxidative burst are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxide ions (OH^-) (Lamb & Dixon, 1997).

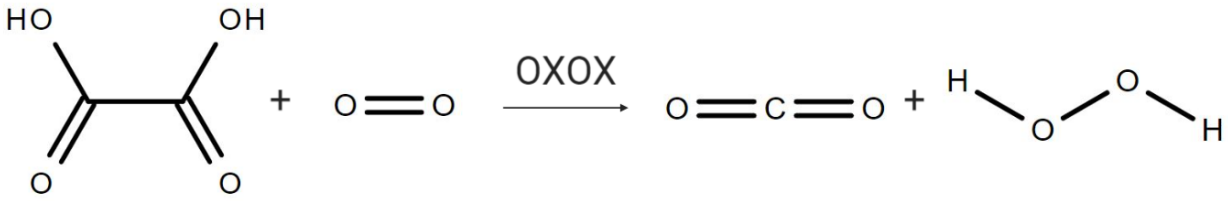
Programmed Cell Death

High concentrations of H_2O_2 lead to the hypersensitive response and cell death (Levine, et al., 1994). After the initiation of the oxidative burst, increased concentrations of ROS play many roles in secondary plant defenses. H_2O_2 has been shown to fortify cell walls by inducing cross-linking of cell wall components (Bradley, et al., 1992, Brisson, et al., 1994). It also

functions to signal adjacent cells that there is a pathogen present (Agrios, 2005). Most importantly, H_2O_2 triggers cell death, which acts to isolate the pathogen infection and prevent its spread to other cells (Levine, et al., 1994). In the case of attack by *C. monteithiana*, which is a necrotrophic fungus, the cell death response fosters increased invasion and growth by the pathogen (Prasad and Shivay, 2017).

Developing Effective C. monteithiana Resistance

Oxalate oxidase is a naturally occuring enzyme that oxidizes oxalic acid into hydrogen peroxide and carbon dioxide.



Equation 1. Oxalic acid plus dioxygen react with oxalate oxidase to form carbon dioxide and hydrogen peroxide. (Created with BioRender.com)

One of the most studied oxalate oxidases to date are germins. Germins are unique developmentally regulated proteins first found during germination of cereals and show remarkable resistance to broad specificity proteases (Patnaik & Khurana, 2001). In wheat, a germin gene known as gf-2.8, has oxalate oxidase activity (Lane et al., 1993). This germin functions in developing embryos to release secondary messengers in the development process (Lane, 1994). Oxalate oxidase is localized around the cell wall, which would be beneficial during pathogen attack, as that is the initial site of infection (Dumas et al., 1995). Lane proposed that this gene could be used to enhance fungal resistance in transgenic plants.

Hydrogen Peroxide Pitfall

While the incorporation of oxalate oxidase to combat fungal attack is intriguing, there are potential limitations. The gf-2.8 gene has been transformed into other species, including soybean (Donaldson et al., 2001), barley (Dumas et al., 1995), poplar (Liang et al., 2001), peanut (Livingstone et al., 2005), tomato (Walz et al., 2008), and canola (Dong et al., 2008). In all cases, the studies show successful integration and expression of *gf-2.8*. However, although these reports do show increased resistance, complete resistance was not observed. The pathogens studied produce oxalic acid, as does *Clarireedia monteithiana.*, and have similar modes of infection. While it may be possible to increase resistance to *Clarireedia monteithiana* in seashore paspalum by overexpressing oxalate oxidase with *gf-2.8*, it remains unlikley that this will result in complete resistance.

Adhereing to Regulatory Normative of Genetic Modification

In 2010, the Scotts Company and Monsanto (now Bayer) submitted an "Am I Regulated" letter of inquiry to the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) for non-regulated status of their genetically engineered (GE) Kentucky bluegrass engineered with a vector free of plant pest derived DNA for glyphosate resistance. APHIS released a statement in 2011 responding that, without posing a plant pest risk, Scotts GE Kentucky bluegrass cannot be regulated by Title 7, Part 340 of the U.S. code of Federal Regulations (Parham, 2011). This case pointed out that APHIS's authority to regulate genetically modified organisms relied on whether the organism contains pest-derived genes. Since the statements release, Scotts has also developed and commeralized St. Augustinegrass

(*Stenotaphrum secundatum* Walt.) cultivars using non-plant-pest-derived contructs. However, changes to these regulations have been added that would elimate this exemption for transgenics developed without pest-derived constructs.

Summary

Dollar spot is a costly and difficult pathogen to manage (Vargas, 1994) and oxalic acid is an important indicator of pathogen virulence. Increased expression of oxalate oxidase in seashore paspalum could improve control of this pathogen. Studies have shown that the wheat germin *gf-2.8* has oxalate oxidase activity (Lane, et al., 1993) and is an effective method for heightening resistance to similar pathogens (Donaldson, et al., 2001). Oxalate oxidase functions to convert oxalic acid secreted by the pathogen into hydrogen peroxide, a reactive oxygen species involved in plant defenses (Lane, 1994, Levine, et al., 1994). Reducing dollar spots ability to trigger cell death could improve disease resistance.

The use of biolistic gene transfer as the method of gene incorporation has been demonstrated in other grass species (Altpeter et al., 2005; King, et al., 2014). With these considerations in mind, research was undertaken to use biolistic transformation with a pest-free construct containing the wheat germin *gf-2.8* gene in an attempt to overexpress oxalate oxidase and thereby improve resistance to dollar spot in seashore paspalum.

Literature Cited

Agrios, G.N. 2005. Plant Pathology. 5th ed. St. Louis, MO. *Academic Press*. pp. 992.

Altpeter, F., Varshney, A., Abderhalden, O., Douchkov, D., Sautter, C., Kumlehn, J., Dudler, R., & Schweizer, P. (2005). Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. *Plant Mol. Biol.*, 57(2), 271-283.

Bateman, D. F., & Beer, S. V. (1965). Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii. Phytopathology,* 55(11), 204-11.

Bradley, D. J., Kjellbom, P., & Lamb, C. J. (1992). Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*, 70(1), 21-30.

Brisson, L. F., Tenhaken, R., & Lamb, C. (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *The Plant Cell*, *6*(12), 1703-1712.

Brosnan, J. T., & Deputy, J. (2008). Seashore Paspalum: Turf Management. *TM-1, Mãnoa, Hawaii: University of Hawaii at Mãnoa, 8p*.

Burpee, L. L. (1997). Control of dollar spot of creeping bentgrass caused by an isolate of *Sclerotinia homoeocarpa* resistant to benzimidazole and demethylation-inhibitor fungicides. *Plant disease*, *81*(11), 1259-1263.

Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L., & Simmonds, D. H. (2001). Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen Sclerotina sclerotiorum. *Physiological and Molecular Plant Pathology*, 59(6), 297-307.

Dong, X., Ji, R., Guo, X., Foster, S. J., Chen, H., Dong, C., Liu, Y., Hu, Q., & Liu, S. (2008). Expressing a gene encoding wheat oxalate oxidase enhances resistance to Sclerotinia sclerotiorum in oilseed rape (*Brassica napus*). *Planta*, 228(2), 331.

Dumas, B., Freyssinet, G., & Pallett, K. E. (1995). Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. *Plant Physiology*, 107(4), 1091-1096.

Duncan, R. R., & Carrow, R. N. (2000). Seashore paspalum: The environmental turfgrass. John Wiley & Sons.

Dutton, M. V., & Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Canadian Journal of Microbiology*, 42(9), 881-895.

Godoy, G., Steadman, J. R., Dickman, M. B., & Dam, R. (1990). Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiological and molecular plant pathology*, 37(3), 179-191.

Hardebeck, G. A., Turco, R. F., Latin, R., & Reicher, Z. J. (2004). Application of *Pseudomonas aureofaciens* Tx-1 through irrigation for control of dollar spot and brown patch on fairway-height turf. *Hort. Sci.,39*(7), 1750-1753.

Jo, Y. K., Niver, A. L., Rimelspach, J. W., & Boehm, M. J. (2006). Fungicide sensitivity of Sclerotinia homoeocarpa from golf courses in Ohio. *Plant Disease*, 90(6), 807-813.

Kim, K. M., Song, I. J., Lee, H. Y., Raymer, P., Kim, B. S., & Kim, W. (2009). Development of seashore paspalum turfgrass with herbicide resistance. *Korean Journal of Crop Science*, 54(4), 427-432.

King, Z. R., Bray, A. L., LaFayette, P. R., & Parrott, W. A. (2014). Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.). *Plant cell reports*, 33(2), 313-322.

Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual review of plant biology*, 48(1), 251-275.

Lane, B. G. (1994). Oxalate, germin, and the extracellular matrix of higher plants. *The FASEB Journal*, *8*(3), 294-301.

Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., & Cuming, A. C. (1993). Germin, a protein marker of early plant development, is an oxalate oxidase. *Journal of Biological Chemistry*, 268(17), 12239-12242.

Lee, G., Duncan, R. R., & Carrow, R. N. (2004). Salinity tolerance of seashore paspalum ecotypes: Shoot growth responses and criteria. *Hort. Sci.*, 39(5), 1138-1142.

Levine, A., Tenhaken, R., Dixon, R., & Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, 79(4), 583-593.

Liang, H., Maynard, C. A., Allen, R. D., & Powell, W. A. (2001). Increased *Septoria musiva* resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant molecular biology*, 45(6), 619-629.

Livingstone, D. M., Hampton, J. L., Phipps, P. M., & Grabau, E. A. (2005). Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. *Plant Physiology*, 137(4), 1354-1362.

Lonard, R. I., Judd, F. W., & Stalter, R. (2015). Biological Flora of Coastal Dunes and Wetlands: *Paspalum vaginatum* Sw. *Journal of Coastal Research*, 31(1), 213-223.

Parham, G. L. (2011). Scotts Miracle-Gro Co.; Regulatory Status of Kentucky Bluegrass Genetically Engineered for Herbicide Tolerance. 76 FR 39812.

Patnaik, D. & Khurana, P. (2001). Germins and germin like proteins: an overview. *NISCAIR-CSIR*, India

Popko Jr, J. T., Sang, H., Lee, J., Yamada, T., Hoshino, Y., & Jung, G. (2018). Resistance of *Sclerotinia homoeocarpa* field isolates to succinate dehydrogenase inhibitor fungicides. *Plant disease*, 102(12), 2625-2631.

Prasad, R., & Shivay, Y. S. (2017). Oxalic acid/oxalates in plants: from self-defence to phytoremediation. *Curr Sci*, 112(8), 110-112.

Punja, Z. K. (1985). The biology, ecology, and control of *Sclerotium rolfsii*. *Annual review of Phytopathology*, 23(1), 97-127.

Rioux, R. A., Shultz, J., Garcia, M., Willis, D. K., Casler, M., Bonos, S., Smith, D., & Kerns, J. (2014). *Sclerotinia homoeocarpa* overwinters in turfgrass and is present in commercial seed. *PLoS One*, 9(10), e110897.

Salgado-Salazar, C., Beirn, L. A., Ismaiel, A., Boehm, M. J., Carbone, I., Putman, A. I., Tredway, L., Clarke, B., & Crouch, J. A. (2018). *Clarireedia*: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. *Fungal biology*, 122(8), 761-773.

Sapkota, S., Martinez-Espinoza, A., Ali, E., Vermeer, B. C., & Bahri, B. A. (2020). Taxonomical identification of *Clarireedia* species causing dollar spot disease of turfgrass in Georgia. *Plant Disease*.

Smiley, R. W., Dernoeden, P. H., & Clarke, B. B. (2005). Compendium of turfgrass diseases (Vol. 1). St. Paul, MN: *APS press*.

Steketee, C. J., Martinez-Espinoza, A. D., Harris-Shultz, K. R., Henry, G. M., & Raymer, P. L. (2017). Evaluation of Seashore Paspalum Germplasm for Resistance to Dollar Spot. *International Turfgrass Society Research Journal*, 13(1), 175-184.

Vargas, J. (1994). Management of Turfgrass Diseases. Boca Raton: *CRC Press*, https://doi.org/10.1201/9780203748374

Walsh, B., Ikeda, S. S., & Boland, G. J. (1999). Biology and management of dollar spot (*Sclerotinia homoeocarpa*); an important disease of turfgrass. *Hort. Sci.*, 34(1), 13-21.

Walz, A., Zingen-Sell, I., Loeffler, M., & Sauer, M. (2008). Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Plant Pathology*, 57(3), 453-458.

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochemical Journal*, 322(3), 681-692.

CHAPTER 2

BIOLISTIC TRANSFORMATION OF SEASHORE PASPALUM (*PASPALUM VAGINATUM* SWARTZ.)[1]

[1] Katherine E. Catching, David Jespersen, Alfredo D. Martinez-Espinoza, Wayne A. Parrott, and Paul L. Raymer. To be submitted to *Crop Science*.

Abstract

Seashore paspalum (*Paspalum vaginatum*) is a warm-season turfgrass with many desirable traits. It shows high susceptibility to dollar spot (*Clarireedia spp.*) with limited host resistance. This study aimed to develop seashore paspalum lines that showed overexpression of a wheat germin gene, *gf-2.8*. This was accomplished by the biolistic transformation of embryogenic callus lines with two vector constructs. One construct had the cauliflower mosaic virus 35s promoter and the other had the *Zea mays* ubiquitin promoter. Both constructs contained the *hph* gene that provided hygromycin resistance and no significant difference in callus growth was seen at concentrations 100, 150, 200, 250, 300, and 350 mg L^{-1}. Bombarded callus of all genotypes was selected at 150 mg L^{-1}. Selected callus was then regenerated and transformation efficiencies were determined by confirming gene integration through PCR. Significant genotypic differences ($P = 0.0136$) in transformation efficiency were observed with breeding lines 17-726 and 17-756 producing higher transformation rates than SS13.

Introduction

Seashore paspalum is an important turfgrass species that is highly susceptible to dollar spot. It was proposed that overexpressing an enzyme for oxalate oxidase would increase disease resistance by breaking down oxalic acid produced by the pathogen. Several other plant species have demonstrated increased fungal resistance through similar enzyme activity (Dong et al., 2008; Dumas et al., 1995; Liang et al., 2001; Livingstone et al., 2005; Walz et al., 2008; Yang, et al., 2019). This study aimed to accomplish this by utilizing particle bombardment to introduce the wheat germin gene *gf-2.8* that is an oxalate oxidase into seashore paspalum. To accomplish this, a cell line was developed *in vitro* to produce embryogenic callus for bombardment. The low

frequency of successful transformations from particle bombardment requires that conditions are optimized for plant regeneration to ensure maximum recovery of positive transformation events.

Callus Induction of Seashore Paspalum

One of the first reports of seashore paspalum grown *in vitro* was in 1997 by Cardona and Duncan. Most prominently, immature inflorescences were used to induce callus. In their study, Duncan and Cardona investigated the effect of plant hormones on callus formation and regeneration. Different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (BAP) were tested on nine ecotypes of seashore paspalum; PI 509021, HI-1, Mauna Key, Adalayd, PI 299042, K-7, SIPV-1, K-3, and AP-6. Using a base of Murashige and Skoog basal salts (Murashige and Skoog, 1962), 1 mg L^{-1} 1000 x Gamborg B5 vitamins (Gamborg et al., 1976), and 30 g L^{-1} sucrose; while different combinations of 2,4-D and BAP were used (Cardona & Duncan, 1997). No differences in callus production per explant were found between BAP and BAP plus 2,4-D treatments in any of the ecotypes tested with the exception of Adalayd where callus production increased with the addition of 2,4-D. Their findings showed that the auxin concentration of the media greatly impacts callus formation, while cytokinin concentration had a lesser effect.

A similar study was conducted by Neibaur et al., 2008 that examined 2,4-D and BAP effects as well as the effect of Dicamba. Their findings showed that the combination of 3 mg L^{-1} dicamba and 1 mg L^{-1} BAP resulted in plant regeneration frequency 12 times higher for the cultivar ‘SeaIsle 1’, compared to 3 mg L^{-1} 2,4-D alone.

