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Phosphite mediated enhancement of defence responses in *Agrostis stolonifera* and *Poa annua* infected by *Microdochium nivale*

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Abstract

The ascomycete fungus *Microdochium nivale* is one of the most damaging pathogens of cool season turfgrass. Prevention of and recovery from infection is costly to many sports facilities each year. In recent years, use of many chemical plant protectants have been restricted and turfgrass managers have increasingly sought alternative measures for disease control. The use of phosphite has been shown to be effective in reducing *M. nivale* disease symptoms in *Agrostis stolonifera* and *Poa annua.* The aims of this research were to assess initial defence responses in *M. nivale* infected turfgrass, specifically total phenolic content and hydrogen peroxide generation, to determine the effect phosphite treatment has on these responses and on suppression of symptoms. Phenolic compounds and H_2O_2 are shown to be components of host responses. Phosphite treatment led to enhanced accumulations of total phenolic content, and that when applied sequentially or singly to greenhouse plants, it led to significant reductions in *M. nivale* disease symptoms compared to phosphate-treated plants or controls. H_2O_2 extractions indicated that while phosphite treatment increased H_2O_2 generation compared to controls, the effect was no different to the responses in phosphate-treated plants.

Keywords

Microdochium nivale, phosphite, turfgrass, phenolic compounds, hydrogen peroxide.

Introduction

Microdochium nivale is an ascomycete pathogen, a direct causal agent and a component of several disease complexes in numerous species of cereals, forage, and turf grasses (Smiley *et al*., 1992). In cool season amenity turfgrasses, *M. nivale* is regarded as the most important pathogen in temperate climates (Smiley *et al.*, 1992; Mann, 2002). The interaction between plants and their pathogens is complex, involving a wide range of defence processes. Upon pathogen challenge, plants initiate an array of induced defences involving numerous interconnected signaling pathways. Amongst these are: the Hypersensitive Response (HR), generation of Reactive Oxygen Species (ROS), programmed cell death (PCD), production of antimicrobial compounds, such as Pathogenesis Related (PR) proteins and phytoalexins, and salicylic acid (SA) which is key for induction of systemic resistance (Campbell and Reece, 2002). Many of these defence compounds are classed as phenols, and synthesis and accumulation of total phenolic content (TPC) upon pathogen challenge has been shown to be an important defence response in plants. For example, the phenolic compound lignin is produced to strengthen cell walls, thus increasing resistance to pathogen ingression, and lignin levels have been shown to increase following pathogen infection in grasses (Panka *et al*., 2013; Wang *et al*., 2016). Pociecha *et al.* (2009) concluded that increased levels of phenolic compounds gave rise to higher resistance to *M. nivale* in *Festulolium* species. Numerous other studies have identified defensive phenolic production in species of gramineae. In barley (*Hordeum vulgare*), phenolic compounds have been identified as phytoalexins in plants challenged with the pathogen *Blumeria graminis* causing powdery mildew (Christensen *et al*., 1998; Kruger *et al*., 2002). Oat (*Avena sativa*) produces phenolic alkaloid avenanthramides as phytoalexins (Okazaki *et al*., 2004), and wheat (*Triticum* spp.) produces hydroxycinnamic acid amides such as feruloylagmatine (Bélanger *et al*., 2003; Remusborel *et al*., 2005). Phenolic compound accumulation was detected at the site of hyphal penetration, and it was concluded that this reaction was part of the triticale defence system

against *M. nivale* (Dubas *et al.,* 2010). Furthermore, Dubas *et al.* (2010) also observed hydrogen peroxide (H_2O_2) accumulations in tritacle closely associated with *M. nivale* infection sites.

H_2O_2 plays a major role in plant responses to both abiotic and biotic challenges, regulating the expression of defence genes, as a key component of ROS and HR and in strengthening cell walls. It also acts as a signal molecule initiating the transcription of resistance genes coding for PR proteins and for enzymes related to phytoalexin biosynthesis. Moreover, H_2O_2 stimulates the synthesis of SA, leading to induction of Systemic Acquired Resistance (SAR) (Campbell and Reece, 2002). The timely production of H_2O_2 and its accumulation in cells is a useful marker for determining the efficacy of a plant's response to pathogen challenge.

Priming and enhancement of plant defence responses by various means has been demonstrated in many plant systems and has led to a decrease in damage by pathogens (Kuc, 1995; Heil and Bostock, 2002). One treatment shown to reduce disease incidence is phosphite (Phi). Phosphite is derived from the alkali metal salts of phosphorous acid, H_3PO_3, which upon disassociation releases the phosphite ion (HPO_3^{2-}) and when fully oxidised PO_3^3. The ability of Phi to control numerous plant diseases caused by Oomycetes, particularly of the genera *Peronospora, Plasmopara, Phytophthora* and *Pythium* has been well documented (Lobato *et al*., 2011; Burra *et al.*, 2014). Phosphite has also been shown to be effective in reducing *M. nivale* infection in amenity turfgrass (Dempsey *et al.,* 2012; Mattox *et al*., 2020). Evidence has been presented indicating that Phi inhibits phytopathogens by both direct fungistatic means and indirectly through stimulation of host defence processes (Fenn and Coffey, 1987; Jackson *et al*., 2000; McCarren *et al*., 2009; Dempsey *et al*., 2018). Daniel and Guest (2005) concluded that Phi induced rapid defence responses, including ROS, PCD and an increase in phenolic compounds in *Arabidopsis thaliana* inoculated with *Phytophthora palmivora*. Lobato *et al*. (2011) showed that potato tubers, following foliar treatment with

Phi, exhibited a reduced susceptibility to *P. infestans*, *Fusarium solani* and *Pectobacterium carotovorum* infections. They suggested that Phi induces a systemic defence response in the treated plants, based partly on their findings of increased levels of phytoalexins. Following Phi treatment and inoculation with *P. cinnamomi*, Eshraghi *et al*. (2011) showed that *A. thaliana* exhibited increased levels of H_2O_2 production, with significant differences in the amount of H_2O_2 production between Phi-treated and untreated control plants.

