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SHORT COMMUNICATION

Turfgrasses as model assay systems for high-throughput *in planta* screening of beneficial endophytes isolated from cereal crops

Hanan R. Shehata[1,2] · **Eric M. Lyons**[1] · **Manish N. Raizada**[1]

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Abstract Cereal crops including maize (*Zea mays* L.) are inhabited by non-disease causing microbes known as endophytes that can promote plant growth, aid in host nutrient acquisition and promote host pathogen resistance. Screening endophytes for beneficial traits *in planta* using large, slow-growing cereals is challenging, thus a rapid but relevant *in planta* system is needed. Here, we propose that turfgrasses can be used as high-throughput assay systems for screening cereal microbes for beneficial nutrient traits. Turfgrasses are genetic relatives of cereals, but small with fast growth rates; they can be grown in test tubes under sterile conditions on defined media. Five turfgrass genotypes were evaluated for traits ideal for assaying endophytes with nutrient acquisition traits. Based on these criteria, annual ryegrass (*Lolium multiflorum*) was selected as a high-throughput assay system. Annual ryegrass was then used to test a collection of maize endophytes for their ability to promote plant biomass in the absence of nitrogen. Out of 75 bacterial endophytes tested, one strain (an *Enterobacter* sp) consistently promoted root and shoot biomass. We discuss the potential of annual ryegrass as a model assay system to test cereal endophytes for acquisition of various nutrients, changes in root/shoot architecture as well as anti-pathogen traits.

Keywords Model assay system · Antifungal · Endophytes · Cereal crops · High-throughput

1 Introduction

Cereal crops including maize (*Zea mays*), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa*) are the most cultivated crops worldwide (Pechanova et al. 2013; Pérez-Montaño et al. 2014). Plants including cereals host large numbers of microbes (endophytes) that can promote plant health, nutrition and pathogen resistance (Reinhold-Hurek and Hurek 2011; Rey and Schornack 2013; Wani et al. 2015). Screening microbial collections, with sufficient replicates, for beneficial activities with large, slow-growing cereals is very challenging, as growing these crops requires considerable space, time, labour and cost (Pliego et al. 2011; Wani et al. 2015). A rapid high-throughput *in planta* system is needed to screen cereal endophytes for beneficial activities (Rey and Schornack 2013) so that primary screens can be conducted *in planta* rather than in vitro only (Shehata et al. 2016b).

Here, we propose that turfgrasses can be used as high-throughput assay systems for screening cereal microbes for nutrient-acquisition activities. Turfgrasses consist of 30 species in 20 genera classified as cool-season grasses (including ryegrasses, bluegrasses, fescues) and warm-season grasses (including bermudagrass) (Budak et al. 2004). Turfgrasses are genetic relatives of cereals, all belonging to the family Poaceae or Gramineae (Budak et al. 2004), and hence their endophyte communities may be compatible. However, turfgrasses are much smaller, have faster growth rate, and can be grown in sterilized test tubes on defined media, and under controlled conditions to prevent microbial cross-contamination. In this study, five turfgrass genotypes were

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✉ Manish N. Raizada
raizada@uoguelph.ca

[1] Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada

[2] Department of Microbiology, School of Pharmacy, Mansoura University, Mansoura, Egypt

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evaluated for their potential to assay nutrient-promoting endophyte traits, and one species was selected as a model assay system for screening maize endophytes.

2 Materials and methods

2.1 Seed and endophyte materials

Annual ryegrass (*Lolium multiflorum*) variety Annuity (~500 seeds/g with Average Germination Time, AGT, of 6 days and germination rate of 98%) was obtained from Seed Research of Oregon (Oregon, USA), while perennial ryegrass (*Lolium perenne*) (~500 seeds/g with AGT of 8 days), Kentucky bluegrass (*Poa pratensis*) (~3000 seeds/g with AGT of 24 days), Bermuda grass (*Cynodon dactylon*) (~4500 seeds/g with AGT of 25 days) (Southern Stav), and tall fescue (*Lolium arundinaceum*) (~500 seeds/g with AGT of 10 days) were courtesy of the Guelph Turfgrass Institute (Guelph, Canada). All seeds were stored at 4°C. The bacterial endophyte collection (Table S1) was previously isolated from seeds of diverse wild, ancient and modern genotypes of maize (Johnston-Monje and Raizada 2011a).