Biolistic transformation

Genetic transformation is accomplished by one of two major methods: bacterial transformation or particle bombardment. Biolistic transformation is a versatile method that can be applied to many species (Altpeter & James, 2005; King et al., 2014; Vain, McMullen, & Finer, 1993). The main difficulty with particle bombardment is obtaining the right tissue type for bombardment. The construct with the gene of interest is adhered to small particles, generally gold or tungsten, and then shot into callus tissue. The workflow for biolistic transformation is shown in Fig. 2-1.

The use of particle bombardment as the method of transformation was chosen for the primary reason that it may be easier to obtain regulatory approval for commercial release if successful. Very little work has been done using biolistic transformation on seashore paspalum. The only report is a dissertation by Neibaur in 2008. It reports a protocol for embryogenic callus induction and a transformation protocol. Stable transformation was confirmed by expression of the *E. coli uidA* gene encoding β–glucuronidase (GUS) (Jefferson, 1987).

Materials and Methods

Plant Material

Embryogenic callus was cultured following the methods described in King et al. (2014) with some modifications. The plant materials used for transformation were mature seeds of 'SeaSpray' (Fricker et al. 2007), germinated in culture and two breeding lines containing sethoxydim resistance, 17-756 and 17-726.

Seed Germination

Mature seeds from 'SeaSpray' were soaked in 0.2% KNO_3^- for 72 h to break dormancy (Shim et al., 2008) and then washed once with 70% ethanol and then 3% sodium hypochlorite and shaken at 250 rpm for thirty minutes. Seeds were then rinsed three times with sterile DI H_2O and allowed to dry on sterile filter paper in a laminar flow hood. Individual seeds were then plated in a 5x5 grid on MSO-B5 lite medium, wrapped with Micropore tape (Microporous Medical Tape Adhesive, D&H Medical, Lot #20181126) and placed in growth room with 23-hr light at 26 °C for two weeks. Germinated seeds were then moved to MSO-B5 rooting medium (Table 2-1) for two to three weeks. Plants that produced three or more strong stems were considered mature enough to collect tissue for callus induction.

Callus Induction

Tissue explants were taken from mature plants and placed on MS-D5-B1 medium (Table 2-1) to induce callus formation. Cultured tissue came from nodal explants which centered at the node of a stem and included approximately 0.5 cm of tissue above and below the node (Fig. 2-2). Explant tissue was incubated at 27 °C in the dark and subcultured at 3-week intervals to induce the growth of embryogenic callus. Embryogenic callus, as described by Praveena & Giri (2012) and Zhang et al. (2007), is friable, opaque yellow, and amenable to biolistic transformation.

Immature inflorescences were used to produce callus for the 17-726 and 17-756 lines. They were removed before emergence and the two racemes were separated. Inflorescences were then surface sterilized with 0.0006% sodium hypochlorite and 0.02% Tween-80 for 10 minutes before rinsing with autoclaved water (Heckart et al., 2010).

Plasmid prep

The coding region of the wheat germin *gf-2.*8 gene was inserted into a binary plasmid that contained either the CaMV35s or ZmUbi promoter (Green et al., 2002) and a catalase terminator from rice (*Oryza sativa*) (Kawahara et al., 2013). The *hph* selection gene was inserted with the *Panicum virgatum* ubiquitin promoter *PvUbi2* (Mann et al., 2011; Fig. 2-3). Both constructs are identical with the exception of the promoter used for the wheat germin *gf*-2.8 gene.

Using NEB 10-beta competent *E. coli* (New England Biolabs, cat. No. C3019), plasmids containing either the CaMV35s or ZmUbi promoter were grown and isolated following the protocol outlined by Epoch Life Sciences for the EconoSpin® All-In-One Plasmid Mini Prep (Epoch Life Science Inc, cat No. NC0789236) and the extracted plasmid was stored at -80 ºC.

Particle Bombardment Parameters for Transformation

Particle bombardment protocols were taken from King et al. (2014) and Trick et al. (1997), with some modifications. Calli were subcultured six hours before bombardment onto MSOsm-DC5-B1 medium (Table 2-1), containing 46.5 g L^{-1} mannitol, 46.5 g L^{-1}, and 5 mg L^{-1} Dicamba, and incubated in the dark at 27 °C. Embryogenic calli were oriented facing upward in a 2.5 cm bulls-eye, being sure that no media or other callus blocked it. Plates were shot with one construct at a time with 650 psi rupture disks, vacuum at 97 kPa (27 in.) Hg, and a 9 cm microcarrier flight distance. After bombardment, plates were allowed to rest in the dark at 27 °C for 18 hours. Shot callus was then transferred to MS-D5-B1 (Table 2-1) medium for 10 days under the same conditions.

Hygromycin Selection of Bombarded Callus

Each genotype was tested for optimum hygromycin concentration with non-bombarded callus. Protocols were developed using Joyce et al. (2010), Visarada & Sarma (2004), and Fakhrana et al. (2019). Sterile 20 mm x 100 mm Petri plates were filled with MS-D5-B1 medium supplemented with filter sterilized hygromycin B at 100, 150, 200, 250, 300, and 350 mg L^{-1}. Hygromycin was added after the medium was autoclaved and allowed to cool for 20 minutes. Each concentration had three replicate plates with 25 pieces of callus in a 5x5 grid pattern and mass was recorded every three days for a month (Fig. 2-4). Shot callus was subcultured onto selection media containing 150 mg·L^{-1} of hygromycin B and transformed callus was determined after 4 weeks. Shoot formation was induced by sub-culturing living tissue onto regeneration medium, MS-B5-N1 (Table 2-1).

Plant Regeneration and Acclimation

After two weeks on regeneration medium, plants with shoots approximately 3 cm tall were subcultured to rooting medium (MSO-B5, Table 2-1) to induce root formation and replaced in the growth chamber for one to two weeks. Plants with three or more roots were then moved to soil in Uline Crystal Clear Plastic Cups with lids (Uline, cat. No. S-22276, S-22281) to increase humidity. Plants were kept at 26 ºC and 11 h/13 h light/dark at 680 µmol·m^{-2}·s^{-1} for a week. After acclimation, plants were moved to the greenhouse. Greenhouse conditions were kept between 20-32 ºC and relative humidity ranged from 50% at night and up to100% during the day.

Confirmation of Gene Presence

Polymerase chain reaction (PCR) and agarose gel electrophoresis were used to determine incorporation of the shot vector. DNA extraction protocol was adapted from Borges et al., 2009. Fresh tissue samples were placed in 500 µL of 2% CTAB (cetyltrimethylammonium bromide) and 2-mercaptoethanol solution before homogenization with a bead mill. Samples were then incubated at 65 ºC for 45 minutes and 500 µL of 24:1 chloroform to isopropanol were added. Next samples were briefly vortexed and centrifuged for 15 minutes at 4000 rpm. Three-hundred µL of the supernatant was removed and 210 µL of ice-cold isopropanol were added with gentle mixing. Then samples were incubated on ice for 15 minutes before centrifuging again for 15 minutes at 4000 rpm. The supernatant was then discarded and the pellet allowed to dry for 30 minutes. Extracted DNA was resuspended in 50 µL of DNase-free water and DNA concentration was quantified using a NanoPhotometer (Implen, Westlake Village, CA, USA). PCR was run using Promega GoTaq® Master Mix following the recommended protocol.

Primer Design

Primers were designed using Primer3Plus (https://primer3plus.com/) to bind at the junction of the promoter and *gf-2.8* gene (Fig. 2-3). A primer set was designed for both promoters and made through Eurofins (https://www.eurofins.com/). Samples of the vector were used with the primers as a positive control in PCR and agarose gels. The sequences used are listed in Table 2-2.

Results

Seed Germination Rates

Though the propagation of seashore paspalum through stolons is straightforward, seed germination is more difficult. Mature seeds underwent several steps of washing to reduce the risk of contamination. In this study, 1,631 seeds were started *in vitro* and 198 germinated. This resulted in a germination rate of 12% and of those plants, seven produced embryogenic callus.

Hygromycin Selection

A standard dose response was determined from three replicate plates of callus on five concentrations of hygromycin; 100, 150, 200, 250, 300 and 350 $mg \cdot L^{-1}$. Each plate was weighed every three days for a month and the mass recorded. The analysis of variance showed no significant difference in mass lost over time between concentrations ($P = 0.9192$). There was a significant difference in mass lost between days ($P = 0.0031$).

Transformation Efficiency

The efficiency of each genotype was evaluated for successfully transformed plants. Efficiency was determined from the number of calli that underwent particle bombardment that survived selection, regenerated, and tested positive for transformation through PCR (Fig. 2-5). Genotype significantly affected transformation efficiency with 17-726 and 17-756 both having higher transformation rates than SS13 (Fig. 2-6).

Discussion

Both germinated seeds and immature inflorescences are commonly used to produce callus in culture. In the case of this study, both methods were used with varying degrees of success. Explant tissue from immature inflorescences typically have a higher rate of embryogenic callus induction. However, it is rare for plants to produce an inflorescence in sterile culture (P. Raymer, personal communication) so they must come from outside sources i.e. the greenhouse or field, and are subject to contamination. Sufficiently washing these inflorescences can be problematic as they are delicate and subject to breakage.

However, germinated seeds pose different problems. While it is easier to disinfest seeds, the germination rate in culture was observed to be 12% which requires a high number of seeds and not all seashore paspalum varieties reliably produce seeds. Additionally, the time from germination to a mature plant can take several weeks and, in that time, only a few nodal explants can be taken at a time. This greatly reduces the amount of material that can go into callus induction.

Previous work has focused on callus induction of seashore paspalum with 2,4-D and found that the percent efficiency was between 12-51% for 6 $mg \cdot L^{-1}$ (Cardona & Duncan, 1997). It was found that after 21 days 2,4-D alone induced callus in 54.4% of inflorescences and dicamba induced callus in 42.3% of inflorescences (Neibaur et al., 2008). However, a direct comparison of 2,4-D to dicamba at this rate was not made and this area could benefit from more investigation. It may be prudent to determine the optimal conditions for embryogenic callus induction for seashore paspalum in culture as well as the regeneration rates and number of shoots produced. Determining the most efficient method of embryogenic callus induction would make the transformation of seashore paspalum more efficient.

After transforming embryogenic callus, the next step is to separate successful events from untransformed callus. A well-known method to do this is to move shot callus to medium with a selectable agent that will kill untransformed tissue. In the constructs used in this study, a gene for hygromycin resistance was added for this purpose.

Hygromycin B is a very well-known selectable marker and is able to reliably select for successful transformation events. Most plants show a low tolerance and are easily selected. Oil palm is selected at 20 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$ is used for wheat (Raja et al., 2010 & Fakhrana et al., 2019). Seashore paspalum has a comparatively higher tolerance. Wu et al., (2018) reported that selection and regeneration rate was not affected at rates below 100 $mg \cdot L^{-1}$ but was greatly affected at 300-400 $mg \cdot L^{-1}$. This was determined from observing 20 embryogenic calli on MS 2.5 medium for 5 weeks. It follows that transformed callus in this study were selected at rates of 150 $mg \cdot L^{-1}$. The mass lost from arrested growth of callus is shown in Figure 2-7 at different concentrations.

The next objective of this study was to determine the integration of the vector cassette in successfully transformed plants which was accomplished by utilizing polymerase chain reactions (PCR). While PCR is a relatively simple process, it is a very powerful tool and provided an efficient way to screen hundreds of plants for successful transformation events. It is important to note that although the calli were screened with hygromycin, not all the plants that survived were transformed. There are multiple reasons for this, such as low selection pressure with the concentration of hygromycin in the media or clumping of the callus which allowed untransformed calli to survive off of transformed calli. No matter what the case, all plants that came out of culture were tested with PCR to confirm a successful transformation event.

Genotype did play a significant role in transformation rate, as SS13 only produced three successfully transformed plants. This could likely be attributed to the difficulty in obtaining viable embryogenic callus from this line. Both 17-726 and 17-756 produced embryogenic callus readily and resulted in more potential transformation events. The SS13 line was germinated in culture and explants all came from the original plant.

Literature Cited

Altpeter, F., & James, V. A. (2005). Genetic transformation of turf-type bahiagrass (*Paspalum notatum* Flugge) by biolistic gene transfer. *Int Turfgrass Soc Res J*, 10, 485-489.

Borges, A., Rosa, M. S., Recchia, G. H., Queiroz-Silva, J. R. D., Bressan, E. D. A., & Veasey, E. A. (2009). CTAB methods for DNA extraction of sweetpotato for microsatellite analysis. *Scientia Agricola*, *66*(4), 529-534.

Cardona, C. A., & Duncan, R. R. (1997). Callus induction and high efficiency plant regeneration via somatic embryogenesis in Paspalum. *Crop science*, *37*(4), 1297-1302.

Cardona, C. A., and R. R. Duncan. "Callus induction and high efficiency plant regeneration via somatic embryogenesis in Paspalum." *Crop Sci.* 37.4 (1997): 1297-1302.

Dong, X., Ji, R., Guo, X., Foster, S. J., Chen, H., Dong, C., ... & Liu, S. (2008). Expressing a gene encoding wheat oxalate oxidase enhances resistance to Sclerotinia sclerotiorum in oilseed rape (Brassica napus). *Planta*, *228*(2), 331.

Dumas, B., Freyssinet, G., & Pallett, K. E. (1995). Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. *Plant Physiology*, *107*(4), 1091-1096.

Fakhrana, I. N., Nurfahisza, A. R., Rasid, O. A., Parveez, G. K. A. 2019. Minimal inhibitory concentration of hygromycin for selecting transformed oil palm embryogenic calli. *Journal of Oil Palm Research* 31: 14-27. doi:https://doi.org/10.21894/jopr.2018.0063.

Fricker, C., Wipff, J. K., & Duncan, R. R. (2007). *U.S. Patent No. 7,262,341*. Washington, DC: U.S. Patent and Trademark Office.

Gamborg, O. L., Murashige, T., Thorpe, T. A., & Vasil, I. K. (1976). Plant tissue culture media. *In vitro*, *12*(7), 473-478.

Green, J., Vain, P., Fearnehough, M. T., Worland, B., Snape, J. W., & Atkinson, H. J. (2002). Analysis of the expression patterns of the *Arabidopsis thaliana* tubulin-1 and *Zea mays* ubiquitin-1 promoters in rice plants in association with nematode infection. *Physiological and molecular plant pathology*, *60*(4), 197-205.

Heckart, D. L., Parrott, W. A., & Raymer, P. L. (2010). Obtaining sethoxydim resistance in seashore paspalum. *Crop science*, 50(6), 2632-2640.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant molecular biology reporter*, *5*(4), 387-405.

Joyce, P., Kuwahata, M., Turner, N., & Lakshmanan, P. (2010). Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. *Plant cell reports*, 29(2), 173-183.

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin. H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa., H, Itoh, T., Buell, C. R., & Matsumoto, T. (2013). Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. *Rice*, *6*(1), 4.

King, Z. R., Bray, A. L., LaFayette, P. R., & Parrott, W. A. (2014). Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.). *Plant Cell Reports*, 33(2), 313-322.

Liang, H., Maynard, C. A., Allen, R. D., & Powell, W. A. (2001). Increased Septoria musiva resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant molecular biology*, *45*(6), 619-629.

Livingstone, D. M., Hampton, J. L., Phipps, P. M., & Grabau, E. A. (2005). Enhancing resistance to Sclerotinia minor in peanut by expressing a barley oxalate oxidase gene. *Plant Physiology*, *137*(4), 1354-1362.

Mann, D. G., King, Z. R., Liu, W., Joyce, B. L., Percifield, R. J., Hawkins, J. S., LaFayette, P. R., Artelt, B. J., Burris, J. N., Mazarei, M., Bennetzen, J. L., Parrott, W. A., & Stewart, C. N. (2011). Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. *BMC biotechnology*, 11(1), 74.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15(3), 473-497.

Neibaur, I., Gallo, M., & Altpeter, F. (2008). The effect of auxin type and cytokinin concentration on callus induction and plant regeneration frequency from immature inflorescence segments of seashore paspalum (*Paspalum vaginatum* Swartz). *In Vitro Cellular & Developmental Biology-Plant*, 44(6), 480.