In the amenity turfgrass industry, Phi products are often marketed on the basis that Phi primes plants prior to disease challenge, leading to reduced disease susceptibility. As mentioned, Phi has been shown to reduce pathogen challenge by enhancing synthesis of defence compounds (Jackson *et al*., 2000; Daniel and Guest, 2005; Lobato *et al.,* 2011; Olivieri *et al.,* 2012). In these published studies however, it is unclear if the increased synthesis of defence compounds was a result of interaction between the pathogen and Phi *in planta*, or whether Phi induced synthesis of defence related compounds prior to pathogen challenge. The consensus of published research is that while Phi can act directly to inhibit many pathogens, the mode of disease suppression also includes a priming or enhancement of defences in treated plants. To determine if Phi enhanced constitutive or inducible defences, research into the responses of *M. nivale* infected turfgrasses was required.

The aims of this research, therefore, were to assess two important defence responses to pathogen challenge: synthesis and accumulation of total phenolic content and generation of hydrogen peroxide, and to assess the effect of Phi treatment had on priming or enhancing these responses.

Materials and methods

Plant material and growth environments

Leaf tissues of *Agrostis stolonifera* and *Poa annua* were collected from *M. nivale* infected and uninfected plants during October and December 2012, 2013, and 2014. Ten randomised

samples were collected during each period from field trial plots at the Royal Curragh Golf Course, Co Kildare, Ireland. Greenhouse samples of *A. stolonifera*, variety Shark, and *P. annua reptans*, variety Truputt, were established and maintained in 110 mm by 300 mm poly-vinyl chloride (PVC) growth vessels, filled with rootzone soil complying with Sports Turf Research Institute (STRI) recommendations for golf green construction in the UK (Baker, 2005). Following seeding at the optimum rate for the species, they were allowed to establish for four months prior to commencement of experimental procedures. All field samples used were allowed to become infected under natural conditions, whilst greenhouse samples were infected using hyphal inoculum. Non-infected turfgrass was used to provide control samples.

Inoculum source and turfgrass inoculation

Hyphal inoculum was prepared by subculturing four *M. nivale* isolates, two obtained from infected golf greens in Ireland, and two from the Sports Turf Research Institute, Bingley, UK, on Potato Dextrose Agar (PDA) (19 g/l^{-1}) at 20° C for five days prior to inoculation of plants. PDA/fungal combinations were removed from the plates, placed in a glass vessel, and blended with 150 ml sterile distilled water (SDW), using a domestic food blender until finely separated. Turfgrass samples were inoculated by spraying with 5 ml of the hyphal suspension to leaf surfaces, which had been wetted with SDW within five minutes prior to inoculation. Non-inoculated controls were prepared by spraying the leaves with SDW. The inoculated and non-inoculated control pots were maintained at high levels of relative humidity in transparent plastic bins, with paper towels moistened with SDW placed along the bottom to prevent drying out.

Determination of total phenolic content in *A. stolonifera* and *P. annua*

Total phenolic content (TPC) was determined by modified extraction methods as described by Singleton *et al.* (1965). Turfgrass tissues were collected and dried for 48 h at 50° C, then ground using a mortar, and pestle, and 0.5 g boiled in 1 ml 80% ethanol; a further 4 ml 80%

ethanol was added and incubated at room temperature for 24 h. The extract solution was filtered and centrifuged for 20 min at 1500 g. Following this, 20 µl was pipetted into separate 20 ml containers and 1.58 ml SDW and 100 µl of Folin-Ciocalteu reagent was added to each 20 ml container. The solutions were vortexed and left for 8 min, after which 300 µl 20% sodium carbonate (Na_2CO_3) solution was added, and the mixture was vortexed again. The solutions were incubated at 20° C for 2 h. Absorbances were read using a Cecil CE 373 spectrophometer at 765 nm and compared with a Gallic acid standard curve. Total phenolic content was calculated and reported as mg/g dry weight of Gallic acid equivalents (GAE).

Determination of hydrogen peroxide generation in *Agrostis stolonifera* and *Poa annua*

Hydrogen peroxide generation in *P. annua* and *A. stolonifera* was determined by forming a titanium hydroperoxide complex, as described by Dagmar *et al*. (2001). Tissue samples (0.2 g) were homogenised in liquid nitrogen, ground with 5 ml cooled acetone and the homogenate centrifuged at 6,000 g for 10 min. The supernatant (1 ml) was put on ice and combined with 0.1 ml 5% titanium oxysulfate and 0.2 ml ammonia. The reaction mixture was centrifuged at 10,000 g for 10 min. The supernatant was discarded, and the precipitate dissolved in 5 ml 2 mM H_2SO_4. The absorbance of the resulting solution was read at 415 nm and H_2O_2 content determined using a standard curve plotted with known quantities of H_2O_2 (Wang *et al.*, 2010).

