Suppression of the *in vitro* growth and development of *Microdochium nivale* by phosphite

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Abstract

The ascomycete fungus *Microdochium nivale* is a major pathogen of many species of the gramineae. Control measures rely heavily on chemical fungicides, making alternative means of disease reduction desirable. Phosphite (PO_3^{3-}) has proven efficacy in reducing susceptibility of different species of gramineae to oomycetes, and has adverse effects on the *in vitro* growth of numerous other pathogens. The effect of phosphorous acid (H_3PO_3), phosphoric acid (H_3PO_4), dihydrogen potassium phosphite (KH_2PO_3), dihydrogen potassium phosphate (KH_2PO_4), and potassium hydroxide (KOH) on the *in vitro* mycelial growth and development of *M. nivale* was determined. Radial growth on amended Potato Dextrose Agar (PDA) was used to calculate mean daily growth and percent inhibition. PO_3^{3-} had a significant inhibitory effect on mycelial growth with EC_{50} values ranging between 35.9 and 40.99 $\mu g/ml^{-1}$, whilst PO_4^{3-} and KOH had no significant inhibitory effect. Microscopic examination of mycelia showed morphological deformities in hyphae growing on PO_3^{3-} amended PDA, whilst hyphal growth was normal on PO_4^{3-} and KOH amended PDA. Conidial germination of *M. nivale* was significantly reduced following immersion in solutions of 50, 100 and 250 µg/ml of PO_3^{3-}, PO_4^{3-} and KOH at same concentrations induced no inhibitory affect. These results show that PO_3^{3-} is a significant inhibitor of the growth of *M. nivale* and may have the potential to be used as a chemical control agent in the field.

Keywords: *Microdochium nivale*, turfgrass, *in vitro*, phosphite, disease suppression

Introduction

Microdochium nivale (teleomorph *Monographella nivalis* (Schafnitt)) is an ascomycete pathogen and causal agent for many disease complexes in numerous graminaceous species (Smiley *et al.*, 1992; Tronsmo *et al.*, 2001). *Microdochium nivale* produces conidia in large numbers which are readily dispersed by wind and rain splash and, along with soil borne mycelium, are the main source of inoculum (Tronsmo *et al.*, 2001). In turfgrasses, *M. nivale* is regarded as the most damaging pathogen of temperate climates, infecting and causing disease in most cool season species, causing pink snow mould and microdochium patch (Vargas, 2005). Chemical protectants represent the foremost tool used to control this pathogen (Smiley *et al.*, 1992; Yang *et al.*, 2011) and while the efficacy and safety of these plant protection products is not disputed, development of alternative means of reducing susceptibility is desirable. Phosphite is an attractive alternative to established turfgrass plant protectants for a number of reasons, to date there has been no issues regarding resistance, it is highly mobile within the plant, its ability to induce plant defence responses and its reported enhancement of turfgrass quality. While phosphite is registered as a fungicide in some legislations, in many it is regarded as a biostimulant. However it is the alternative mode of action in suppressing numerous plant pathogens that is of interest here.

Phosphite (PO_3^{3-}) is a reduced form of phosphorus (P) derived from the alkali metal salts of phosphorous acid (H_3PO_3) (Guest and Grant, 1991). The pH of phosphorous acid is modified to prevent phytotoxicity, commonly by combining with potassium hydroxide (KOH), forming potassium dihydrogen phosphite (KH_2PO_3) or dipotassium hydrogen phosphite (K_2HPO_3). Phosphite is chemically similar to phosphate (PO_4^{3-}), but the different tetrahedral molecular structure of phosphite ensures that enzymes, which react with phosphate to catalyse metabolic processes, do not bind to phosphite in the same manner ensuring that phosphite does not supply a metabolically usable form of P (Mcdonald *et al.*, 2001). Phosphite, however, has significant properties as an inhibitor of plant pathogens (Fenn and Coffey,

1984). The mode of suppression remains a subject of debate (Abbasi and Lazarovits, 2006) with research showing it as acting both directly on the pathogen and indirectly by stimulating host defences (Guest and Grant, 1991).

The use of *in vitro* studies is an established method to assess a compound's ability either to reduce or inhibit the growth of, or to kill plant pathogenic organisms (Mann, 2002; Glynn *et al.*, 2008; Hofgaard *et al.*, 2010). When compiling a disease protection programme an important factor is determining whether a compound is fungicidal or fungistatic. It is possible that at sufficient concentrations, fungistatic compounds will prevent fungal growth and sporulation fully but, upon removal, the effects are reversed and growth will re-commence. This would have a significant bearing on the application rate and interval.