2.2 Selecting a turfgrass genotype for use as a model assay system for nutrient promoting endophytes

To select a turfgrass to conduct endophyte assays, five turfgrass genotypes were evaluated for four criteria:

Germination, uniformity and rate of growth Fifteen seeds per genotype were germinated on wet paper towels (water only) in Petri dishes, and growth was observed for 10 days. There were three replicate dishes per genotype. Seeds were also germinated on 0.5 strength Murashige & Skoog (MS) medium in glass tubes and observed for growth over 3 weeks. Each glass tube (15 cm × 25 cm, C5916, Sigma, USA), capped (C5791, Sigma, USA) contained 15 ml of autoclaved 0.5 strength MS (pH 5.8), consisting of (per L): half-strength modified basal MS salt (M571, Phytotech, USA), 250 μl nicotinic acid (1 mg/ml), 500 μl pyridoxine HCl (0.5 mg/ml), 5 ml thiamine HCl (100 mg/l), 500 μl glycine (2 mg/ml) and 2 g Phytagel (P8169, Sigma, USA) in double distilled water (Shehata et al. 2016b). To solidify Phytagel, 0.166 g/l $CaCl_2$ and 90 mg/l $MgSO_4$ were added. The plant growth conditions were previously described (Shehata et al. 2016b).

Surface sterilization efficiency Two protocols were used: (Method 1) washing seeds in 70% ethanol for 1 min, then washing in bleach (5.25% sodium hypochlorite) for 10 min and six washes with water; (Method 2) washing seeds in 70% ethanol for 1 min, then washing in bleach for 20 min and six washes with water. Twenty microliters from the last wash were spotted on R-2A agar (18.12 g/l) (17209, Sigma, USA) to test for microbial growth.

Growth on different media Annual ryegrass and tall fescue seeds were surface sterilized using Sterilization Method 2 then grown on four different media: 1.5% bacto-agar (DF0140, Fisher), 1.5% R-2A agar (17209, Sigma, USA), sand (15 g sand and 4 ml of water), and Phytagel (0.5 strength MS). There were three seeds/tube and three replicate tubes/treatment. Growth was observed over 3 weeks. The plant growth conditions were previously described (Shehata et al. 2016b).

Responsiveness to mineral nutrients Annual ryegrass and tall fescue seeds were surface sterilized using Sterilization Method 2 and grown at three different nutrient concentrations (0 MS, 0.1 strength MS and 0.5 strength MS). There were four tubes/treatment and three seeds/tube. After 1 month, plants were removed, rinsed, and air-dried for 20 min. Shoots and roots were dissected and weighed. The 0 strength MS medium (pH 5.8) consisted of (per L): 2 g Phytagel, 0.33 g/l $CaCl_2$ and 180 mg/l $MgSO_4$ in double distilled water. The plant growth conditions were previously described (Shehata et al. 2016b).

2.3 Screening a collection of maize endophytes for growth promotion of annual ryegrass in the absence of nitrogen

A modified 0.5 strength MS medium was prepared as described above except that the modified MS basal salt mixture contained no nitrogen (M531, Phytotech, USA). Maize endophytes were coated onto annual ryegrass seeds as previously described (Shehata et al. 2016b). Seven seeds were germinated per tube and each endophyte was tested in triplicate. After 4 weeks, plants were removed, rinsed and air-dried for 20 min. Shoots and roots were dissected and weighed as pools per tube, then divided by the number of plants to calculate the mean weight per tube. Positive candidates were rescreened in two additional trials with seven plants/tube and seven replicate tubes per endophyte.

Taxonomic identification of maize endophytes with growth promotion ability Taxonomic analysis employed 16S rRNA universal primers 799f and 1492r as previously described (Shehata et al. 2016a).