Assessment of the effects of *M. nivale* infection on total phenolic content (TPC) and hydrogen peroxide generation in *A. stolonifera* and *P. annua.*

Accumulation of TPC in response to *M. nivale* infection in *A. stolonifera* and *P. annua* was determined from leaf tissues collected from infected field trial plots and inoculated greenhouse plants, between September 2012 and March 2014. Tissue samples from the boundary zone between green, visually healthy, and dead desiccated parts of leaves were

154 collected on four separate occasions during each of the three years, and mean values of TPC
155 were determined and compared with TPC in uninfected samples.
156 Hydrogen peroxide generation in response to infection was determined by analyses of
157 infected greenhouse tissues. Field samples were not used, as the generation of H_2O_2 upon
158 infection is rapid and transitory and would have been difficult to assess under field conditions.
159 Following hyphal inoculation of greenhouse grown *A. stolonifera* and *P. annua* plants,
160 generation of H_2O_2 was determined over the course of 72 h post-inoculation (hpi) and
161 compared with H_2O_2 in uninfected plants in the same greenhouses. This was performed on
162 four separate occasions, with ten replications (n=10); the data were then pooled.
163 **Assessment of the effect of phosphite treatment on total phenolic content and hydrogen**
164 **peroxide generation in *M. nivale* infected and uninfected *Agrostis stolonifera* and *Poa***
165 ***annua***
166 The effects of phosphite treatment on TPC and H_2O_2 generation in healthy, uninfected *Poa*
167 *annua* and *A. stolonifera* were assessed by treating trial plots and greenhouse samples with
168 Phi and Pi at 0.35g/m^2, and SDW (control), using 20 l knapsack sprayers fitted with flat
169 fan nozzles, operating at 4 bar, calibrated to deliver 300 l/ha. Treatments were either
170 sequential, as six treatments applied at four-week intervals, or applied as a single
171 treatment. Leaf tissues were harvested, following the final sequential treatment application
172 and after the single treatment at 0, 1, 6, 24, 48 and 72 h post application (hpa). Total
173 phenolic content and H_2O_2 generation were determined, and comparisons made between
174 the Phi, Pi and SDW control levels.
175 Treatment effects on *M. nivale* infection diameters, and on TPC and H_2O_2 generation in *M.*
176 *nivale* infected *Poa annua* and *A. stolonifera,* was assessed on greenhouse samples, which
177 had been treated previously either sequentially or singly as above. Following infection

with *M. nivale* hyphal inoculum, TPC levels and infection diameters were determined over 10 dpi, H_2O_2 generation was determined over 72 hpi, and comparisons made between the Phi, Pi and SDW control levels.

Data analysis

All treatments were randomised with ten replications. Prior to analyses, residuals were tested to ensure the assumptions of the one-way Anova were satisfied. Outliers were assessed by inspection of a boxplots, Shapiro-Wilk's test determined normality (Shapiro and Wilke, 1965) and homogeneity of variances assessed by Levene's test (Levene, 1960). Two-way Anova determined statistical differences, using dependent variables of TPC and H_2O_2 levels in infected and uninfected tissues, and *M. nivale* infection diameters (mm) in inoculated turfgrass samples, with turfgrass species and treatments as independent variables. Following significant interactions, pairwise comparisons using 95% confidence intervals and Bonferroni-adjusted p-values determined significant differences. All data analyses were performed using the statistical program SPSS Statistics 22.

Results

Total phenolic content and H_2O_2 generation in *Agrostis stolonifera* and *Poa annua* as influenced by *M. nivale* infection

Mean levels of TPC were determined as GAE mg/g dw in *M. nivale* infected tissues of *A. stolonifera* and *P. annua* and compared with TPC levels in uninfected samples. Assessments were made over a three-year period between 2012 and 2014, in trial plots and greenhouse samples, with mean levels of TPC shown Table 1. In both species, during each year of assessment, TPC was significantly greater ($p < 0.05$) in the infected tissues compared with the uninfected. In field samples, TPC in the infected tissues of *A. stolonifera* ranged between 2.29 and 2.42 mg/g, compared to 1.78 and 2.08 mg/g in uninfected tissues. Similarly, in *P.*

annua TPC in the infected tissues ranged between 2.25 and 2.45 mg/g, compared to 1.82 and 2.04 mg/g in uninfected tissues.

In greenhouse samples, TPC was also significantly greater ($p < 0.05$) each year of assessment in the infected tissues compared to the uninfected in both turfgrass species (Table 1). Total phenolic content in the infected tissues of *A. stolonifera* ranged between 2.34 and 2.55 mg/g, compared to 1.94 and 2.07 mg/g in uninfected samples. In *P. annua*, TPC ranged between 2.23 and 2.42 mg/g in the infected tissues, while the uninfected levels ranged between 1.75 and 1.89 mg/g.

Hydrogen peroxide generation levels in response to *M. nivale* infection were measured only on inoculated greenhouse samples of *A. stolonifera* and *P. annua* over 72 hpi. In both *A. stolonifera* and *P. annua* there were significantly greater amounts generated in infected tissues compared to uninfected tissues at 1 and 6 hpi (Table 2). At all other time periods there were no statistical differences between infected and uninfected tissues.