Praveena, M., & Giri, C. C. (2012). Plant regeneration from immature inflorescence derived callus cultures of salt tolerant kallar grass (*Leptochloa fusca* L.). *Physiology and Molecular Biology of Plants*, 18(4), 345-356.

Raja, N. I., Bano, A., Rashid, H., Chaudhry, Z., & Ilyas, N. (2010). Improving *Agrobacterium*-mediated transformation protocol for integration of XA21 gene in wheat (*Triticum aestivum* L.). *Pak J Bot*, 42(5), 3613-3631.

Shim, S. I., Moon, J. C., Jang, C. S., Raymer, P., & Kim, W. (2008). Effect of potassium nitrate priming on seed germination of seashore paspalum. *Hortic. Sci.*, *43*(7), 2259-2262.

Steketee, C. J., Martinez-Espinoza, A. D., Harris-Shultz, K. R., Henry, G. M., & Raymer, P. L. (2016). Effects of genotype and isolate on expression of dollar spot in seashore paspalum. *Hortic. Sci.*, 51(1), 67-73.

Trick, H. N., Dinkin, R. D., Santarem, E. R., Di, R., Samoylov, V., Meurer, C. A., Walker, D. R., Parrott, W. A., Finer, J. J., & Collins, G. B. (1997). Recent advances in soybean transformation. *Plant Tissue Cult. Biotech*., 3, 9-26.

Vain, P., McMullen, M. D., & Finer, J. J. (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports*, 12(2), 84-88.

Visarada, K. B. R. S., & Sarma, N. P. (2004). Transformation of Indica rice through particle bombardment: factors influencing transient expression and selection. *Biologia plantarum*, 48(1), 25-31.

Walz, A., Zingen-Sell, I., Loeffler, M., & Sauer, M. (2008). Expression of an oxalate oxidase gene in tomato and severity of disease caused by Botrytis cinerea and Sclerotinia sclerotiorum. *Plant Pathology*, *57*(3), 453-458.

Wu, X., Shi, H., Chen, X., Liu, Y., & Guo, Z. (2018). Establishment of *Agrobacterium*-mediated transformation of seashore paspalum (*Paspalum vaginatum* O. Swartz). *In Vitro Cellular & Developmental Biology-Plant*, 54(5), 545-552.

Yang, X., Yang, J., Wang, Y., He, H., Niu, L., Guo, D., ... & Li, Q. (2019). Enhanced resistance to sclerotinia stem rot in transgenic soybean that overexpresses a wheat oxalate oxidase. *Transgenic research*, *28*(1), 103-114.

Zhang CX, Li Q, Kong L (2007) Induction, development and maturation of somatic embryos in Bunge's pine (*Pinus bungeana* Zucc. ex Endl.). *Plant Cell Tissue Organ Cult.* 91:273–280.

Table 2-1. Tissue Culture Media Used in Development of Paspalum Cell Lines.

Nutrients	MSO-B5 lite	MSO-B5 rooting	MS-D5-B1	MSOsm-DC5-B1	MS-B5-N1
Macronutrients			$mg \cdot ml^{-1}$		
NH_4NO_3	1.65	1.65	1.65	1.65	1.65
KNO_3	1.9	1.9	1.9	1.9	1.9
$MgSO_4 \cdot 7H_2O$	0.37	0.37	0.37	0.37	0.37
KH_2PO_4	0.17	0.17	0.17	0.17	0.17
$CaCl_2 \cdot 2H_2O$	0.44	0.44	0.44	0.44	0.44
$Na_2EDTA \cdot 2H_2O$	0.0372	0.0372	0.0372	0.0372	0.0372
$FeSO_4 \cdot 7H_2O$	0.0278	0.0278	0.0278	0.0278	0.0278
Micronutrients			$\mu g \cdot ml^{-1}$		
H_3BO_3	3.1	3.1	3.1	3.1	3.1
$MnSO_4 \cdot H_2O$	8.45	8.45	8.45	8.45	8.45
$ZnSO_4 \cdot 7H_2O$	4.3	4.3	4.3	4.3	4.3
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.0125	0.0125	0.0125	0.0125	0.0125
$CoCl_2 \cdot 6H_2O$	0.0125	0.0125	0.0125	0.0125	0.0125
KI	0.415	0.415	0.415	0.415	0.415
Vitamins			$\mu g \cdot ml^{-1}$		
Thiamine·HCl	5.0	5.0	5.0	5.0	5.0
Nicotinic Acid	0.5	0.5	0.5	0.5	0.5
Pyridoxine·HCl	0.5	0.5	0.5	0.5	0.5
Myo-Inositol	50	50	50	50	50
Carbohydrate			$g \cdot L^{-1}$		
Sucrose	15	30	30	30	30
Mannitol				46.5	
Sorbitol				46.5	
Plant hormone			$\mu g \cdot ml^{-1}$		
2,4-D			2.0		
BAP			0.045		0.1
Dicamba			5		
NAA					1
Gelrite	$2\ g \cdot L^{-1}$	$2\ g \cdot L^{-1}$	$2\ g \cdot L^{-1}$	$2\ g \cdot L^{-1}$	$2\ g \cdot L^{-1}$

Table 2-2. PCR Primer Sequences for Transgene Confirmation

Primer Symbol	Vector Name	5'-Primer Sequence (Forward/Reverse)-3'	Amplicon Length (bp)
Z1	pZmUbi-TaOXOH	TTTAGCCCTGCCTTCATACG/GCCGGAGCTAGTAACAGCAT	153
Z2	pZmUbi-TaOXOH	TTTAGCCCTGCCTTCATACG/AAGTCCTGGAGAGGGTCTGG	189
Z3	pZmUbi-TaOXOH	GATGCTCACCCTGTTGTTTG/GCCAACTTGGACGAGAAGAG	239
Cam1	p35s-TaOXOH	CGCACAATCCCACTATCCTT/GCCGGAGCTAGTAACAGCAT	175
Cam2	p35s-TaOXOH	TTCGCAAGACCCTTCCTCTA/GCCGGAGCTAGTAACAGCAT	157
Cam3	p35s-TaOXOH	CGCACAATCCCACTATCCTT/AAGTCCTGGAGAGGGTCTGG	211

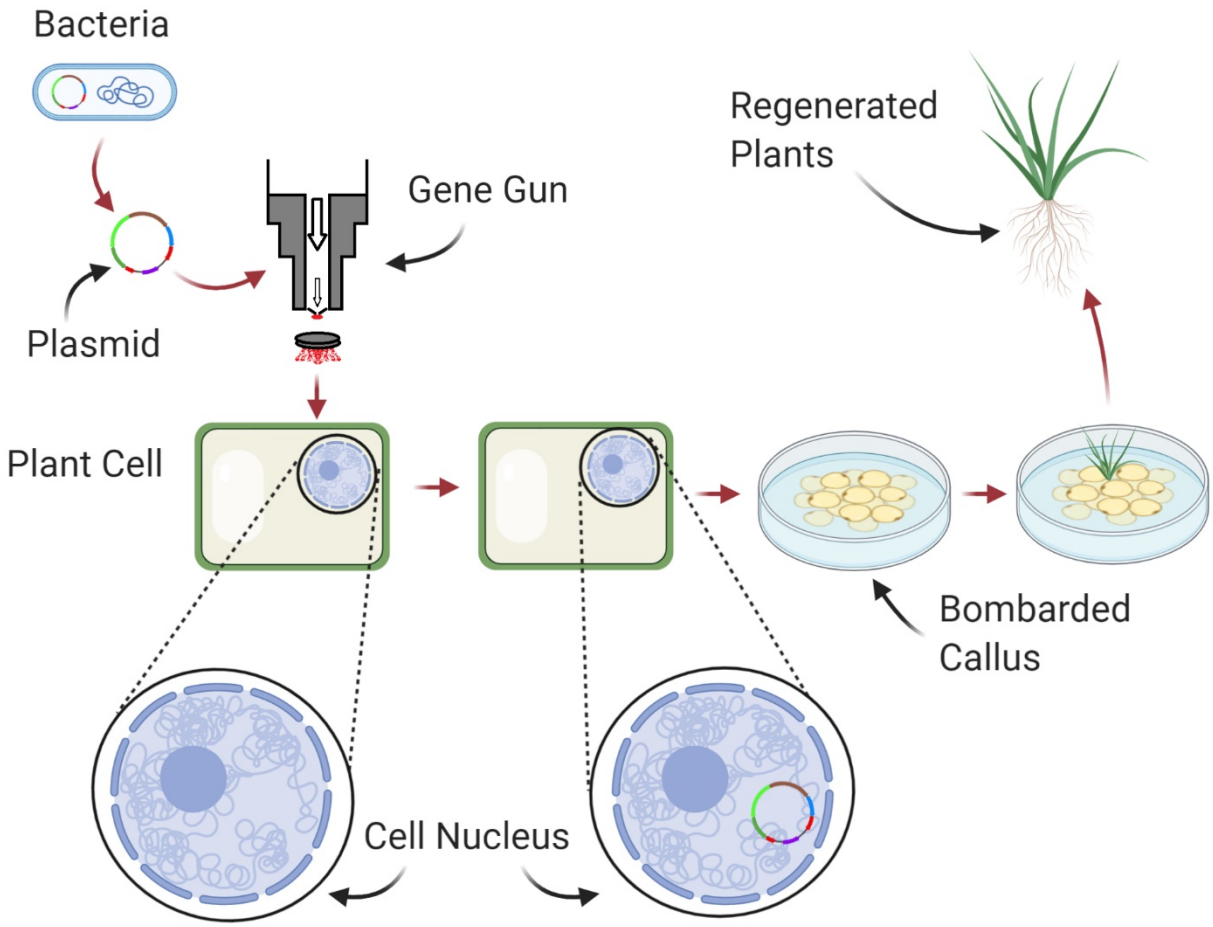


Figure 2-1. Biolistic Transformation Pipeline. The gene of interest was inserted into bacterial cells and bulked before being isolated and prepped for particle bombardment. The isolated vector was then adhered to gold microparticles and shot into embryogenic plant cells. Cultured cells were then allowed to rest for 10 days before being moved to selection material. Callus that survived selection was then moved to regeneration medium and transformed plantlets were moved to soil after one to two weeks. (Created with BioRender.com)

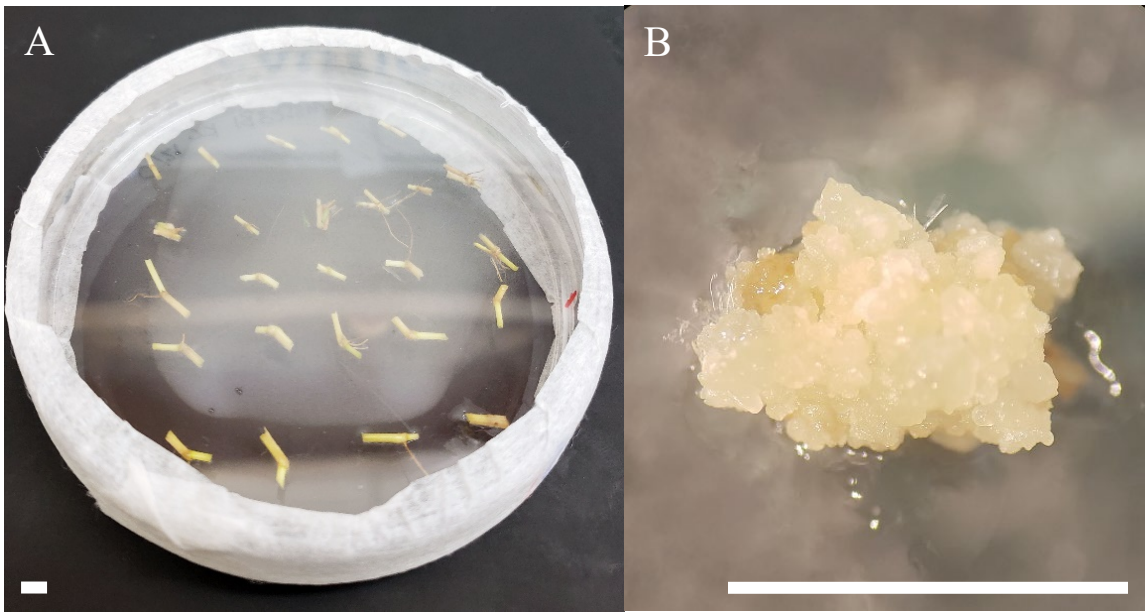


Figure 2-2. Seashore paspalum tissue culture pipeline. (A) ~1 cm cut stem segments on induction medium to induce callus formation from the nodes. (B) Type II embryogenic callus derived from stem segments on induction medium. Each measurement bar represents 5 mm.

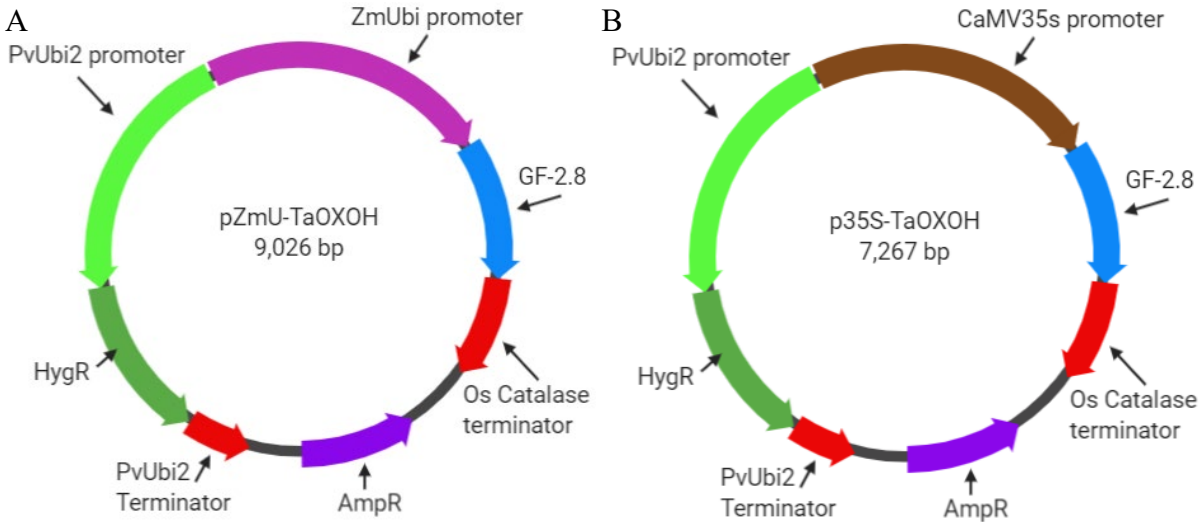


Figure 2-3. Construct diagram. Both constructs are identical except for the promoter for the wheat germin gf-2.8 gene. The *hph* gene functions as a selectable marker and induces hygromycin resistance with the *Panicum virginatum* Ubiquitin 2 promoter. A) pZMU-TaOXOH vector with the *Zea mays* ubiquitin promoter for the wheat germin *gf*-2.8 gene and *Oryza sativa* catalase terminator. B) p35s-TaOXOH vector with the cauliflower mosaic virus 35s promoter for the wheat germin *gf-2.8* gene and *Oryza sativa* catalase terminator. (Created with BioRender.com)

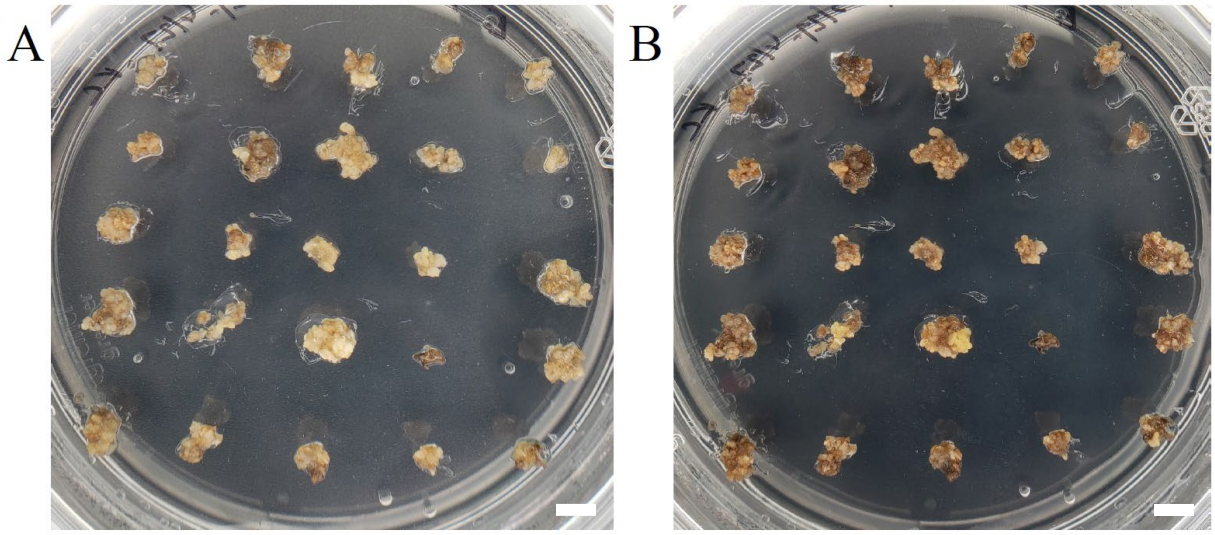


Figure 2-4. Bombarded Callus on Hygromycin Selection Medium. A) Bombarded callus arranged in a five by five grid on agarose medium with 150 $mg \cdot L^{-1}$ 10 days after bombardment. B) Bombarded callus on hygromycin selection medium with 150 $mg \cdot L^{-1}$ hygromycin B 30 days after bombardment. Surviving callus remains yellow and is resistant to hygromycin. Each measurement bar represents 5 mm.