Statistical analysis and graphs Microsoft Excel 2011 and GraphPad Prism 7 were used for graphing and for statistical analysis (Student t-test).

3 Results and discussion

3.1 Selecting a turfgrass genotype for use as a model assay system

Germination, uniformity and rate of growth Five turfgrass genotypes (annual ryegrass, perennial ryegrass, tall fescue, Bermuda grass, Kentucky bluegrass) were tested for germination and growth rate on paper towels and in tubes with 0.5 strength MS media. Annual ryegrass, perennial ryegrass and tall fescue were fast growing while Bermuda grass and Kentucky bluegrass were slow growing (Fig. 1a-j, Table 1).

Efficiency of surface sterilization As endophyte assays often involve coating of surface sterilized seeds, the ability to efficiently surface sterilize the turfgrass genotypes was evaluated using two protocols. Washing seeds in 70%

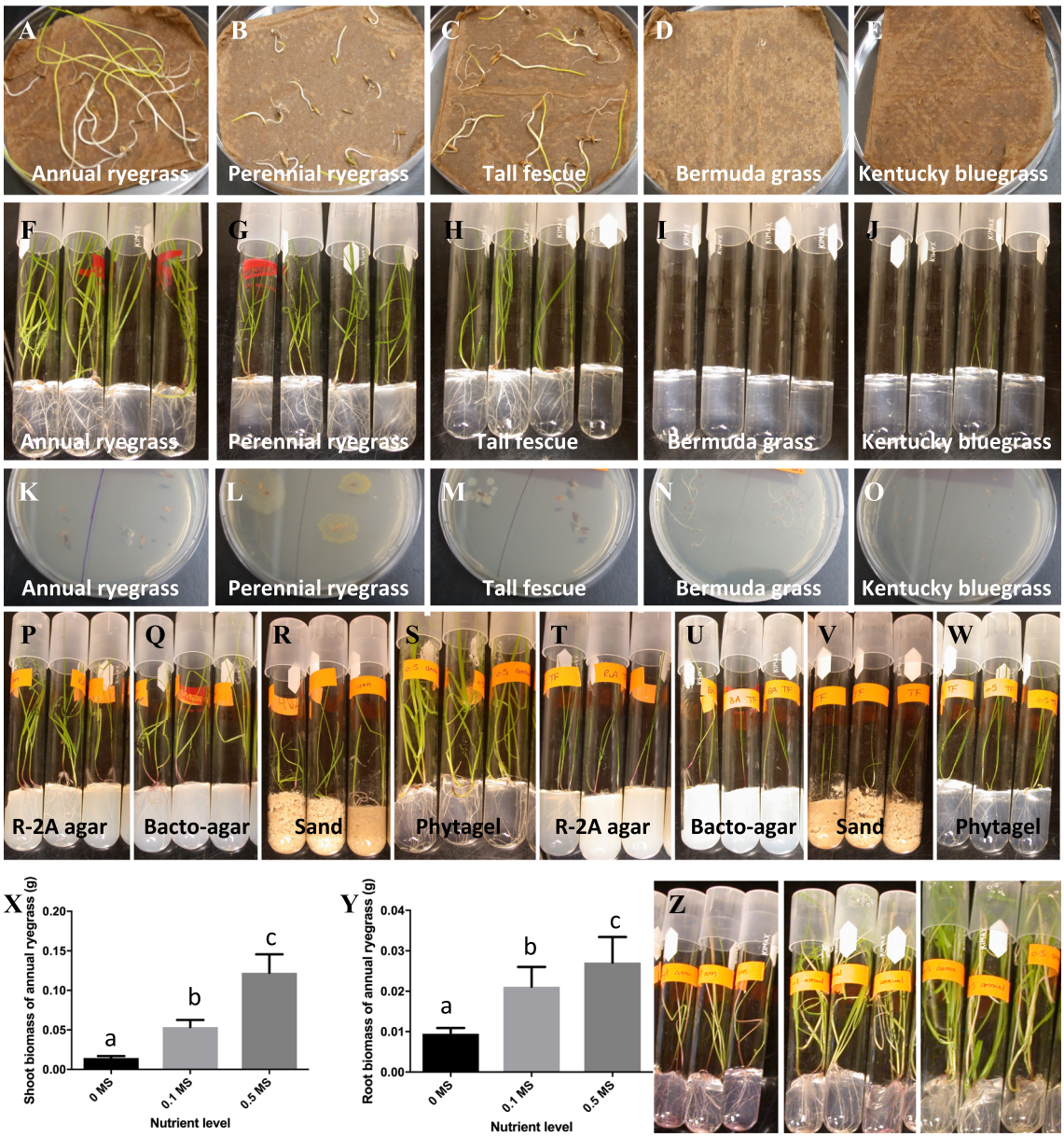

Fig. 1 Selecting a turfgrass genotype as a model assay system. **a-e** Germination and growth rate of turfgrass seeds on wet paper towels (water only) after 10 days. **f-j** Growth rate of turfgrass seeds in tubes after 3 weeks. **k-o** Testing for efficiency of seed surface sterilization on R_2A agar after 10 days: Sterilization Method 1 is on the left side of each plate and Method 2 is on the right side of each plate. **p-w** Testing growth of (**p-s**) annual ryegrass and (**t-w**) tall fescue on different media. **x-z** Response of annual ryegrass to different nutrient levels after 1 month with respect to (**x**) shoot biomass and (**y**) root biomass, with (**z**) corresponding representative pictures (from L-R): plants grown at 0 strength MS, 0.1 strength MS and 0.5 strength MS. In panels **x** and **y**, the error bars represent the standard error of the mean (SEM), while different letters (a, b, c) above each histogram denotes that the means are significantly different from one another

Table 1 Summary of tests conducted to select a turfgrass genotype as a model system