Effect of phosphite treatment on TPC and H_2O_2 generation in uninfected *Agrostis stolonifera* and *Poa annua*

There were significant ($p < 0.05$) treatment effects in TPC in both field and greenhouse samples of *P. annua* and *A. stolonifera* following six sequential control, Pi and Phi treatments applied at four-week intervals. Analyses of field samples, collected one-week post-final treatment, showed that in *A. stolonifera,* TPC in Phi treated plants was 3.06 mg/g, significantly greater ($p < 0.05$) than the Pi treated plants at 2.41 mg/g, which were significantly greater ($p < 0.05$) than the controls with 2.13 mg/g. In *P. annua,* TPC was 2.89 mg/g following Phi treatment, significantly greater ($p < 0.05$) than the Pi and control levels which were statistically the same at 2.26 and 2.19 mg/g respectively (Table 3).

Assessment of *A. stolonifera* and *P. annua* infected tissues, collected one week post treatment from greenhouse samples, gave similar results as those collected from the field. In *A.*

227 *stolonifera,* levels in Phi treated plants were significantly greater (2.85 mg/g) ($p < 0.05$), than
228 both the Pi and control levels which were statistically the same at 2.01 and 2.00 mg/g,
229 respectively. In *P. annua*, TPC was 3.18 mg/g following Phi treatment, significantly greater
230 ($p < 0.05$) than the Pi treated plants at 2.32 mg/g, which were significantly greater ($p < 0.05$)
231 than the controls with 2.08 mg/g (Table 3).
232 Phi treatment applied as a single application also had a significant ($p < 0.05$) effect on TPC
233 compared to Pi and controls, in both field and greenhouse samples of *P. annua* and *A.*
234 *stolonifera.* Total phenolic content in field samples, following a single Phi treatment and
235 collected one-week post-treatment, showed significantly greater ($p < 0.05$) amounts
236 compared to controls in *A. stolonifera* at 1, 6, 12, 24, 48 and 72 hpa and in *P. annua* at 12, 24,
237 48 and 72 hpa. There were also significantly greater ($p < 0.05$) levels of TPC in Phi compared
238 to Pi treated tissues of *A. stolonifera* at 1, 6 and 12 hpa and in *P. annua* tissues at 12, 24 and
239 72 hpa (Table 4). Phosphate treatments led to significantly higher ($p < 0.05$) levels of TPC
240 compared to controls in *A. stolonifera* at 12, 24, 48 and 72 hpa and in *P. annua* at 12, 24, 48
241 and 72 hpa, with significantly greater ($p < 0.05$) TPC compared to Phi treatment at 24 hpa in
242 *P. annua* (Table 4).
243 In the greenhouse samples, following a single Phi treatment, TPC in *A. stolonifera* was
244 significantly greater ($p < 0.05$) than controls at 12, 24, 48 and 72 hpa, and in *P. annua* at 1,
245 12, 24, 48 and 72 hpa. There were also significantly greater ($p < 0.05$) TPC in Phi treated
246 tissues compared to Pi in *A. stolonifera* at 48 hpa and in *P. annua* at 1, 12, 24 and 48 hpa.
247 Phosphate treatments led to significantly higher ($p < 0.05$) TPC compared to controls in *A.*
248 *stolonifera* at 12, 24, 48 and 72 hpa and in *P. annua* at 12, 24, 48 and 72 hpa, with
249 significantly greater levels compared to Phi treatments at 24 hpa (Table 4).
250 Generation of H_2O_2 in *A. stolonifera* and *P. annua* was determined as µmol H_2O_2/g fw, over
251 72 h in greenhouse samples, following control, Pi, and Phi treatments. There was a clear

spike in H_2O_2 levels at 6 hpa in Pi and Phi treated tissues in both turfgrass species. During each time period, there were no significant ($p > 0.05$) differences in H_2O_2 generation between Pi and Phi treated tissues in either turfgrass species (Table 5).

Effect of phosphite treatments on TPC and H_2O_2 generation in *M. nivale* infected *Agrostis stolonifera* and *Poa annua*

Following hyphal inoculation of greenhouse samples of *A. stolonifera* and *P. annua*, conditions were ideal for *M. nivale* infection, which led to rapid development of disease symptoms. By 10 dpi, disease patch diameters in *A. stolonifera* were: control 97.67 mm, Pi 91.34 mm, Phi (single treatment) 58.85 mm and Phi (6 sequential treatments) 46.85 mm. Similarly, with *P. annua*, at 10 dpi patch diameters were: control 99.55 mm, Pi 100.00 mm, Phi (single treatment) 69.35 mm and Phi (6 sequential treatments) 62.30 mm (Table 6). Phosphite, applied either as a single treatment or sequentially over six applications, led to significant reductions ($p < 0.05$) in patch diameters, compared to controls and Pi treatments at 2, 4, 6, 8 and 10 dpi in both turfgrass species. Phosphite applied sequentially, also gave rise to significant reductions ($p < 0.05$) in patch diameters compared to a single Phi treatment at 2, 4, 6, 8 and 10 dpi in both turfgrass species (Table 6).