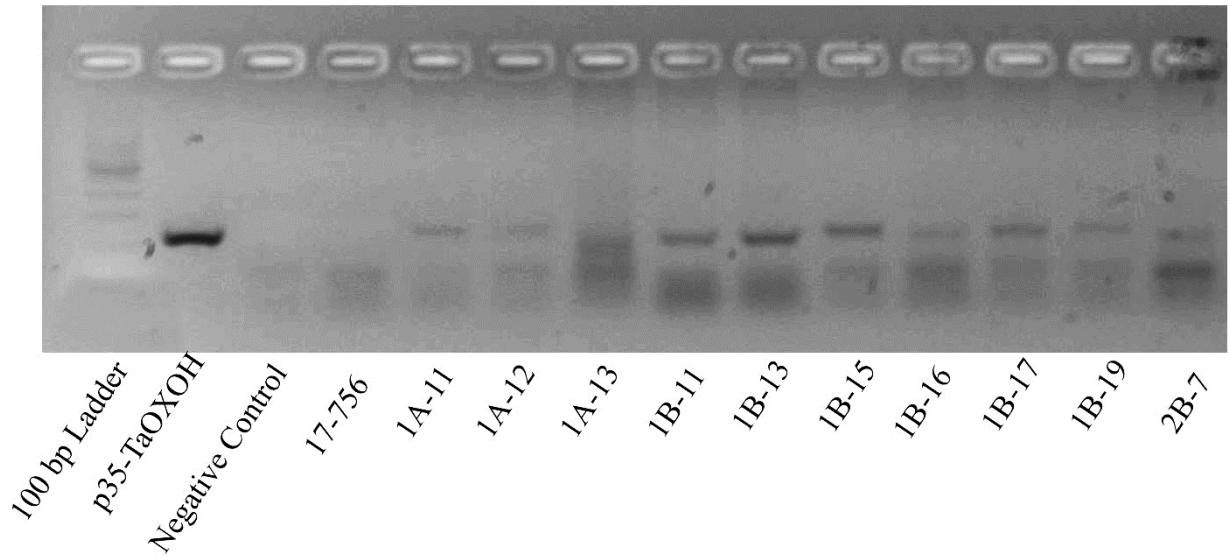


Figure 2-5. PCR Results. Agarose gel electrophoresis of PCR product.

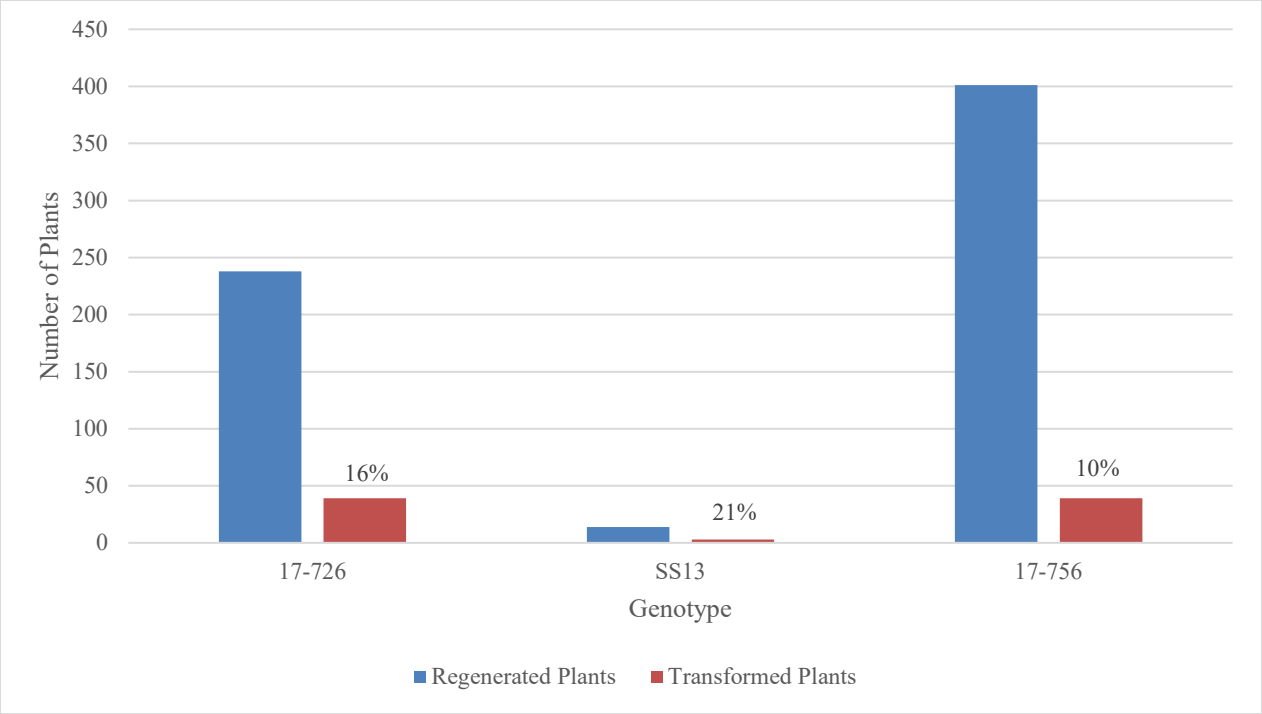


Figure 2-6. Transformation Efficiencies by Genotype. Statistical analysis was performed on all genotypes. Plants regenerated from culture were tested with PCR for gene presence. The effect of genotype had a significant impact on the total number of regenerated plants and the transformation efficiency (P = 0.0136.)

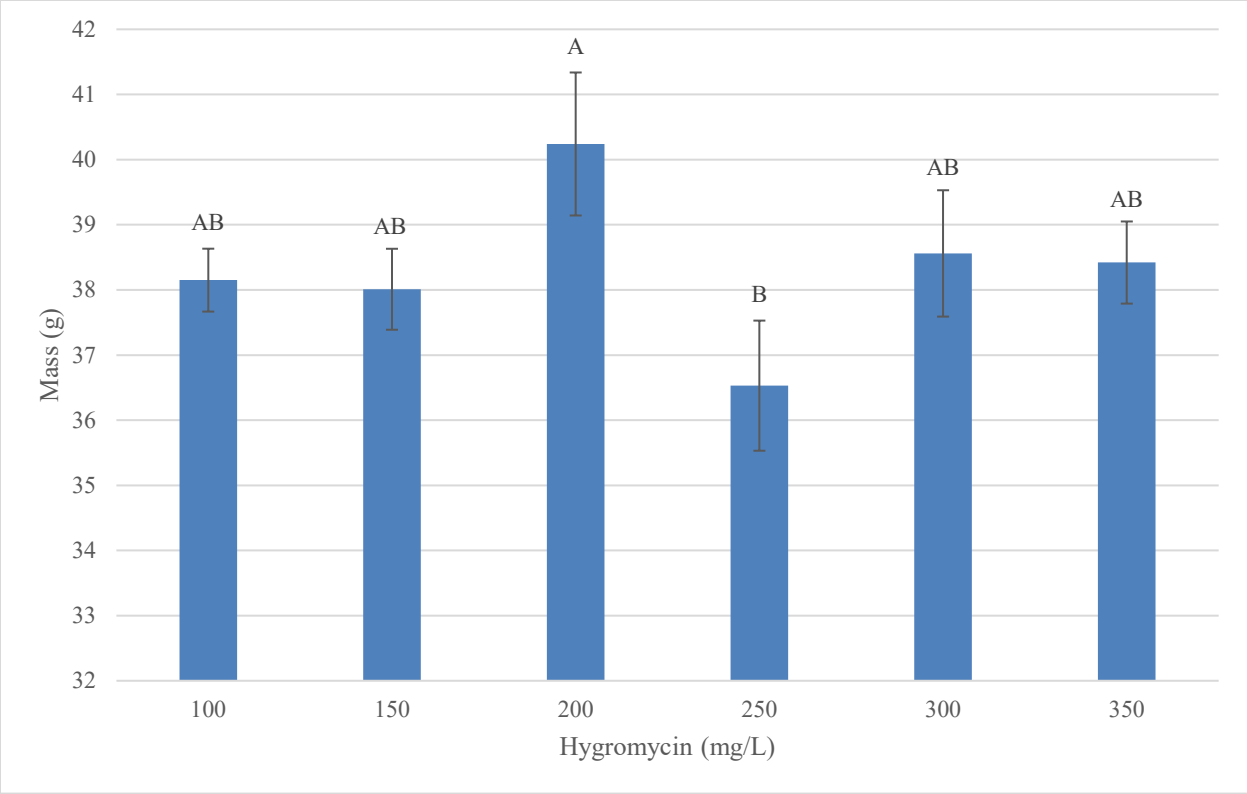


Figure 2-7. Change in Mass in Response to Hygromycin Concentration. The difference in mass of callus lost at different hygromycin concentrations was not statistically significant ($P = 0.9192$). The greatest difference between day 1 and day 30 was seen at 100 mg·L^{-1} and callus was selected at 150 mg·L^{-1}. Bars with the same letter are significantly different.

CHAPTER 3

CONFIRMATION OF GENE INTEGRATION AND EXPRESSION IN BIOLISTICALLY TRANSFORMED SEASHORE PASPALUM[2]

[2] Katherine E. Catching, David Jespersen, Alfredo D. Martinez-Espinoza, Wayne A. Parrott, and Paul L. Raymer. To be submitted to *Crop Science*.

Abstract

Seashore paspalum is a warm-season turfgrass species that is often used on golf courses and putting greens. While it has many desirable traits as a turfgrass, it also is highly susceptible to dollar spot, caused by *Clarireedia spp.* (Salgado-Salazar et al., 2018). It was proposed that by overexpressing an oxalate oxidase enzyme, seashore paspalum may have increased disease resistance. After using particle bombardment to transform embryogenic cell lines with the wheat germin gene *gf-2.8*, transformed plants were evaluated for gene integration. In a colorimetric oxalate oxidase enzyme assay, transformed plants derived from SeaSpray and 17-756 showed significant differences from their parental lines. Lines R2-1B, R2-2BA, and 4-CC had higher enzyme levels than the parental controls. Lines derived from 17-756 and 17-726 with the p35s-TaOXOH cassette did not show significant increases from their respective parent lines (*P*-values of 0.2508 and 0.8019, respectively). Additionally, a secondary screen of leaf tips on hygromycin-supplemented medium confirmed that all transformed lines expressed hygromycin resistance indicating an intact and functional cassette. Using qRT-PCR, the increase in gene expression was quantified and showed thirteen lines overexpressing *gf-2.8*. The successfully transformed lines were evaluated for disease resistance through a detached leaf assay. The inoculum used for the detached leaf was quantified and it was found that the pH and biomass were not affected by the number of agar plugs used as the source of inoculum. Due to difficulties in phenotyping whole plant expression of disease resistance, it is still unclear if overexpressing oxalate oxidase increases disease resistance to dollar spot in seashore paspalum.

Introduction

Seashore paspalum is a warm-season turfgrass that is commonly used on golf courses. While its native range is coastal, its growing popularity has expanded its range into areas with higher disease pressure. Current breeding lines of seashore paspalum show susceptible to moderately resistant responses to dollar spot, a fungal pathogen caused by *Clarireedia spp.* (Salgado-Salazar et al., 2018). This study aimed to increase disease resistance by overexpressing a wheat germin oxalate oxidase gene, *gf-2.8* through biolistic transformation. The oxalate oxidase enzyme catalyzes the breakdown of oxalic acid and conversion to carbon dioxide and hydrogen peroxide (Lane et al., 1993; Liang, et al., 2001). The pathogen uses oxalic acid to reduce the pH of the intercellular space and create an optimal environment for pectolytic enzymes to degrade the cell wall (Agrios, 2005; Dutton and Evans, 1996).

The previous chapter reported that potentially transformed calli coming out of the transformation pipeline were initially tested for gene integration through PCR to confirm presence of the construct. Additional tests to confirm successful transformation and function of the construct are typically conducted following novel trait transformation. Using agarose-based media with varying concentrations of hygromycin, leaves can be assayed for hygromycin resistance (Wang and Waterhouse, 1997). Leaf tips are inserted into the media and discoloration of leaf tissue is measured after 3 days. Untransformed control leaves show bleaching while leaves from transformed plants remain green due to the expression of the *hph* gene in the vector cassette for resistance to hygromycin. If the leaves remain green, then it is likely that the transformed plant contains the intact vector and is expressing the *hph* gene.

The oxalate oxidase enzyme has been well studied and its properties characterized. The colorimetric enzyme assay was developed from previous work done on the oxalate oxidase

enzyme found in barley. The chemical reaction used to visualize enzyme activity was described in a study that extracted and purified oxalate oxidase from barley seedlings (Sugiura et al., 1979). The reaction is catalyzed by two enzymes; the extracted oxalate oxidase and a peroxidase that is added to the developing solution. The optimal pH range for oxalate oxidase is 3.5 (Sugiura et al., 1979) and for horseradish peroxidase is 6.0 to 6.5 (Schomburg and Salzmann, 1991).

The assay is based on the chemical reaction between the oxalic acid in the solution and the oxalate oxidase enzyme in the transformed plants. The initial buffer exposes oxalate oxidase to oxalic acid and provides an acidic environment for optimal enzyme activity to convert the oxalic acid to hydrogen peroxide. Next, the hydrogen peroxide is oxidatively coupled with 4-aminoantipyrine and *N,N*-dimethylaniline by the horseradish peroxidase (Hu et al., 2009). This results in the formation of chromogen which is purple and can be measured spectrophotometrically. The study by Liu measured the absorbance of this reaction every half hour for 3 hours and showed no difference after thirty minutes. The study focused on the breakdown of the oxalate ion by a known concentration of purified oxalate oxidase, but the reaction can also be measured with a known amount of oxalate to estimate oxalate oxidase activity.

The final confirmation of gene expression was conducted with quantitative reverse-transcriptase PCR (qRT-PCR). PCR amplifies sections of DNA by binding primers to strands of DNA and amplifying them with *Taq* polymerase. The primers are designed specifically to bind to the section of interest in the DNA sample. RT-PCR uses the same basic principle with the exception that it begins with RNA extracted from the sample which is then converted to cDNA by reverse transcriptase. Next primers bind to the specific site in the cDNA and it is replicated through the same process as traditional PCR. The method used in this study is referred to as

qRT-PCR because it allows for the quantification of the product. Using the ΔΔCt method, the fold increase in gene expression can be determined by comparing a reference gene to the gene of interest and also by comparing the transformed sample to the untransformed parent line.