Selection criteria	Turfgrass species	Replicate 1		Replicate 2		Replicate 3		Conclusion
Percentage seed germination after 10 days	Annual ryegrass	87%		80%		93%		Acceptable
	Perennial ryegrass	67%		40%		47%		Acceptable
	Tall fescue	80%		60%		33%		Acceptable
	Kentucky bluegrass	0		0		0		Excluded
	Bermuda grass	13%		0		0		Excluded
Rate of growth after 3 weeks (+++ = fastest)	Annual ryegrass	+++		+++		+++		Acceptable
	Perennial ryegrass	+		+		+		Acceptable
	Tall fescue	+		+		+		Acceptable
	Kentucky bluegrass	–		–		–		Excluded
	Bermuda grass	–		–		–		Excluded
Microbial contamination observed after seed sterilization		Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	
	Annual ryegrass	–	–	–	–	–	–	Selected
	Perennial ryegrass	+	+	+	–	+	+	Excluded
	Tall fescue	+	+	+	–	+	–	Acceptable
	Kentucky bluegrass	–	–	–	–	–	–	Acceptable
	Bermuda grass	+	–	–	+	–	–	Acceptable

ethanol for 1 min, then washing in bleach for 20 min and six washes with water (Method 2), was efficient at surface sterilizing all genotypes except perennial ryegrass (Fig. 1k-o, Table 1).

Growth on different media The ability of plants to grow on different media offers distinct opportunities. The two promising turf genotypes that passed the above criteria (annual ryegrass, tall fescue) were grown on four different media (bacto-agar, R-2A agar, sand, Phytagel). Both genotypes grew well on all media types but Phytagel had the advantage of being transparent, allowing visualization of plant root growth without removing plants from tubes (Fig. 1p-w).

Responsiveness to mineral nutrition To use a turfgrass as a model assay system, it must be responsive to increasing nutrients. Annual ryegrass and tall fescue were grown at three different nutrient concentrations (0 MS, 0.1 strength MS, and 0.5 strength MS). After 1 month, annual ryegrass showed significant differences in both shoot and root biomass on all three nutrient concentrations (Fig. 1x-z) while tall fescue only showed a significant difference in shoot biomass which was limited to 0 MS versus 0.5 strength MS (data not shown).