Analyses of infected tissues over the course of 10 dpi showed there was increasing accumulation of TPC in all samples with statistically significant ($p < 0.05$) differences between treatments in each time (Table 7). Both turfgrass species treated with either a single or six sequential treatments of Phi accumulated significantly ($p < 0.05$) higher levels of TPC than Pi treated or control tissues, at all measurement periods from 2 to 10 dpi (Table 7). In *A. stolonifera,* Phi (six sequential treatments) induced the greatest TPC which was significantly greater ($p < 0.05$) than all other treatments at each time. Total phenolic content in tissues treated with Phi (single treatment) was significantly greater ($p < 0.05$) than Pi and control treatments at all time periods, except for at 0 dpi. The Pi treatment induced TPC

277 significantly more ($p < 0.05$) than controls at 2 and 6 dpi, with the control TPC significantly
278 greater ($p < 0.05$) than the Pi treatments at 8 and 10 dpi (Table 7).
279 In *P. annua,* Phi (six sequential treatments) also induced the largest TPC, significantly
280 greater ($p < 0.05$) than all other treatments, at each time. Total phenolic content in tissues
281 treated with Phi (single treatment) was significantly greater ($p < 0.05$) than Pi and control
282 treatments at all time periods, except for 0 dpi. The Pi treatment induced TPC significantly
283 greater ($p < 0.05$) than controls at 2, 6, 8 and 10 dpi (Table 7).
284 Following *M. nivale* inoculation, there were significant treatment effects on H_2O_2 generation
285 in Phi (single treatment), and Phi (six sequential treatments), treated tissues in both turfgrass
286 species. In *A. stolonifera,* H_2O_2 accumulation in the Phi (single treatment), and Phi (six
287 sequential treatments) was statistically the same at all time periods but was significantly
288 greater at 1 and 6 hpi than both the control and Pi treated plants. There were no significant
289 differences between treatments at any other time period. In *P. annua*, there were no
290 significant differences between treatments at any time period with the exception of the Phi
291 (six sequential treatments) treated plants where H_2O_2 accumulations were significantly
292 greater than all other treatments at 1 and 6 hpi.

293 **Discussion**

294 Plants produce a broad, complex array of induced defences and interconnected signaling
295 pathways, which combine to combat invading microorganisms. The goals of this study,
296 therefore, were to compare host defences in *A. stolonifera* and *P. annua* in response to *M.*
297 *nivale* infection by measuring total phenolic content and H_2O_2 generation before and during
298 the infection process, and to assess the effect of Phi treatment on these induced responses and
299 subsequent effects on *M. nivale* disease suppression.
300 Accumulation of TPC is a common response to pathogen challenge, and the speed of
301 accumulation of phenolics prior to or following infection can influence the plant's level of

susceptibly or resistance to a particular pathogen. TPC accumulation is also a response to abiotic stresses in response to mechanical injury, drought, UV radiation and low temperature. It was therefore important to sample turfgrass tissues during a wide range of conditions and that treated turfgrasses and controls were harvested under identical situations. This ensured that TPC levels due to non-disease related pressures did not influence the assessments. In this study, mean levels of TPC were assessed in infected and non-infected turfgrass tissues grown in the field and greenhouse. As shown in Table 1, *M. nivale* infection led to increased TPC accumulations in both situations, and that levels in infected tissues were significantly higher ($p<0.01$) than in non-infected plants. Hydrogen peroxide generation in response to infection was measured in *M. nivale* inoculated greenhouse samples only. In a similar manner to TPC generation, infection in both species led to significantly higher accumulations of H_2O_2 in infected plants compared to uninfected at 1 and 6 hpa (Table 2). These data confirm that in *A. stolonifera* and *P. annua* increased TPC and H_2O_2 generation are components of defence responses following biotic challenge. This accumulation of TPC in response to pathogen challenge and its importance in plant resistance has been documented previously in other graminaceous species (Ishihara *et al.*, 1999; Jin and Yoshida, 2000; Okazaki *et al.*, 2004; Remusborel *et al.*, 2005). A major aim of this research was to determine if Phi treatment could stimulate or enhance these accumulations. Results here showed that a single Phi treatment did influence TPC accumulations, leading to significantly higher levels, compared to controls in both field and greenhouse samples. Following Phi treatment TPC levels in field samples were significantly ($p < 0.05$) higher in *A. stolonifera* tissues from 1 to 72 hpa and in *P. annua* from 12 to 72 hpa (Table 4). The increases in TPC were similar in treated greenhouse turfgrasses, with levels in *A. stolonifera* significantly higher than controls from 12 to 72 hpa, and in *P. annua* at 1 hpa and from 12 to 72 hpa (Table 4). While these data indicate that a single Phi treatment

stimulated TPC accumulation, it can be argued that regarding induced defence responses in plants it was not different to the response elicited from the Pi treatments. Phosphate treatment, in both field and greenhouse turfgrasses, at over 72 hpa also led to significantly higher ($p < 0.05$) TPC levels compared to controls, from 12 to 72 hpa, in a similar manner to Phi treatment (Table 4).

However, while a single treatment of Phi and Pi elicited similar responses in TPC levels, sequentially applied treatments, over a period of six months, gave rise to significantly higher levels in Phi treated tissues compared with both Pi and control tissues (Table 3). These data, therefore, would indicate that Phi can prime plants for stress responses, but also that several applications prior to infection are required for Phi mediated disease suppression to be successful.

This, however, is not fully supported when the data in relation to the suppression of disease incidence in greenhouse inoculated plants is examined. *Microdochium nivale* infected greenhouse plants, following a single application of Phi prior to inoculation, gave rise to higher accumulations of TPC (Table 7) and significantly reduced the size of infection patches compared with Pi and untreated controls (Table 6).

A factor that should be considered is that Phi has been shown to have a direct fungistatic effect on *M. nivale* (Dempsey *et al.,* 2018). The presence of Phi in the tissues directly inhibits pathogen development, thus increasing the plants' reaction time and its synthesis of defence compounds. Disease suppression as noted here following a single Phi treatment could be construed as evidence of the fungistatic properties of Phi. But the data here show significantly higher levels of TPC following a single Phi treatment, which leads to the conclusion that Phi may have a dual role, in that it can have a fungistatic effect but also enhances TPC prior to, and during, biotic stress. This would infer that Phi not only

stimulates TPC accumulations in uninfected plants but that during infection allows for a more rapid response than the Pi treated plants.