Phenotyping disease resistance in turfgrass still poses many issues. The primary ways of evaluating disease resistance in turfgrass include in vitro detached leaf assays, greenhouse/growth chamber screens, and field trials. While field trials provide the most comprehensive information on resistance, they take several years and a significant amount of work to create enough plant material, establish field trails, and conduct disease assessment experiments. Greenhouse or growth chamber screens could offer a more efficient method of assessing disease response, but there are several issues associated with disease screening in a greenhouse/growth chamber environment. There are many variables such as temperature, relative humidity, and light intensity that have not been optimized for disease screening and the conditions necessary to induce fungal growth without overwhelming the plants are still unclear. A clear understanding of the pathogen-host plant interaction and the environmental conditions necessary for disease expression is key in clearly defining disease resistance assay protocols.

Inherited disease resistance to dollar spot has been evaluated for some turfgrass species such as tall fescue (*Festuca arundinacea* Schreb.), creeping bentgrass (*Agrostis stolonifera* L.), and seashore paspalum (*Paspalum vaginatum* Swartz) with the bulk of the work being done on creeping bentgrass (Brede, 1991; Bonos et al., 2003; Steketee, 2014). In all cases, turfgrass cultivars show variation in susceptibility. Specifically, for seashore paspalum, while some cultivars show less susceptibility, high levels of resistance to dollar spot have not been identified (Steketee, 2014).

Determining dollar spot resistance has been a subject of intense study in recent years. Field trials have been used to evaluate resistance to artificial inoculation under natural conditions. Isolates of dollar spot grown on sterilized Kentucky bluegrass seeds were used to inoculate field plots of creeping bentgrass by a drop spreader at a rate of 1.75 g m^{-2} (Bonos et al., 2003). Visual ratings were made two weeks after inoculation and plants were rated every week during the growing season on a scale of 1-9. A similar method was used to inoculate field plots of seashore paspalum. Dollar spot isolates were grown on a grain mixture of wheat, barley, and oat seed and spread evenly by hand onto the field plots (Steketee, et al., 2016). Visual ratings were made in addition to digital image analysis to evaluate disease response. Images were captured using a Canon PowerShot G6 digital camera (Canon USA, Melville, NY) and a light box to provide consistent lighting. Images were processed using Assess version 2.2 (American Phytopathological Society, St. Paul, MN. Using this method with the visual ratings, disease progress curves were constructed for dollar spot on seashore paspalum.

Greenhouse inoculation and disease ratings were conducted with isolates of *Clarireedia monteithiana* and *Clarireedia jacksonii* on bermudagrass (*Cynodon dactylon* x *transvaalensis* 'Riviera') and creeping bentgrass (*Agrostis palustris* 'Penncross') (Aynardi et al., 2019). Isolates were grown on PDA and used to infest flasks of rye grain (*Secale cereale* L.). Two grains of each isolate were used to inoculate 11.4 cm square pots of bermudagrass and bentgrass and polyethylene bags were used to cover the plants in promote high humidity. Disease ratings were made seven days after inoculation. Additional work is still needed to increase the accuracy and efficiency of greenhouse disease screens and to develop methods for screening seashore paspalum. After confirming transgene presence, the main objectives of this study were to evaluate gene expression and enzyme activity.

Materials and Methods

Plant Material

The plants tested were derived from three genotypes referred to as 17-726, 17-756, and SeaSpray. From these genotypes, transformed plants were created that had one of the two vectors used and resulting plants contained the *gf-2.8* gene with either the CaMV35s promoter or the *Zea mays* Ubiquitin promoter. The line 17-726 produces four transgenic lines: 5A-3, 5A-10, 6A-2, and 7A-53, all with the CaMV35s promoter. Line 17-756 produced five lines: 1B-11, 1B-13, 1B-15, 1B-17, and 1B-19, with the CaMV35s promoter and one line, 4-CC, with the ZmUbi promoter. SeaSpray produced two transformed lines, R2-1B and R2-2BA both with the CaMV35s promoter.

Hygromycin Whole Plant Expression

Transformed lines were checked for expression of the hygromycin marker through an assay developed by Wang and Waterhouse (1997). The only changes to the protocol were the conditions under which the plates incubated. All plates were kept at 25 ^{o}C in a 11 h/13h light/dark regime. The medium consisted of Murashige and Skoog salts (Murashige and Skoog, 1962), 0.5 mg$\cdot$L^{-1} 6-benzylaminopurine, and 2.5 g$\cdot$L^{-1} agarose and 200 mg$\cdot$L^{-1} hygromycin. All transformed lines and their respective untransformed parents were checked using four replicate plates containing five leaves each. Leaf tips were inserted into the medium and discoloration measured by digital image analysis three days later.

Oxalate Oxidase Colorimetric Assay

A colorimetric enzyme assay was used to measure enzyme expression in transformed events. The protocol used was modified from previous work by Livingstone et al., 2005, Sugiura et al., 1979, Donaldson et al., 2001, Lane et al., 1993, and Zhang et al., 1995. Fresh leaf tissue samples, 10 mg per replicate, were taken from confirmed transformants and their untransformed parents and placed in 500 µL of incubation buffer. The incubation buffer consisted of 2mM oxalic acid in 50 mM succinate buffer. Tissue samples were ground in a bead mill homogenizer (Bead Ruptor 12, Omni International, 19-050A) for 20 seconds at 5 $m \cdot s^{-1}$ and then centrifuged at 4,000 rpm for 10 seconds. Next, 500 µL of development buffer were added and samples were left to develop at room temperature for 20 minutes. The development buffer consisted of 8 mg of 4-aminoantipyrene, 20 µL *N,N*-dimethylaniline, 1.5 mg horseradish peroxidase in a 0.1 M Tris buffer pH 7.0 . After development, samples were centrifuged at 4,000 rpm for 5 minutes and then read in a UV spectrophotometer at 550 nm. Four replicates were tested for each plant and absorbance was recorded for each.

A standard curve was constructed from serial dilutions of incubation buffer with added H_2O_2. Solutions with 9.765, 19.53, 39, 78, 156.25, and 312.5 µM of H_2O_2 were measured spectrophotometrically and their absorbances plots (Fig. 3-3). The resulting trendline had an equation of $y = 397.25x - 2.6305$, which was used to determine H_2O_2 generated from oxalate oxidase activity for transformed and untransformed samples.

qRT-PCR Gene Expression

Gene expression was analyzed by extracting RNA using the PureLink® RNA Mini Kit (ThermoFisher, Cat.12183020) in conjunction with TRIzol™ Plus RNA Purification Kit

(Invitrogen, Cat. 12183555) and following the protocols outlined for these reagents. Fresh tissue samples were used for RNA extraction and then converted to cDNA with ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich, Cat. RDRT). The cDNA was then run with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Cat. A25742). Each sample was run with three technical replicates and gene expression was quantified using the ΔΔCt method (Pfaffl, 2006). The expression of two genes were measured; the inserted gene *gf-2.8* and a F-box/kelch-repeat protein (Liu et al., 2017) which served as a reference for stable gene expression. For each transformed line the expression level for the F-box/kelch-repeat and *gf-2.*8 oxalate oxidase gene were measured and compared to the expression of both genes in that line's parent using the primers listed in Table 3-2.

Inoculum Quantification

Clarireedia monteithiana was grown on petri plates with potato dextrose agar (PDA) and transferred every 3-4 days to obtain pure and fresh cultures. Agar plugs were made using a sterilized 0.5 cm cork borer to cut agar plugs from the outer ring of actively growing mycelium for transfer to the center of the new plate. After 4 days, plugs were taken from the outer ring of growth and placed in 100 mL of autoclaved potato dextrose broth (PDB). Liquid cultures were placed on an orbital shaker and stirred at 100 rpm at room temperature for 7 days. Cultures were then homogenized in a standard blender and pH was recorded. Samples of 1 ml were taken of the homogenized culture and optical density was spectrophotometrically measured at 550 nm. Filtrate from unhomogenized samples was obtained through vacuum filtration through autoclaved and pre-weighed Whatman filter paper (Whatman 1001-055 Grade 1 Qualitative Filter Paper Standard Grade, Cytiva, 09-805D) and weighed to determine wet weight. Samples

were then dried in an oven at 50 ºC for 24 hours and then weighed to determine dry weight of mycelia mass.

Detached Leaf Assay

A detached leaf assay was developed to screen for disease resistance with liquid inoculum following the protocol outlined by Chang, 2011 with some modification. The protocol was developed using two breeding lines; SeaIsle 1 and SeaStar, that varied in their susceptibility to dollar spot. One of the main objectives was to determine a standard volume and concentration of inoculum that displayed the differences in disease expression. Too much inoculum would overwhelm the most resistance cultivars but too little would show no difference between the lines. Before running this experiment, the plants to be tested were trimmed and allowed to grow in the greenhouse with no fertilization for two weeks. The leaves were collected and immediately placed in tap water. They were then transferred to a 3% sodium hypochlorite solution and washed for 10 minutes. Then they underwent three rinses in tap water and a final rinse in sterile DI water with 0.01% Tween-20. Under sterile conditions, leaves were cut to 3 cm and placed in Petri dishes (Nunc® petri dishes, Nunc, P7741-1CS) on sterile Whatman filter paper with 2 ml of autoclaved DI water. Each plate contained five leaves and for each volume tested there were six plates; three controls with only uninoculated PDB and three treated plates with inoculum.

The amount of inoculum to be used in the assay was determined by testing volumes of 0, 10, 20, 40, and 80 µL of liquid inoculum per leaf. Three replicates of each volume were run for both cultivars and the plates were sealed and placed in a growth chamber at 26 ºC, 11 h light/ 13 h dark for four days. Then the images of the leaves were recorded and analyzed for damage through Assess (Version 2.0; Lamari, 2008). From this data, 50 µL of homogenized liquid

inoculum was selected as the most appropriate inoculum dosage to maximize differences in disease response between the two cultivars (Fig 3-1).

Following this determination, ten transformed lines and three parent lines; 17-756, 17-726, and SeaSpray, were examined. For all lines, six plates were used consisting of three controls with 50 µL uninoculated PDB and three plates with 50 µL of homogenized inoculum per leaf. Each plate contained five leaves of approximately the same size with either the treatment or control liquid pipetted onto the center of the leaf. Percent disease was calculated using the software Assess 2.0 (American Phytopathological Society, St. Paul, MN). The total area of the leaf was determined and the percent of that area which showed disease was calculated.

Digital Image Analysis

Images were taken with a Canon Powershot G9x Mark II (Canon USA, Melville, NY) in JPEG (joint photographic experts group, or ".jpg") format. Images were then processed using Assess version 2.0 from APS Press (American Phytopathological Society, St. Paul, MN). In leaf mode, the hue threshold was set to 31-255 and in lesion mode the threshold was set to 31-98. These thresholds were determined by visual confirmation of leaf and lesion area. A calculation of percent injury or percent disease was determined from total lesion pixels/total pixels.

Statistical Analysis

Analysis of variance was determined from least squares means in JMP (Sall, 2018) with a significance at $\alpha = 0.05$. Student's t-test was used to determine statistical differences between means.

Results

Whole Plant Hygromycin Screen

The ten transformed plants and three parental lines were screened for hygromycin resistance using four replicates of five leaf tips each. Analysis showed that the transformed lines had higher hygromycin resistance than their respective parents SeaSpray, 17-756, and 17-726 (P = <.0001, <0.0001, and <0.0001, respectively). Lines R2-2BA, R2-1B, and 5A-3 had very little injury with means of 0.23, 0.04, and 0.05% respectively (Fig. 3-2).

Colorimetric Enzyme Assay

Transformed plants were tested in comparison to their untransformed parent lines (Fig. 3-4). The two transformed lines derived from SeaSpray; R2-1B and R2-2BA, had higher levels of H_2O_2 generated (241.94 µM and 216.25 µM) indicating higher oxalate oxidase activity. The six lines from 17-756 with the p35s-TaOXOH promoter had H_2O_2 levels ranging from 39.68 µM to 55.63 µM and were not different from 17-756 (P=0.2508). Line 4-CC generated 107.51 µM of H_2O_2 which was higher than the parent line, 17-756. The four lines from 17-726 with p35s-TaOXOH all had higher absorbances than 17-726.

Quantitative Gene Expression

By using qRT-PCR and the F-box gene expression as a reference for stable gene expression and the corresponding parental lines as the baseline for oxalate oxidase expression, the relative expression of *gf-2.8* in transformed plants was quantified. Using the ΔΔCt method (Table 3-2), the level of expression was determined as a fold change compared to each parent

line. Oxalate oxidase expression in line R2-1B from SeaSpray did not show an increase in expression (0.93) while R2-2BA, also from SeaSpray, showed increased expression by 1.67 times that of the parent (Table 3-3). Lines from 17-756 with p35s-TaOXOH all showed increased expression of *gf-2.8*, with fold increases ranging from 1.16 to 6.27-fold. Line 4-CC from 17-756 increased by 3.72-fold over its parent. The four lines from 17-726 with p35s-TaOXOH had the highest increase in expression with values ranging from 27.48 to 109,427-fold greater than the parent (Fig. 3-5).

Inoculum Quantification

In order to develop a protocol to screen for disease resistance, the source of inoculum was standardized. The biomass of fungal colonies grown for ten days in PDB was determined in relation to the number of 0.5 cm plugs of agar and inoculum added. There was not a difference between the number of plugs added and the dry weight of mycelium produced. It was determined that adding four plugs of inoculum laden agar to 100 mL of PDB created an inoculum concentration of 15.19 ± 0.06 g·L^{-1} after 10 days.

Detached Leaf

The detached leaf assay was first performed on two cultivated lines; SeaStar and SeaIsle 1, which are known to be susceptible and moderately resistant to dollar spot, respectively (Steketee, 2014). From the preliminary screen of these two lines it was determined that the greatest difference in disease response between these two cultivars occurred when between 40 and 80 µL of inoculum was used (Fig. 3-1), which correlates to $6 \times 10^{-4} \pm 0.06$ to $1.12 \times 10^{-3} \pm$

0.06 grams dry mycelium per leaf. All subsequent detached leaf assays were conducted with 50 µL of inoculum ($7.6 \times 10^{-4} \pm 0.06$ grams dry mycelia).

Ten of the transformed lines were examined in the detached leaf assay. Lines 1B-11 and 1B-15 were not tested as there was not enough plant material to conduct the test. Lines R2-1B and R2-2BA had a lower disease response than the parent, SeaSpray ($P = 0.001$, Fig. 3-6). However, the lines derived from 17-726 showed no improvement in disease over the parent line ($P = 0.1904$). Additionally, lines 1B-13, 1B-17, 1B-19, and 4-CC showed no improvement in disease response over the parent, 17-756 ($P = 0.9466$).

Discussion

After transforming three genotypes of seashore paspalum, it was necessary to determine if the plants were expressing the inserted construct, which contained the gene of interest, *gf-2.8*. This was tested using a hygromycin screen, oxalate oxidase enzyme assay, qRT-PCR, and a detached leaf assay. Each of these tests provide additional information about each line and its ability to express the gene and resist dollar spot.

Initial PCR on all potentially transformed plants identified 76 plants. However, during a secondary PCR screen that focused on smaller batches of samples, not all plants tested positive the second time. After removing the plants that were not confirmed, transformed plants were moved to the greenhouse. Some plants did not survive the transition and still others died in the greenhouse due to poor fitness that may be related to the transformation process.

Hygromycin was used as an indicator of construct integration and activity. Plants that remained green after four days in 200 $mg \cdot L^{-1}$ of hygromycin medium were considered to be resistant to hygromycin and therefore expressing the *hph* gene in the inserted vector. The method

for the protocol was adapted from Wang and Waterhouse (1997). The results of this screen confirmed that all lines tested were resistant to hygromycin as they were statistically different from their parental line in level of injury. Lines R2-1B, R2-2BA, and 5A-3 had the lowest damage ratings which indicated that they have the highest expression of *hph*.