3.2 Use of annual ryegrass as an *in planta* assay system to screen maize endophytes

Based on the above criteria, the annual ryegrass tube system was selected as a high-throughput model assay system to test nutrient activities of cereal endophytes. Annual ryegrass is an economically important crop used as cattle feed (Venuto et al. 2002) and for remediation of manure phosphate (Li et al. 2014). However, in future studies involving annual ryegrass as a model assay system, it is advised to test additional surface sterilization techniques as microbes and native endophytes may exist under the seed coat, which can vary by the seed source, soil type and geographic location. De-husking may be required in some cases to achieve surface sterilization (Kim et al. 2012).

There is significant interest in discovering endophytes that allow crops including maize to grow with reduced nitrogen fertilizers (Johnston-Monje and Raizada 2011b). Thus, this new assay system was used to screen a maize bacterial endophyte collection (Johnston-Monje and Raizada 2011a) for plant growth promotion in the absence of nitrogen. Out of 75 endophytes tested (Fig. 2a-c), strain 3D9 was found to consistently promote root biomass in three independent trials ($p = 0.04, 0.007, 0.03$). The growth promotion effect was weaker but promising for shoots ($p = 0.07, 0.12, 0.08$) (Fig. 2d, e). Based on 16S rRNA sequencing, strain 3D9 was found to most closely resemble *Enterobacter* (99% similarity). We hypothesize that the growth promotion activity may be attributed to biological nitrogen fixation (de Souza et al. 2015; Santi et al. 2013), root scavenging of secreted nitrogen metabolites normally used to support the rhizosphere (Tkacz and Poole 2015), and/or improved recycling of nitrogen metabolites from senescing tissues to growing tissues. Future experiments are needed to understand the mechanism of action of strain 3D9 but the results demonstrate that the annual ryegrass tube system has the potential to rapidly identify potentially