The priming of TPC levels and subsequent disease suppression by Phi pre-treatment is further supported by the data presented in Tables 3 and 6. Phosphite, when applied sequentially over a six-month period, allowed for significantly greater cumulative TPC in treated turfgrasses, when compared to Pi and controls. This priming and increased TPC accumulation subsequentially led to significantly greater *M. nivale* suppression. Total Phenolic Content in both *A. stolonifera* and *P. annua,* as shown in Table 7, following six sequential Phi treatments, was significantly greater prior to *M. nivale* inoculation and, as infection progressed, remained significantly greater than those in Pi, Phi (single treatment) and controls. These significant differences in TPC subsequently led to reduced disease incidence, as determined by the infection diameters shown in Table 6. Disease incidence was significantly less in plants following sequential treatments of Phi than in those in Pi, Phi (single treatment) and controls. The increased levels of TPC following either a single or sequential treatments of Phi shows a direct correlation between increased levels of TPC and suppression of *M. nivale* disease symptoms. The results here are significant in that not only did Phi treatment suppress disease symptoms and increased TPC following pathogen challenge, but sequential treatments also primed the plants by increasing accumulations of TPC, prior to infection, thus allowing a more rapid and efficient defence response.

Hydrogen peroxide plays a major role in a plant's response to pathogen challenge, with the speed of generation and accumulation at infection sites providing a measure of a plant's level of resistance or susceptibility to a particular pathogen. As well as having direct antimicrobial properties, H_2O_2 is a component of the hypersensitive response, which produces a rapid, localised, but transient, oxidative burst, directly impairing the pathogen. In this study, when H_2O_2 generation was compared between *M. nivale* infected and uninfected tissues, as shown

376 in Table 2, there was clear evidence of this, with significantly greater accumulations in
377 infected tissues of both *A. stolonifera* and *P. annua*. The transient nature of the role H_2O_2 plays
378 as part of initial stress responses was also shown here. When measured over a 72 h period
379 following *M. nivale* inoculation, there was a rapid generation of H_2O_2 at 1 hpi, with peak
380 accumulations at 6 hpi. Following this, accumulations diminished and were statistically the
381 same as those in the uninfected plants.
382 The stimulatory effect of Phi treatment on H_2O_2 generation was examined here and as can be
383 seen in Table 5, while there were statistically greater accumulations at 1 and 6 hpa, in both
384 turfgrass species compared to controls, there was no differences between the Phi and Pi
385 treatments. This stimulation of H_2O_2 generation, following all treatments, could be attributed
386 to abiotic stress responses, induced by the salt content of the nutrient solutions.
387 Whether Phi pre-treatment could enhance H_2O_2 in response to infection was also studied here.
388 Table 8 shows the effect treatments had on H_2O_2 generation following *M. nivale* inoculation.
389 The greatest effect was observed with *A. stolonifera* where Phi treatment, either applied
390 singly or sequentially, led to significantly greater amounts of H_2O_2 at 1 and 6 hpi compared to
391 either the controls or Pi treatments. In *P. annua* however it was only the sequentially applied
392 Phi that had this effect. It would appear therefore that Phi pre-treatment does have a
393 beneficial effect on stimulating early defence responses. This would be supported by the
394 conclusions of Eshraghi *et al.* (2011) who showed increased levels of H_2O_2 in response to
395 pathogen challenge in *A. thaliana,* and that there were significant differences evident between
396 the amount of H_2O_2 production between the Phi-treated and non-Phi- treated plants.
397 Why there was a significantly greater accumulation of H_2O_2 during the infection period, in
398 the Phi treated plants compared to the Pi treatments, is of interest. It was shown here (Table
399 5) that there were no differences in H_2O_2 accumulations between Pi and Phi treatments in
400 uninfected tissues. Despite this, Phi treatments led to greater H_2O_2 generation during

infection, and this may indicate a Phi/pathogen interaction. Phosphite, when applied to turfgrass, remains stable in the leaf, and has fungistatic properties (Dempsey, *et al.,* 2022), which may have stressed the pathogen allowing for increased production of elicitors, thus increasing the plants' reaction time and greater induction of H_2O_2.

Induced generation of H_2O_2 is concentrated at sites of infection and sampling of whole leaves to determine H_2O_2 via extraction methodology may not be sensitive enough to quantify functional changes in levels. Investigation of localised H_2O_2 generation during pathogen challenge has used fluorescence microscopy to visualise H_2O_2 at sites of infection (Huckelhoven *et al*.,1999; Dubas *et al.,* 2010). This may be a useful method to further study the Phi, turfgrass and *M. nivale* interaction in the future.

This study showed that phenolic compounds and H_2O_2 are components of responses to *M. nivale* infection in *A. stolonifera* and *P. annua*. Phosphite treatment led to enhanced TPC accumulations in both infected and uninfected plants and subsequently reduced severity of infection. Results of H_2O_2 extractions indicated that while Phi treatment increased H_2O_2 generation compared to controls, the effect was no different to the Pi treated plants.

It was evident that Phi treatment increased total phenolic content, but how this occurs is not clear. The possibility that it is by induction of physiological stress or interference with the Pi uptake mechanisms merits investigation. Whilst this study has provided novel data on the process of *M. nivale* infection of turfgrass and plant responses, the mechanisms by which Phi interacts with and influences these responses requires further research.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request (Dempsey, 2022).