A potential issue with the colorimetric enzyme assay is variation in background H_2O_2. In samples without oxalic acid, a positive reaction was still seen suggesting that H_2O_2 exists in the plant tissue independent of the oxalate oxidase enzyme. Additionally, there was variation between the three parent lines. Line 17-726 had much higher absorbances than 17-756 or SeaSpray. This variation could reduce the accuracy of this test to determine oxalate oxidase activity.

Gene expression of *gf-2.8* was also confirmed using qRT-PCR. The fold change in expression level was determined using the equations listed in Table 3-2. By taking the difference in expression between the reference gene (F-box) and gene of interest (*gf-2.8)* for both the transformed and untransformed parent plants, the relative expression levels of the two genes can be determined for all samples. The gene expression level of *gf-2.8* for tested samples ranged from 0.9 to 109,427.5-fold of the reference gene. Sample 5A-10 had the highest *gf-2.8 e*xpression, these results are shown in Fig. 3-5, but in preliminary disease screens, it did not show a vast increase in disease resistance.

Line R2-1B showed a decrease in oxalate oxidase expression with a fold change of 0.9. In the colorimetric assay, this line had the highest absorbance indicating higher oxalate oxidase activity followed by R2-2BA and 5A-10. This raises the concern about how accurate the colorimetric enzyme assay is. It seems likely that the qRT-PCR is a more accurate indication of

gene expression since it directly measured expression of *gf-2.8* while the colorimetric enzyme assay did not specifically target *gf-2.8*.

A standardization of inoculum was necessary to efficiently evaluate disease responses *in vitro* and could help with future screens in the greenhouse. It was determined that the number of plugs used to start a culture did not have a significant effect on biomass of the colony. As all cultures were started with the volume of PDB, it seems likely that the amount of biomass is determined by available nutrients and not the amount of mycelium used to start the culture. A very similar study analyzed the filtrate of liquid colonies by HPLC and determined that oxalic acid was present as a result of a fungal colony (Venu et al., 2009). The methods of growing colonies in this study and that of Venu (2009) are similar enough that it stands to reason that the colonies grown for this study also produced oxalic acid. This is important as oxalic acid has been determined to be an important pathogenicity factor and is a crucial factor in determining if overexpression of the wheat germin gene improves disease resistance.

The detached leaf assay underwent many iterations to identify the amount of inoculum necessary to see differences in disease response between susceptible and resistant lines. The addition of Tween 20 to the wash regime was done to improve adherence of the mycelia to the leaves. Tween 20 has been demonstrated to improve adherence of spore suspensions to rice leaves (Jia et al., 2003). There was little uniformity in replicates tested without Tween 20, likely due to variation in the leaf epidermis.

The two lines used to determine inoculum dosage were SeaStar, which is known to be susceptible, and SeaIsle 1, which shows more resistance. However, the conditions necessary for the assay are not favorable for all plant lines. In some cases, the SeaIsle 1 controls showed more chlorosis than the inoculated leaves, making it difficult to determine what damage was caused by

the pathogen. However, only inoculated leaves showed necrosis and so that was the benchmark used for determining the level of inoculum in addition to calculating the difference in leaf damage between treated leaves and controls. Further analysis to improve the detached leaf assay by determining if there are better conditions that prevent the chlorosis on SeaIsle 1 and other genotypes would be beneficial. Additionally, more cultivated lines could be tested to investigate if the results of the assay are similar to what is seen in the field.

Literature Cited

Agrios, G.N. 2005. Plant Pathology. 5th ed. St. Louis, MO. *Academic Press*. pp. 992.

Bonos, S. A., Casler, M. D., & Meyer, W. A. (2003). Inheritance of dollar spot resistance in creeping bentgrass. *Crop science*, *43*(6), 2189-2196.

Boulter, J. I., Boland, G. J., & Trevors, J. T. (2002). Evaluation of composts for suppression of dollar spot (*Sclerotinia homoeocarpa*) of turfgrass. *Plant disease*, 86(4), 405-410.

Brede, A. D. (1991). Interaction of management factors on dollar spot disease severity in tall fescue turf. *Hortic. Sci.*, *26*(11), 1391-1392.

Cessna, S. G., Sears, V. E., Dickman, M. B., & Low, P. S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell*, 12(11), 2191-2199.

Chang, T. H. (2011). Difference of susceptibility on bentgrass cultivars to pink snow mold caused by *Microdochium nivale*. *Asian J. Turfgrass Sci*, *25*(2), 177-183.

Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L., & Simmonds, D. H. (2001). Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotina sclerotiorum*. Physiological and Molecular Plant Pathology, 59(6), 297-307.

Dutton, M. V., & Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Canadian journal of microbiology*, *42*(9), 881-895.

Godoy, G., Steadman, J. R., Dickman, M. B., & Dam, R. (1990). Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiological and molecular plant pathology*, *37*(3), 179-191.

Guimaraes, R. L., & Stotz, H. U. (2004). Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant physiology*, 136(3), 3703-3711.

Hu, Y., & Guo, Z. (2009). Purification and characterization of oxalate oxidase from wheat seedlings. *Acta physiologiae plantarum*, *31*(2), 229-235.

Jia, Y., Valent, B., & Lee, F. N. (2003). Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Disease*, *87*(2), 129-133.

Lamari, L. (2008) Assess 2.0 Image Analysis Software for Plant Disease Quantification [Computer Software]. Winnipeg, Canada: *APS Press*.

Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., & Cuming, A. C. (1993). Germin, a protein marker of early plant development, is an oxalate oxidase. Journal of Biological Chemistry, 268(17), 12239-12242.

Lesley, A. R. (Ed.). (2020). *Handbook of integrated pest management for turf and ornamentals*. CRC Press.

Liang, H., Maynard, C. A., Allen, R. D., & Powell, W. A. (2001). Increased Septoria musiva resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant molecular biology*, *45*(6), 619-629.

Liu, Y., Liu, J., Xu, L., Lai, H., Chen, Y., Yang, Z., & Huang, B. (2017). Identification and validation of reference genes for seashore paspalum response to abiotic stresses. International journal of molecular sciences, 18(6), 1322.

Livingstone, D. M., Hampton, J. L., Phipps, P. M., & Grabau, E. A. (2005). Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. Plant Physiology, 137(4), 1354-1362.

Miller, G. L., Stevenson, K. L., & Burpee, L. L. (2002). Sensitivity of *Sclerotinia homoeocarpa* isolates to propiconazole and impact on control of dollar spot. *Plant disease*, 86(11), 1240-1246.

Pfaffl, M. W. (2006). Relative quantification. Real-time PCR, 63, 63-82.

Salgado-Salazar, C., Beirn, L. A., Ismaiel, A., Boehm, M. J., Carbone, I., Putman, A. I., ... & Crouch, J. A. (2018). *Clarireedia*: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. *Fungal biology*, 122(8), 761-773.

Sall, J. (2018) JMP [Computer software] SAS Institute Inc.

Schomburg, D., & Salzmann, M. (1991). Enzyme handbook. In Enzyme Handbook (pp. 1-1175). Springer, Berlin, Heidelberg.

Steketee, C. J. (2014). *Characterization of dollar spot resistance in seashore paspalum* (Doctoral dissertation, University of Georgia).

Sugiura, M., Yamamura, H., Hirano, K., Sasaki, M., Morikawa, M., & Tsuboi, M. (1979). Purification and properties of oxalate oxidase from barley seedlings. Chemical and Pharmaceutical Bulletin, 27(9), 2003-2007.

Vargas, J. (1994). Management of Turfgrass Diseases. Boca Raton: *CRC Press*, https://doi.org/10.1201/9780203748374

Venu, R. C., Beaulieu, R. A., Graham, T. L., Martinez Medina, A., & Boehm, M. J. (2009). Dollar Spot Fungus *Sclerotinia homoeocarpa* Produces Oxalic Acid.

Wang, M. B., & Waterhouse, P. M. (1997). A rapid and simple method of assaying plants transformed with hygromycin or PPT resistance genes. Plant Molecular Biology Reporter, 15(3), 209-215.

Zhang, Z., Collinge, D. B., & Thordal-Christensen, H. (1995). Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. The Plant Journal, 8(1), 139-145.

Table 3-1. qRT-PCR Primer Sequences for Confirmation of Gene Expression

Primer Symbol	Gene Name (GenBank Accession)	5'-Primer Sequence (Forward/Reverse)-3'	Amplicon Length (bp)
GF_2.8_1_F/R	Oxalate oxidase GF-2.8 (XM_020301682)	ATTGTCTTCGTGCCCCTCAC/AAAACCCAGCGGCAAACTTG	127
GF_2.8_2_F/R	Oxalate oxidase GF-2.8 (XM_020301682)	TGCACTTCCAGTTCAACGTC/TGAGGGGCACGAAGACAATG	90
GF_2.8_3_F/R	Oxalate oxidase GF-2.8 (XM_020301682)	TCTCCTTCAACAGCCAGAACC/CGGCAAACTTGGACTTGAGAAG	144
FBOX_F/R	F-box/kelch-repeat protein (KX268097)	GTGCTAGCCAGCTCTGCAATAG/ACACATCCGACATCAACGATTC	184

Table 3-2. ΔΔCt Method for Determination of Gene Expression

$$\Delta Ct = Ct_{(OXO)} - Ct_{(Fbox)}$$

$$\Delta\Delta Ct = \Delta \mathrm{Ct}_{(untransformed\ parent)} - \Delta Ct_{(transformed\ sample)}$$

$$Fold\ Change\ from\ Untransformed\ Parent = 2^{-(\Delta\Delta Ct)}$$

Table 3-3. Quantitative Gene Expression for Transformed Lines as Determined Through qRT-PCR and the ΔΔCt Method.

Parent Line	Promoter	Line	Fold Increase ($2^{-\Delta\Delta Ct}$)
Seaspray	p35s-TaOXOH	R2-1B	0.934223
Seaspray	p35s-TaOXOH	R2-2BA	1.669074
17-756	p35s-TaOXOH	1B-11	6.270268
17-756	p35s-TaOXOH	1B-13	1.157959
17-756	p35s-TaOXOH	1B-15	2.451309
17-756	p35s-TaOXOH	1B-17	1.180983
17-756	p35s-TaOXOH	1B-19	1.630619
17-726	p35s-TaOXOH	5A-10	109,427.5
17-726	p35s-TaOXOH	5A-3	1,387.442
17-726	p35s-TaOXOH	6A-2	1,647.902
17-726	p35s-TaOXOH	7A-53	27.47658
17-756	pZmUbi-TaOXOH	4-CC	3.723639

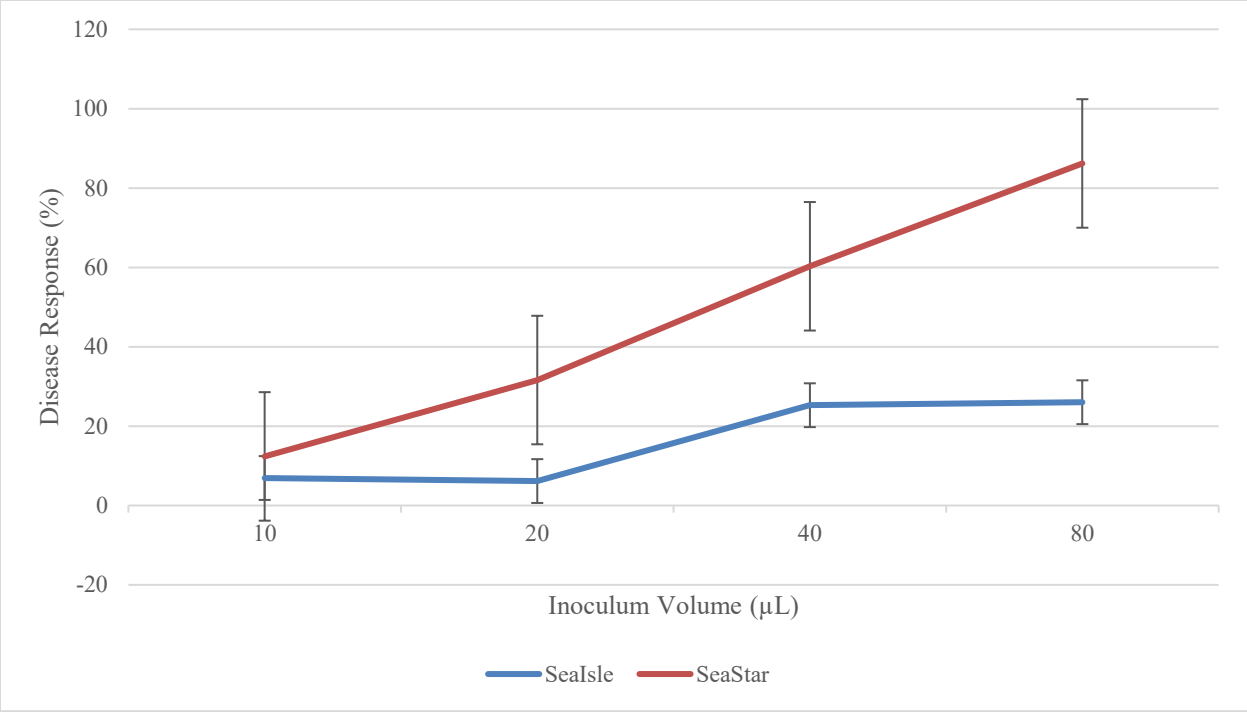


Figure 3-1. Analysis of Disease Response to Dollar Spot on Detached Leaf. Leaves of approximately 3 cm in length were sterilized and plated with the corresponding volumes of liquid inoculum. Digital image ratings were taken four days later and the percent disease was calculated by subtracting the percent injury of the controls from the treated plates after analysis through Assess (Lamari, 2008).

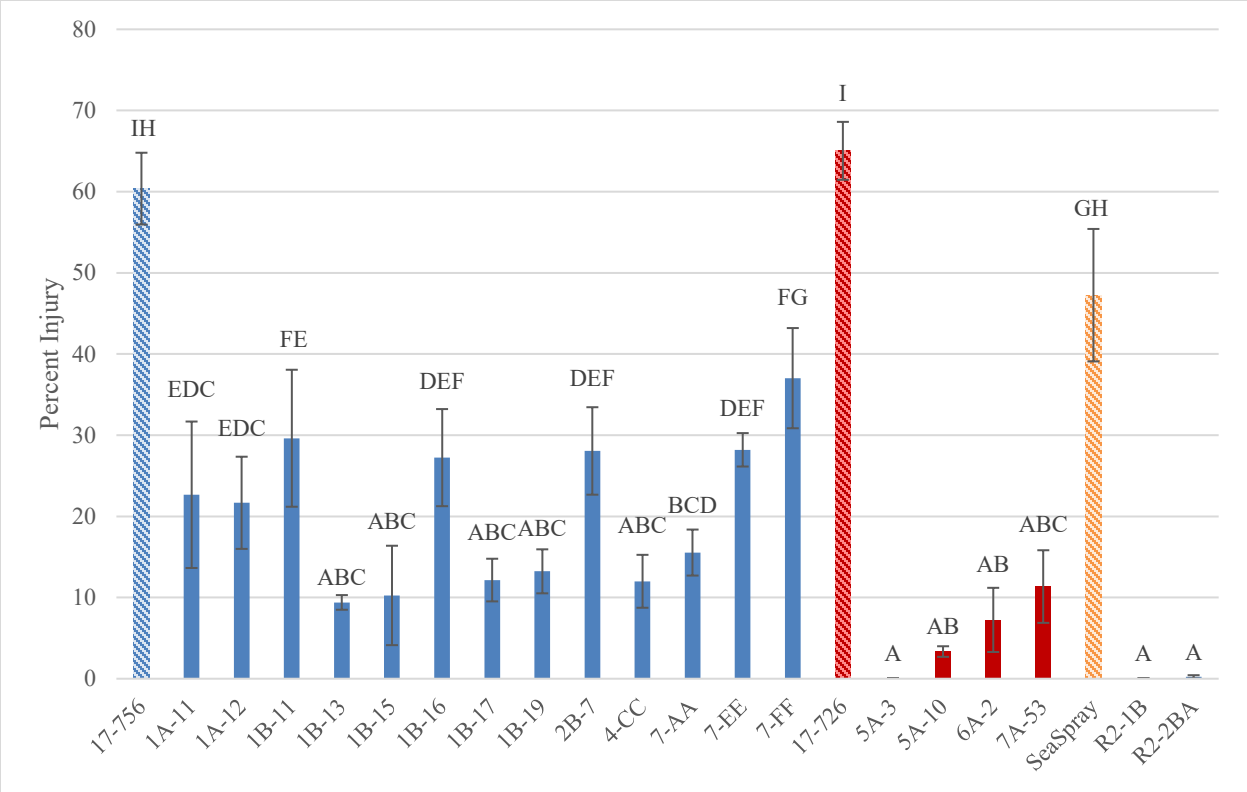


Figure 3-2. Whole Plant Hygromycin Screen. Lines R2-2BA, R2-1B, and 5A-3 had average injury scores of 0.23, 0.04, and 0.05%, respectively. Lines with the same letter above the bar are not statistically different according to pairwise Student's t-test at the $\alpha = 0.05$ level.