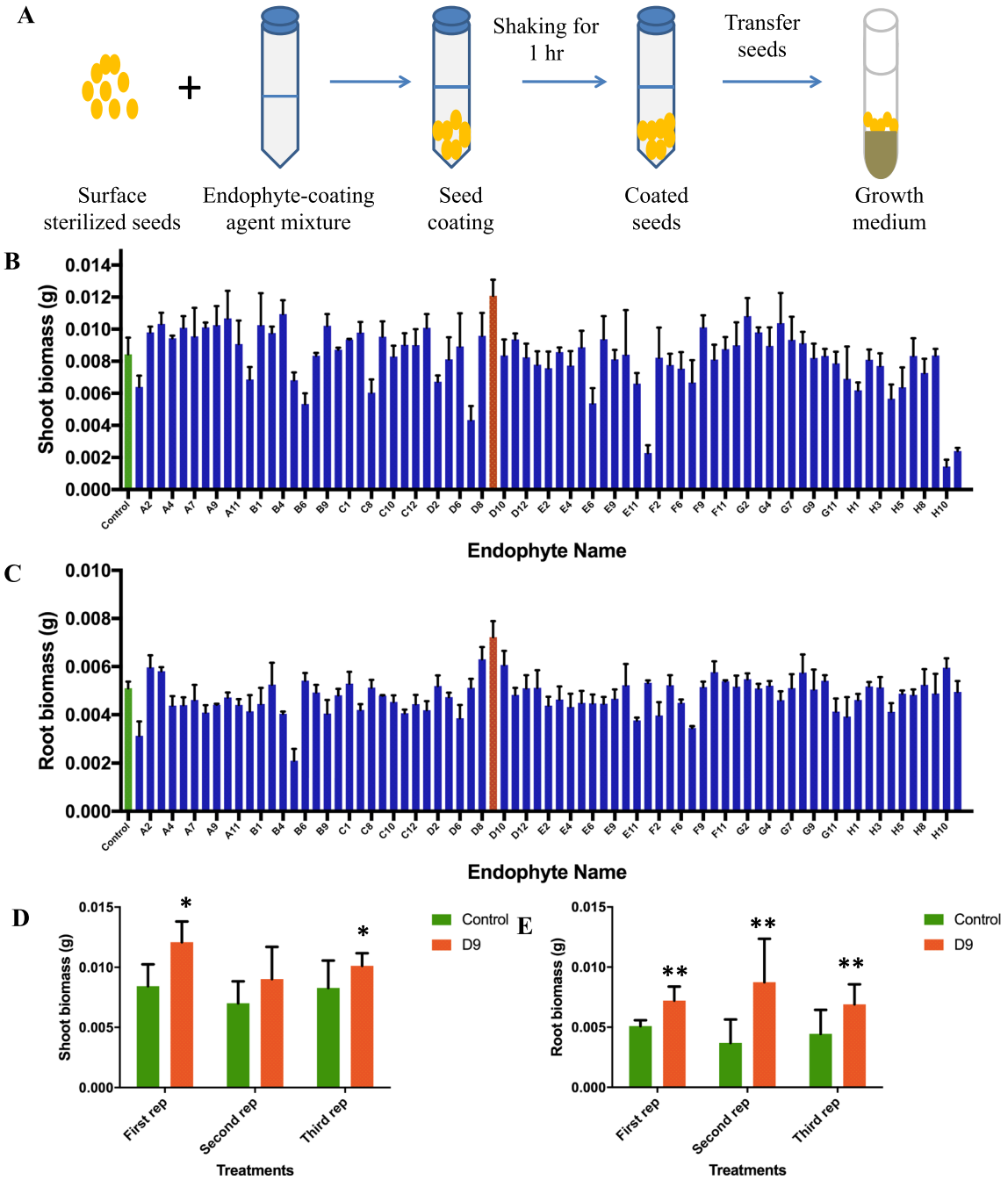

Fig. 2 Screening of maize endophytes for growth promotion of annual ryegrass on media without nitrogen. **a** The seed coating methodology for bacterial inoculation. **b** Shoot biomass and (**c**) root biomass of annual ryegrass at 4 weeks after bacterial inoculation. (D-E) Replicated trials of the growth promotion effect of strain 3D9 on annual ryegrass with respect to: **d** shoot biomass and **e** root biomass. Shown are three independent replicates at 4 weeks after bacterial inoculation. First replicate (n = 3 tubes, each with 7 plants) and second and third trials (n = 7 tubes, each with 7 plants). The error bars represent the standard error of the mean (SEM). Two asterisks denote that the endophyte treatment is significantly different from the buffer only control at p = 0.05, while one asterisk notes that the treatments are significantly different at p = 0.10 (student t-test)

important cereal endophytes. The ability to translate the results from annual ryegrass to corn and other cereals should be viewed with caution, however, as the beneficial phenotype in annual ryegrass may have been the result of the candidate endophyte interacting with the native annual ryegrass microbial community as a consortium. Furthermore, cereal crops have their own native endophyte and rhizosphere communities that may interact with any microbial candidate of interest, which may prevent its beneficial activity in the real world, though it is also possible that they will act as a beneficial consortium especially in its native host.

3.3 Future applications

In the future, annual ryegrass could be used to screen endophytes that promote other nutrients including phosphorous (Arcand and Schneider 2006; Richardson et al. 2009; Sharma et al. 2013) as it is a phosphorous-hyperaccumulator (Sharma and Sahi 2005; Sharma et al. 2004), or for traits such as root/shoot phenotyping caused by microbial production of phytohormones (Glick 2012; Kurepin et al. 2014; Ludwig-Müller 2015). The transparency of the Phytagel based media enables non-destructive monitoring of root growth. In addition, annual ryegrass and other turfgrasses hold potential as model assay systems to screen microbes with anti-pathogen activities when the target pathogen affects both the cereal as well as the turfgrass. We demonstrated the utility of creeping bentgrass to screen for maize endophytes that suppress the fungal pathogen *Rhizoctonia solani*, which affects both turfgrass and maize (Shehata and Raizada 2017).

Author contributions HRS helped to design the study, carried out all experiments, and wrote the manuscript. EML helped to design the study and provided seed materials. MNR helped to design the study and edited the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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