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Table 1. Total phenolic content as GAE mg/g dw in *M. nivale* infected and uninfected *A. stolonifera* and *P. annua* leaf tissues, sampled from field trial plots and greenhouse plants over three years. Letters indicate significant differences at each time determined by pairwise comparisons using Bonferroni correction at $p < 0.05$, n=10

Table 2. H_2O_2 concentrations as µmol H_2O_2/g fresh weight (fw) in *M. nivale* infected and uninfected *A. stolonifera* and *P. annua* leaf tissues, sampled from greenhouse plants over 72 h post-inoculation (hpi). Data are pooled from 4 infection periods. Letters indicate significant differences at each time determined by pairwise comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 3. Total phenolic content as GAE mg/g dw collected from greenhouse tissue samples of *A. stolonifera* and *P. annua*. Analyses carried out 48 h post final treatment of six, monthly sequential applications of SDW (control), Pi and Phi. Letters indicate significant differences determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 4. Total phenolic content as GAE mg/g dw of turfgrass tissues sampled from trial plots and greenhouse samples of *A. stolonifera* and *P. annua* over 72 h following SDW (control), Pi and Phi treatment. Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 5. H_2O_2 concentrations as µmol H_2O_2/g fw in *A. stolonifera* and *P. annua* leaf tissues collected from greenhouse samples over 72 h following SDW (control), Pi and Phi treatment. Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 6. *M. nivale* infection diameters in mm at 10 dpi in greenhouse samples of *A. stolonifera* and *P. annua* treated with SDW (control), Pi, Phi (1 treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 7. Total phenolic content as GAE mg/g dw in *M. nivale* infected *A. stolonifera* and *P. annua* tissues over 10 dpi in greenhouse samples treated with SDW (control), Pi, Phi (single treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 8. H_2O_2 concentrations as µmol H_2O_2/g fw in *M. nivale* infected tissues of *A. stolonifera* and *P. annua* over 72 hpi, following treatments with SDW (control), Pi, Phi (single treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Table 1. Total phenolic content as GAE mg/g dw_{-5} in *M. nivale* infected and uninfected *A. stolonifera* and *P. annua* leaf tissues, sampled from field trial plots and greenhouse plants over three years. Letters indicate significant differences at each time determined by pairwise comparisons using Bonferroni correction at $p < 0.05$, n=10

Total phenolic content as GAE mg/g dw of leaf tissues.						
Field plots						
	A. stolonifera			*P. annua*		
	2012	2013	2014	2012	2013	2014
Uninfected	1.78a	1.89a	2.08a	1.82a	1.97a	2.04a
Infected	2.42b	2.35a	2.29b	2.45b	2.25b	2.38b
Greenhouse samples						
	A. stolonifera			*P. annua*		
	2012	2013	2014	2012	2013	2014
Uninfected	1.94a	2.07a	1.95a	1.82a	1.89a	1.75a
Infected	2.34b	2.46b	2.55b	2.23b	2.42b	2.27b

Table 2. H_2O_2 concentrations as µmol H_2O_2/g fresh weight (fw) in *M. nivale* infected and uninfected *A. stolonifera* and *P. annua* leaf tissues, sampled from greenhouse plants over 72 h post inoculation (hpi). Data are pooled from 4 infection periods. Letters indicate significant differences at each time determined by pairwise comparisons using Bonferroni correction at $p < 0.05$, n=10.

H_2O_2 as µmol H_2O_2/g fresh weight in infected and uninfected greenhouse plants							
Agrostis stolonifera							
	0 hpi	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Uninfected	19.01a	19.70a	21.50a	20.77a	22.00a	20.28a	19.65a
Infected	18.71a	22.65b	26.58b	22.07a	21.19a	19.85a	20.25a
Poa annua							
	0 hpi	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Uninfected	18.80a	18.73a	20.16a	18.30a	19.81a	19.75a	18.74a
Infected	18.60a	20.84b	24.18b	17.60a	19.26a	19.87a	19.90a

Table 3. Total phenolic content as GAE mg/g dw collected from greenhouse tissue samples of *A. stolonifera* and *P. annua*. Analyses carried out 48 h post final treatment of six, monthly sequential applications of SDW (control), Pi and Phi. Letters indicate significant differences determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Total phenolic content as GAE mg/g dw of leaf tissues.		
Agrostis stolonifera		
	Field plots	Greenhouse
Control	2.13a	2.00a
Pi	2.41b	2.01a
Phi	3.06c	2.85b
Poa annua		
	Field plots	Greenhouse
Control	2.19a	2.08a
Pi	2.26a	2.32b
Phi	2.89b	3.18c

Table 4. Total phenolic content as GAE mg/g dw of turfgrass tissues sampled from trial plots and greenhouse samples of *A. stolonifera* and *P. annua* over 72 hours following SDW (control), Pi and Phi treatment. Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Total phenolic content as GAE mg/g dw of turfgrass tissues sampled from field trial plots							
				Agrostis stolonifera			
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	2.00a	1.94a	2.09a	1.95a	2.05a	2.07a	2.02a
Pi	2.01a	1.97a	2.15a	2.15b	2.26b	2.27b	2.19b
Phi	1.95a	2.07b	2.27b	2.22c	2.24b	2.25b	2.16b
				Poa annua			
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	1.93a	1.88a	2.02a	1.89a	1.98a	2.00a	1.95a
Pi	1.94a	1.91a	1.98a	2.08b	2.19b	2.20b	2.12b
Phi	1.89a	1.91a	2.00a	2.18c	2.12c	2.20b	2.17c