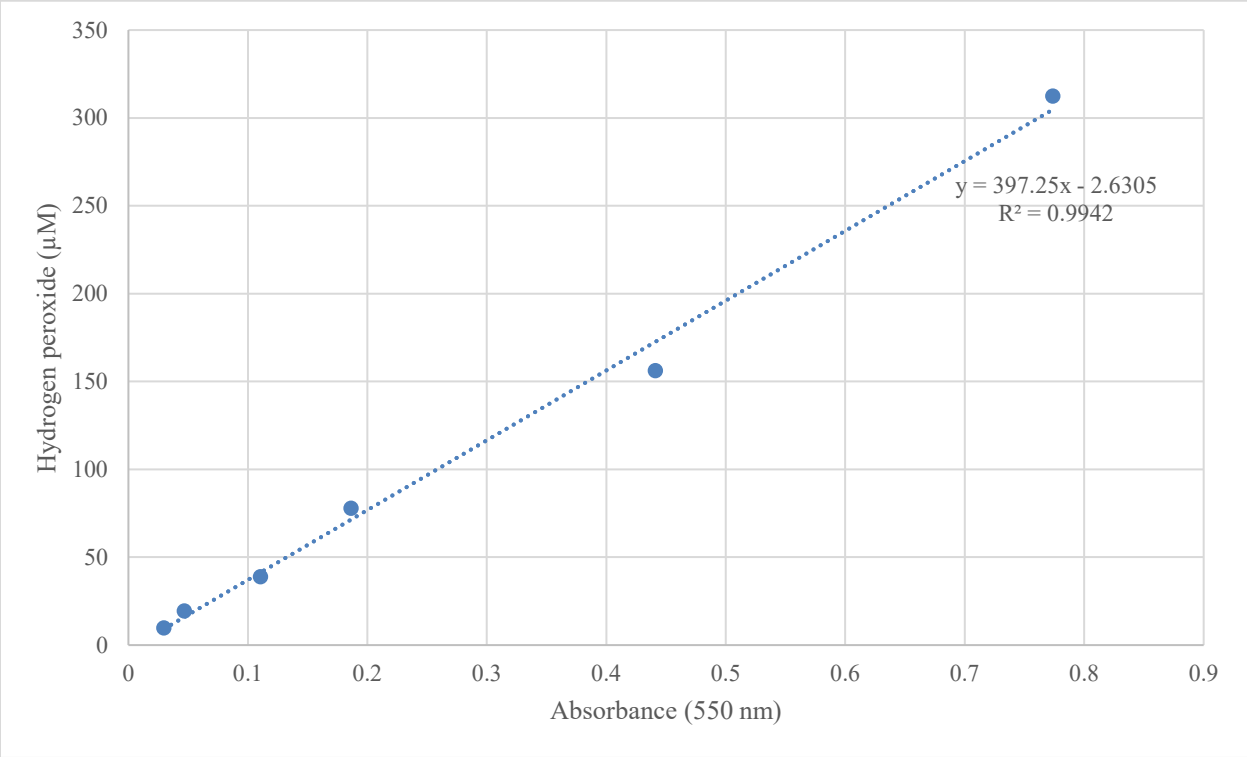


Figure 3-3. Enzyme Assay Trendline. The optical density of known concentrations of H_2O_2 was measured by spectrophotometer and values used created a curve that unknown samples could be measured against to estimate H_2O_2 generated by oxalate oxidase activity. The equation for the trendline is $y = 397.25x - 2.6305$, $R^2 = 0.9942$.

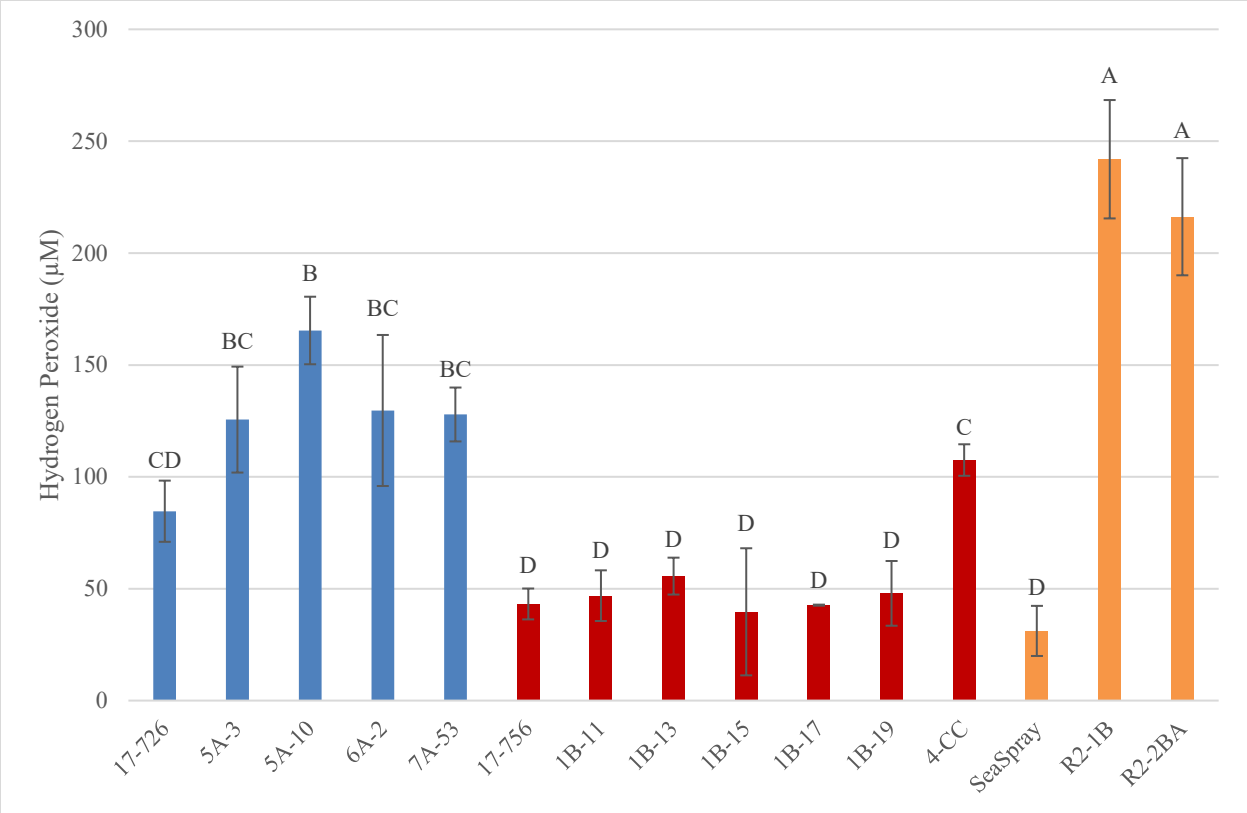


Figure 3-4. Colorimetric Oxalate Oxidase Enzyme Assay. Four replicates were made for each line and absorbance measured for each at 550 nm. Using the equation determined by the standard curve, $y = 397.25x - 2.6305$, the amount of H_2O_2 generated was calculated.

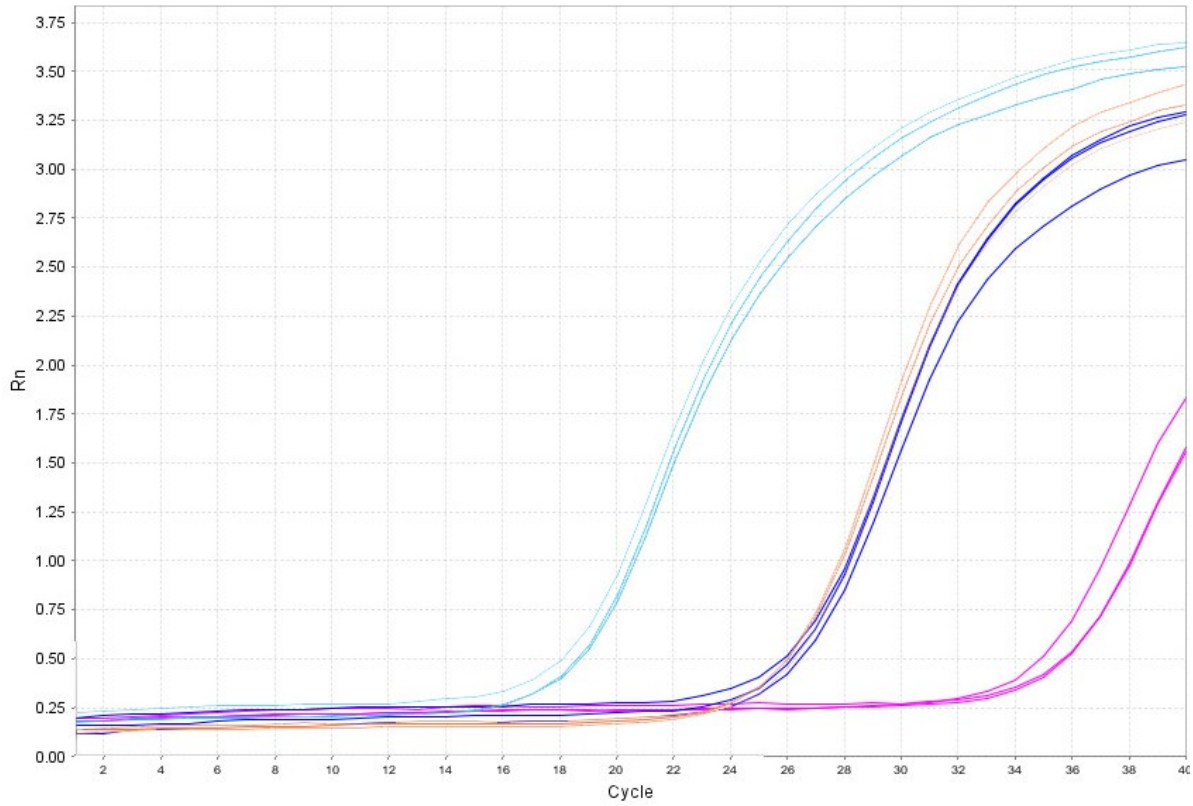


Figure 3-5. Example Plot of Gene Expression Curves from qRT-PCR for parent line 17-726 and transformed line 5A-10. Dark blue and tan lines show relative expression of the reference gene, F-box/kelch-repeat protein for 5A-10 and 17-726, respectively. Light blue and pink lines show the expression of the *gf-2.8* oxalate oxidase gene in the 5A-10 and 17-726, respectively.

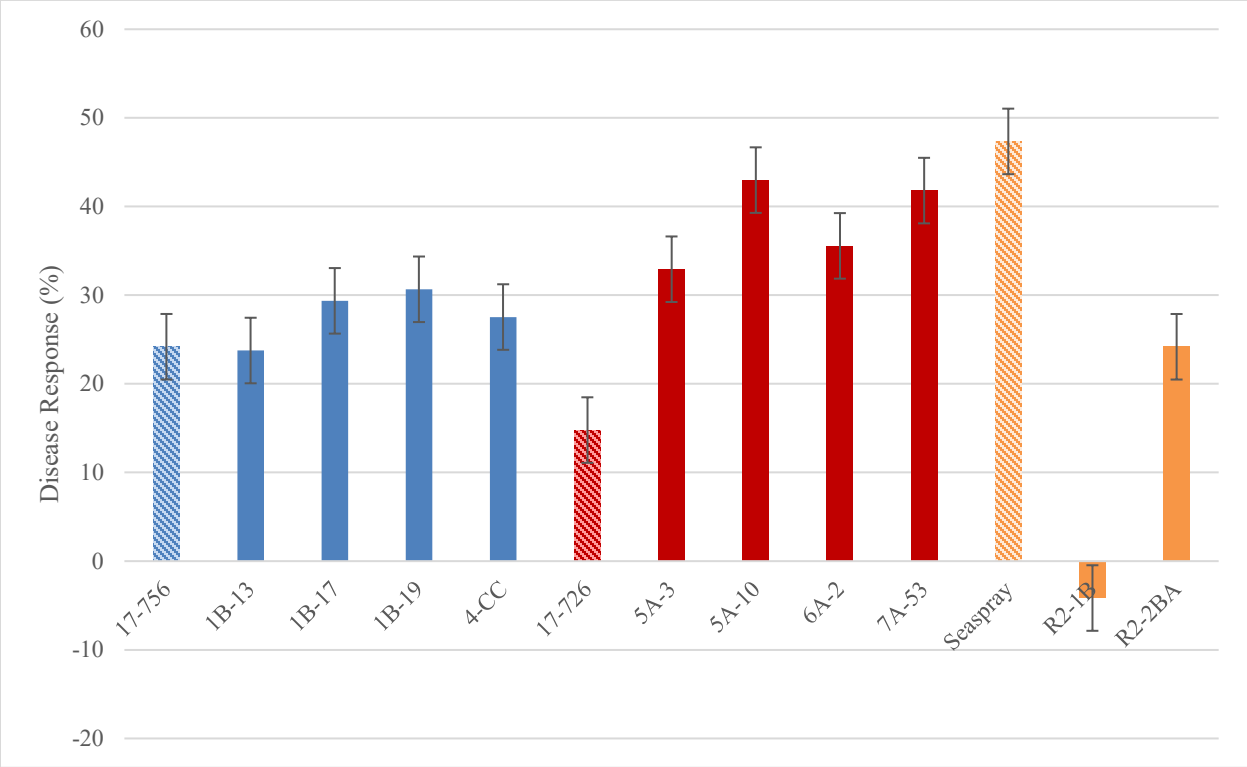


Figure 3-6. Analysis of Disease Response to Dollar Spot on Transformed Plant Lines. Leaves of approximately 3-cm length were sterilized and plated with the corresponding volumes of liquid inoculum. Digital image ratings were taken four days afterward and the percent disease was calculated as the difference between inoculated leaves and control leaves using Assess (Lamari, 2008). Line R2-1B is an outlier that showed more injury in the controls than the inoculated leaves and resulted in a negative value.

CONCLUSION

Seashore paspalum (*Paspalum vaginatum* Swartz.) is a warm-season turfgrass commonly used for golf courses and is often damaged by a fungal pathogen, dollar spot (*Clarireedia monteithiana* Salgado-Salazar). The overall goal of this study was to develop lines of seashore paspalum that overexpressed a wheat germin gene to potentially improve disease resistance. Embryogenic callus lines were developed for three genotypes; 17-756, 17-726, and SeaSpray. Using particle bombardment, the wheat germin gene *gf-2.8* was inserted into the callus. The callus was then screened with hygromycin and regenerated before being transferred to the greenhouse.

Successful transformation events were determined through PCR and additional testing was done through a secondary hygromycin screen, an oxalate oxidase enzyme assay, and qRT-PCR. The hygromycin screen confirmed that all transformed lines were resistant to hygromycin and the enzyme assay provided an initial indication of enzyme activity. Results from qRT-PCR indicated that seven lines were overexpressing *gf-2.8* relative to the untransformed parents. To quickly verify improved disease resistance, a detached leaf assay was developed using two cultivated lines; SeaStar and SeaIsle 1. When applied to the transformed lines, there was some variation in the responses seen. It is likely that this assay will need further testing to optimize the conditions to reduce chlorosis of the leaves not associated with disease response. The studies conducted are not able to conclusively determine if the overexpression of *gf-2.8* in seashore paspalum increases disease resistance. Further screening in the greenhouse as well as the field are recommended with transformed lines that showed upregulation of the gene through qRT-PCR.

REFERENCES

Agrios, G.N. 2005. Plant Pathology. 5th ed. St. Louis, MO. *Academic Press*. pp. 992.

Altpeter, F., Varshney, A., Abderhalden, O., Douchkov, D., Sautter, C., Kumlehn, J., Dudler, R., & Schweizer, P. (2005). Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. *Plant Mol. Biol.*, 57(2), 271-283.

Bateman, D. F., & Beer, S. V. (1965). Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii. Phytopathology,* 55(11), 204-11.

Bonos, S. A., Casler, M. D., & Meyer, W. A. (2003). Inheritance of dollar spot resistance in creeping bentgrass. *Crop science*, *43*(6), 2189-2196.

Borges, A., Rosa, M. S., Recchia, G. H., Queiroz-Silva, J. R. D., Bressan, E. D. A., & Veasey, E. A. (2009). CTAB methods for DNA extraction of sweetpotato for microsatellite analysis. *Scientia Agricola*, *66*(4), 529-534.

Boulter, J. I., Boland, G. J., & Trevors, J. T. (2002). Evaluation of composts for suppression of dollar spot (*Sclerotinia homoeocarpa*) of turfgrass. *Plant disease*, 86(4), 405-410.

Bradley, D. J., Kjellbom, P., & Lamb, C. J. (1992). Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*, 70(1), 21-30.

Brede, A. D. (1991). Interaction of management factors on dollar spot disease severity in tall fescue turf. *Hortic. Sci.*, *26*(11), 1391-1392.

Brisson, L. F., Tenhaken, R., & Lamb, C. (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *The Plant Cell*, *6*(12), 1703-1712.

Brosnan, J. T., & Deputy, J. (2008). Seashore Paspalum: Turf Management. *TM-1, Mãnoa, Hawaii: University of Hawaii at Mãnoa, 8p*.

Burpee, L. L. (1997). Control of dollar spot of creeping bentgrass caused by an isolate of *Sclerotinia homoeocarpa* resistant to benzimidazole and demethylation-inhibitor fungicides. *Plant disease*, *81*(11), 1259-1263.

Cardona, C. A., & Duncan, R. R. (1997). Callus induction and high efficiency plant regeneration via somatic embryogenesis in Paspalum. *Crop science*, *37*(4), 1297-1302.

Cessna, S. G., Sears, V. E., Dickman, M. B., & Low, P. S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell*, 12(11), 2191-2199.

Chang, T. H. (2011). Difference of susceptibility on bentgrass cultivars to pink snow mold caused by *Microdochium nivale*. *Asian J. Turfgrass Sci*, *25*(2), 177-183.

Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L., & Simmonds, D. H. (2001). Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotina sclerotiorum*. Physiological and Molecular Plant Pathology, 59(6), 297-307.

Dong, X., Ji, R., Guo, X., Foster, S. J., Chen, H., Dong, C., Liu, Y., Hu, Q., & Liu, S. (2008). Expressing a gene encoding wheat oxalate oxidase enhances resistance to Sclerotinia sclerotiorum in oilseed rape (*Brassica napus*). *Planta*, 228(2), 331.

Dumas, B., Freyssinet, G., & Pallett, K. E. (1995). Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. *Plant Physiology*, *107*(4), 1091-1096.

Duncan, R. R., & Carrow, R. N. (2000). Seashore paspalum: The environmental turfgrass. John Wiley & Sons.

Dutton, M. V., & Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Canadian journal of microbiology*, *42*(9), 881-895.

Fakhrana, I. N., Nurfahisza, A. R., Rasid, O. A., Parveez, G. K. A. 2019. Minimal inhibitory concentration of hygromycin for selecting transformed oil palm embryogenic calli. *Journal of Oil Palm Research* 31: 14-27. doi:https://doi.org/10.21894/jopr.2018.0063.

Fricker, C., Wipff, J. K., & Duncan, R. R. (2007). *U.S. Patent No. 7,262,341*. Washington, DC: U.S. Patent and Trademark Office.

Gamborg, O. L., Murashige, T., Thorpe, T. A., & Vasil, I. K. (1976). Plant tissue culture media. *In vitro*, *12*(7), 473-478.

Godoy, G., Steadman, J. R., Dickman, M. B., & Dam, R. (1990). Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiological and molecular plant pathology*, *37*(3), 179-191.

Green, J., Vain, P., Fearnehough, M. T., Worland, B., Snape, J. W., & Atkinson, H. J. (2002). Analysis of the expression patterns of the *Arabidopsis thaliana* tubulin-1 and *Zea mays* ubiquitin-1 promoters in rice plants in association with nematode infection. *Physiological and molecular plant pathology*, *60*(4), 197-205.

Guimaraes, R. L., & Stotz, H. U. (2004). Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant physiology*, 136(3), 3703-3711.

Hardebeck, G. A., Turco, R. F., Latin, R., & Reicher, Z. J. (2004). Application of *Pseudomonas aureofaciens* Tx-1 through irrigation for control of dollar spot and brown patch on fairway-height turf. *Hort. Sci.,39*(7), 1750-1753.

Heckart, D. L., Parrott, W. A., & Raymer, P. L. (2010). Obtaining sethoxydim resistance in seashore paspalum. *Crop science*, 50(6), 2632-2640.

Hu, Y., & Guo, Z. (2009). Purification and characterization of oxalate oxidase from wheat seedlings. *Acta physiologiae plantarum*, *31*(2), 229-235.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant molecular biology reporter*, *5*(4), 387-405.

Jia, Y., Valent, B., & Lee, F. N. (2003). Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Disease*, *87*(2), 129-133.

Jo, Y. K., Niver, A. L., Rimelspach, J. W., & Boehm, M. J. (2006). Fungicide sensitivity of Sclerotinia homoeocarpa from golf courses in Ohio. *Plant Disease*, 90(6), 807-813.

Joyce, P., Kuwahata, M., Turner, N., & Lakshmanan, P. (2010). Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. *Plant cell reports*, 29(2), 173-183.

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin. H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa., H, Itoh, T., Buell, C. R., & Matsumoto, T. (2013). Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. *Rice*, *6*(1), 4.

Kim, K. M., Song, I. J., Lee, H. Y., Raymer, P., Kim, B. S., & Kim, W. (2009). Development of seashore paspalum turfgrass with herbicide resistance. *Korean Journal of Crop Science*, 54(4), 427-432.

King, Z. R., Bray, A. L., LaFayette, P. R., & Parrott, W. A. (2014). Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.). *Plant Cell Reports*, 33(2), 313-322.

Lamari, L. (2008) Assess 2.0 Image Analysis Software for Plant Disease Quantification [Computer Software]. Winnipeg, Canada: *APS Press*.

Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual review of plant biology*, 48(1), 251-275.

Lane, B. G. (1994). Oxalate, germin, and the extracellular matrix of higher plants. *The FASEB Journal*, *8*(3), 294-301.

Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., & Cuming, A. C. (1993). Germin, a protein marker of early plant development, is an oxalate oxidase. Journal of Biological Chemistry, 268(17), 12239-12242.

Lee, G., Duncan, R. R., & Carrow, R. N. (2004). Salinity tolerance of seashore paspalum ecotypes: Shoot growth responses and criteria. *Hort. Sci.*, 39(5), 1138-1142.

Lesley, A. R. (Ed.). (2020). *Handbook of integrated pest management for turf and ornamentals*. CRC Press.

Levine, A., Tenhaken, R., Dixon, R., & Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, 79(4), 583-593.

Liang, H., Maynard, C. A., Allen, R. D., & Powell, W. A. (2001). Increased Septoria musiva resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant molecular biology*, *45*(6), 619-629.

Liu, Y., Liu, J., Xu, L., Lai, H., Chen, Y., Yang, Z., & Huang, B. (2017). Identification and validation of reference genes for seashore paspalum response to abiotic stresses. International journal of molecular sciences, 18(6), 1322.

Livingstone, D. M., Hampton, J. L., Phipps, P. M., & Grabau, E. A. (2005). Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. Plant Physiology, 137(4), 1354-1362.

Lonard, R. I., Judd, F. W., & Stalter, R. (2015). Biological Flora of Coastal Dunes and Wetlands: *Paspalum vaginatum* Sw. *Journal of Coastal Research*, 31(1), 213-223.

Mann, D. G., King, Z. R., Liu, W., Joyce, B. L., Percifield, R. J., Hawkins, J. S., LaFayette, P. R., Artelt, B. J., Burris, J. N., Mazarei, M., Bennetzen, J. L., Parrott, W. A., & Stewart, C. N. (2011). Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. *BMC biotechnology*, 11(1), 74.

Miller, G. L., Stevenson, K. L., & Burpee, L. L. (2002). Sensitivity of *Sclerotinia homoeocarpa* isolates to propiconazole and impact on control of dollar spot. *Plant disease*, 86(11), 1240-1246.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15(3), 473-497.

Neibaur, I., Gallo, M., & Altpeter, F. (2008). The effect of auxin type and cytokinin concentration on callus induction and plant regeneration frequency from immature

inflorescence segments of seashore paspalum (*Paspalum vaginatum* Swartz). *In Vitro Cellular & Developmental Biology-Plant*, 44(6), 480.

Parham, G. L. (2011). Scotts Miracle-Gro Co.; Regulatory Status of Kentucky Bluegrass Genetically Engineered for Herbicide Tolerance. 76 FR 39812.

Patnaik, D. & Khurana, P. (2001). Germins and germin like proteins: an overview. *NISCAIR-CSIR*, India

Pfaffl, M. W. (2006). Relative quantification. Real-time PCR, 63, 63-82.

Popko Jr, J. T., Sang, H., Lee, J., Yamada, T., Hoshino, Y., & Jung, G. (2018). Resistance of *Sclerotinia homoeocarpa* field isolates to succinate dehydrogenase inhibitor fungicides. *Plant disease*, 102(12), 2625-2631.

Prasad, R., & Shivay, Y. S. (2017). Oxalic acid/oxalates in plants: from self-defence to phytoremediation. *Curr Sci*, 112(8), 110-112.

Praveena, M., & Giri, C. C. (2012). Plant regeneration from immature inflorescence derived callus cultures of salt tolerant kallar grass (*Leptochloa fusca* L.). *Physiology and Molecular Biology of Plants*, 18(4), 345-356.

Punja, Z. K. (1985). The biology, ecology, and control of *Sclerotium rolfsii*. *Annual review of Phytopathology*, 23(1), 97-127.

Raja, N. I., Bano, A., Rashid, H., Chaudhry, Z., & Ilyas, N. (2010). Improving *Agrobacterium*-mediated transformation protocol for integration of XA21 gene in wheat (*Triticum aestivum* L.). *Pak J Bot*, 42(5), 3613-3631.

Rioux, R. A., Shultz, J., Garcia, M., Willis, D. K., Casler, M., Bonos, S., Smith, D., & Kerns, J. (2014). *Sclerotinia homoeocarpa* overwinters in turfgrass and is present in commercial seed. *PLoS One*, 9(10), e110897.

Salgado-Salazar, C., Beirn, L. A., Ismaiel, A., Boehm, M. J., Carbone, I., Putman, A. I., Tredway, L., Clarke, B., & Crouch, J. A. (2018). *Clarireedia*: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. *Fungal biology*, 122(8), 761-773.

Sall, J. (2018) JMP [Computer software] SAS Institute Inc.

Sapkota, S., Martinez-Espinoza, A., Ali, E., Vermeer, B. C., & Bahri, B. A. (2020). Taxonomical identification of *Clarireedia* species causing dollar spot disease of turfgrass in Georgia. *Plant Disease*.

Schomburg, D., & Salzmann, M. (1991). Enzyme handbook. In Enzyme Handbook (pp. 1-1175). Springer, Berlin, Heidelberg.

Shim, S. I., Moon, J. C., Jang, C. S., Raymer, P., & Kim, W. (2008). Effect of potassium nitrate priming on seed germination of seashore paspalum. *Hortic. Sci.*, *43*(7), 2259-2262.

Smiley, R. W., Dernoeden, P. H., & Clarke, B. B. (2005). Compendium of turfgrass diseases (Vol. 1). St. Paul, MN: *APS press*.

Steketee, C. J. (2014). *Characterization of dollar spot resistance in seashore paspalum* (Doctoral dissertation, University of Georgia).

Steketee, C. J., Martinez-Espinoza, A. D., Harris-Shultz, K. R., Henry, G. M., & Raymer, P. L. (2016). Effects of genotype and isolate on expression of dollar spot in seashore paspalum. *Hortic. Sci.*, 51(1), 67-73.

Steketee, C. J., Martinez-Espinoza, A. D., Harris-Shultz, K. R., Henry, G. M., & Raymer, P. L. (2017). Evaluation of Seashore Paspalum Germplasm for Resistance to Dollar Spot. *International Turfgrass Society Research Journal*, 13(1), 175-184.

Sugiura, M., Yamamura, H., Hirano, K., Sasaki, M., Morikawa, M., & Tsuboi, M. (1979). Purification and properties of oxalate oxidase from barley seedlings. *Chemical and Pharmaceutical Bulletin*, 27(9), 2003-2007.

Trick, H. N., Dinkin, R. D., Santarem, E. R., Di, R., Samoylov, V., Meurer, C. A., Walker, D. R., Parrott, W. A., Finer, J. J., & Collins, G. B. (1997). Recent advances in soybean transformation. *Plant Tissue Cult. Biotech*., 3, 9-26.

Vain, P., McMullen, M. D., & Finer, J. J. (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports*, 12(2), 84-88.

Vargas, J. (1994). Management of Turfgrass Diseases. Boca Raton: *CRC Press*, https://doi.org/10.1201/9780203748374

Venu, R. C., Beaulieu, R. A., Graham, T. L., Martinez Medina, A., & Boehm, M. J. (2009). Dollar Spot Fungus *Sclerotinia homoeocarpa* Produces Oxalic Acid. *International Turfgrass Society Research Journal,* (11).

Visarada, K. B. R. S., & Sarma, N. P. (2004). Transformation of Indica rice through particle bombardment: factors influencing transient expression and selection. *Biologia plantarum*, 48(1), 25-31.

Walsh, B., Ikeda, S. S., & Boland, G. J. (1999). Biology and management of dollar spot (*Sclerotinia homoeocarpa*); an important disease of turfgrass. *Hort. Sci.*, 34(1), 13-21.

Walz, A., Zingen-Sell, I., Loeffler, M., & Sauer, M. (2008). Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Plant Pathology*, 57(3), 453-458.

Wang, M. B., & Waterhouse, P. M. (1997). A rapid and simple method of assaying plants transformed with hygromycin or PPT resistance genes. *Plant Molecular Biology Reporter*, 15(3), 209-215.

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochemical Journal*, 322(3), 681-692.

Wu, X., Shi, H., Chen, X., Liu, Y., & Guo, Z. (2018). Establishment of *Agrobacterium*-mediated transformation of seashore paspalum (*Paspalum vaginatum* O. Swartz). *In Vitro Cellular & Developmental Biology-Plant*, 54(5), 545-552.

Yang, X., Yang, J., Wang, Y., He, H., Niu, L., Guo, D., ... & Li, Q. (2019). Enhanced resistance to Sclerotinia stem rot in transgenic soybean that overexpresses a wheat oxalate oxidase. *Transgenic research*, *28*(1), 103-114.

Zhang CX, Li Q, Kong L (2007) Induction, development and maturation of somatic embryos in Bunge's pine (*Pinus bungeana* Zucc. ex Endl.). *Plant Cell Tissue Organ Cult.* 91:273–280

Zhang, Z., Collinge, D. B., & Thordal-Christensen, H. (1995). Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *The Plant Journal*, 8(1), 139-145.