Total phenolic content as GAE mg/g dw of turfgrass tissues sampled from greenhouse samples							
				Agrostis stolonifera			
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	2.12a	2.21a	2.24a	2.19a	2.25a	2.28a	2.20a
Pi	2.09a	2.22a	2.29a	2.32b	2.40b	2.34b	2.31b
Phi	2.13a	2.25a	2.27a	2.36b	2.34c	2.40c	2.29b
				Poa annua			
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	2.10a	2.01a	2.15a	2.10a	2.01a	1.96a	1.99a
Pi	2.06a	1.99a	2.16a	2.18b	2.10b	2.22b	2.19b
Phi	2.07a	2.11b	2.14a	2.23c	2.19c	2.27c	2.16b

Table 5. H_2O_2 concentrations as µmol H_2O_2/g fw, in *A. stolonifera* and *P. annua* leaf tissues collected from greenhouse samples over 72 hours following SDW (control), Pi and Phi treatment. Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

H_2O_2 concentrations as µmol H_2O_2/g fw, in turfgrass leaf tissues

Agrostis stolonifera							
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	18.80a	18.73a	20.16a	15.30a	18.81a	21.75a	18.44a
Pi	18.60a	19.64a	24.18b	16.03a	19.26a	21.87a	19.90a
Phi	17.99a	19.18a	22.78b	15.66a	19.72a	20.99a	20.99a
Poa annua							
	0 hpa	1 hpa	6 hpa	12 hpa	24 hpa	48 hpa	72 hpa
Control	19.20a	19.70a	21.50a	20.77a	22.00a	20.28a	19.65a
Pi	20.12a	22.65b	25.58b	21.77a	23.19b	19.85a	20.25a
Phi	19.66a	21.68b	26.03b	21.26a	23.95b	20.25a	21.21a

Table 6. *M. nivale* infection diameters in mm, 10 dpi, observed in greenhouse samples of *A. stolonifera* and *P. annua* treated with SDW (control), Pi, Phi (1 treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time period as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Greenhouse infection diameters (mm)					
Agrostis stolonifera					
Treatment	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi
Control	14.91a	40.72a	64.97a	81.32a	97.67a
Pi	11.24b	36.39b	72.09b	80.11a	91.34b
Phi 1 treatment	7.12c	22.37c	37.38c	48.95b	58.85c
Phi 6 treatments	4.35d	15.65d	30.38d	37.95c	46.85d
Poa annua					
Treatment	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi
Control	19.25a	48.15a	73.15a	87.75a	99.55a
Pi	14.55b	40.20b	79.85b	94.55b	100.00b
Phi 1 treatment	7.65c	33.45c	48.65c	61.74c	69.35c
Phi 6 treatments	4.55d	22.55d	42.35d	53.25d	62.30d

Table 7. Total phenolic content as GAE mg/g dw, in *M. nivale* infected *A. stolonifera* and *P. annua* tissues over 10 dpi in greenhouse samples treated with SDW (control), Pi, Phi (single treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

Total phenolic content as GAE mg/g dw, in *M. nivale* infected tissues

Agrostis stolonifera						
	0 dpi	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi
Control	1.79a	2.08a	2.42a	2.44a	2.60a	2.47a
Pi	1.73a	2.40b	2.46a	2.63b	2.51b	2.43b
Phi 1 treatment	1.78a	2.53c	2.58b	2.84c	2.71c	2.74c
Phi 6 treatments	2.03b	2.59d	2.72c	2.94d	2.81d	2.80d
Poa annua						
	0 dpi	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi
Control	1.92a	2.13a	2.34a	2.43a	2.22a	2.18a
Pi	1.99a	2.22b	2.37a	2.53b	2.32b	2.24b
Phi 1 treatment	1.95a	2.35c	2.50b	2.69c	2.70c	2.51c
Phi 6 treatments	2.12b	2.44d	2.61c	2.77d	2.79d	2.55d

Table 8. H_2O_2 concentrations as µmol H_2O_2/g fw, in *M. nivale* infected tissues of *A. stolonifera* and *P. annua* over 72 hpi, following treatments with SDW (control), Pi, Phi (single treatment) and Phi (6 sequential treatments). Letters indicate significant differences at each time as determined by Post hoc comparisons using Bonferroni correction at $p < 0.05$, n=10.

H_2O_2 concentrations as µmol H_2O_2/g fw							
Agrostis stolonifera							
	0 hpi	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Control	18.05a	23.21a	24.65a	20.65a	20.45a	19.35a	18.22a
Pi	19.86a	22.23a	23.95a	19.33a	19.45a	20.12a	18.85a
Phi 1 treatment	18.82a	24.69b	26.43b	19.65a	20.65a	18.75a	18.94a
Phi 6 treatments	18.33a	24.86b	27.85b	20.37a	20.64a	20.75a	20.05a
Poa annua							
	0 hpi	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Control	18.46a	21.66a	25.22a	21.15a	20.92a	19.79a	18.64a
Pi	20.32a	21.72a	24.85a	20.77a	19.90a	20.58a	19.28a
Phi 1 treatment	19.26a	22.23a	26.02a	20.10a	21.12a	19.19a	19.37a
Phi 6 treatments	18.76a	25.43b	27.77b	19.88a	21.11a	21.23a	20.51a