Most studies on phosphite mediated inhibition of plant pathogens have been on its effects on oomycetes. Suppression of *Pythium* by phosphite under field conditions was reported by Sanders (1983), but when no *in vitro* inhibition was demonstrated it was concluded that control resulted from enhanced host defences. However, Fenn and Coffey (1984, 1987) demonstrated that phosphite inhibited four *Pythium* spp. and *Phytophthora cinnamomi in vitro*. *Phytophthora cinnamomi* exhibited sensitivity to phosphite with EC_{50} values (Effective Concentration which reduces growth by 50% of control growth) ranging from 4 to 148 μg ml^{-1} (Wilkinson *et al.*, 2001). In a later study *Pythium* spp. were inhibited with EC_{50} values between 38.7 and 220.8 $\mu g/ml^{-1}$ (Cook *et al.*, 2009). This direct mode of inhibition seems to involve disruption of the pathogen's metabolism. For example, a study with three *Phytophthora* species showed that phosphite interfered with phosphate metabolism in pathogen cells by causing an accumulation of polyphosphate and pyrophosphate, diverting ATP from other metabolic pathways, resulting in reduced growth (Niere *et al.*, 1994). Other studies determined that phosphite inhibited enzymes of the glycolytic and phosphogluconate

pathways, disrupting phosphorus metabolism in *P. palmivora* by competing with phosphate as an allosteric regulator on several enzymes (Stehmann and Grant, 2000).

Less has been published on the *in vitro* effects of phosphite on fungal pathogens. Reuveni *et al*. (2003) showed inhibition of *Alternaria alternata* mycelial growth and conidial germination, while Burpee (2005) reported suppression of *in vitro* growth of *Colletotrichum cereale* (*Colletotrichum graminicola*). Mills *et al*. (2004) demonstrated that H_2PO_3 not only reduced mycelial growth but caused complete inhibition of sporulation of *A. alternata*, *Botrytis cinerea* and *Fusarium solani.* Growth of *F. culmorum* and *F. graminearum* was reduced on KH_2PO_3 amended PDA (Hofgaard *et al.*, 2010). The same study included the effects of phosphite on *Microdochium majus,* and found that mycelial growth was reduced by more than 90% at the lowest KH_2PO_3 concentration used (10 μg ml^{-1}), with full inhibition at concentrations of 100 μg ml^{-1} (Hofgaard *et al.*, 2010)(Hofgaard *et al.*, 2010)(Hofgaard *et al.*, 2010). However, there has been no published data on the *in vitro* effect phosphite may have on *M. nivale.*

Data from turfgrass field trials conducted to evaluate *M. nivale* suppression by KH_2PO_3, determined that phosphite significantly ($p < 0.05$) suppressed disease symptom expression (Dempsey *et al.*, 2012). The success of these trials led to this current research to discover possible modes of suppression. The aims of this research, therefore, were to determine the effect phosphite may have on the *in vitro* mycelial growth and conidial germination of *M. nivale*, and to determine if phosphite has fungistatic or fungicidal properties.

Materials and methods

Microdochium nivale mycelial and conidial inoculum

Four isolates of *M. nivale* were assessed. Two isolates were obtained from infected *Poa annua* golf greens on Irish golf courses, the remainder from the Sports Turf Research Institute, Bingley, UK. The isolates were confirmed as *M. nivale* by Crops Research, Oak Park, Teagasc,

Carlow, using molecular biology techniques as described by Glynn *et al.* (2005). Conidiation was induced by incubating mycelia in darkness for 48 hours and then exposing to UV light (Jewell and Hsiang, 2013). Conidia were then collected by flooding the plate with sterile distilled water (SDW) and scraping with a sterile rod, immediately before use in experiments.

PDA amendments, H_3PO_3, H_3PO_4, KH_2PO_3, KH_2PO_4 and KOH

Phosphorous acid (H_3PO_3) and phosphoric acid (H_3PO_4), were obtained from 1 M reagent grade solutions (supplied by Lennox Laboratory Supplies, Dublin). Dihydrogen potassium phosphite (KH_2PO_3) and dihydrogen potassium phosphate (KH_2PO_4) amendments were prepared by titrating 1 M solution phosphorus and phosphoric acids with 6 M reagent-grade potassium hydroxide (KOH) to pH 6.5. KOH amendments were prepared from 6 M potassium hydroxide, and all amendments were serial diluted to required concentrations. Unamended PDA, containing no additional chemicals, were used as controls. All experimental compounds were filter sterilised and added to autoclaved Potato Dextrose Agar (PDA, 19 g/l, Himedia Potato Dextrose Agar, Sparks Laboratory Supplies, Dublin), after cooling to 50^{o} C to ensure no oxidation of phosphite to phosphate (Komorek and Shearer, 1997).

Measurement of mycelial growth on solid media

Experiments were a randomised complete design with six replications. Measurement of mycelial growth of *M. nivale* isolates, incubated on PDA amended with 0 (unamended control), 10, 50, 100 and 250 µg/ml of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH were used to calculate mean daily growth (MDG), percent relative growth (PRG), percent inhibition and colony diameters. Agar plugs, 5 mm in diameter, were cut from margins of actively-growing colonies of *M. nivale*, and transferred to the centre of plates of amended PDA then incubated in darkness in a growth chamber maintained at 18° +/- 2^0 C. Mycelial growth rate was determined by measuring the colony radius at four points on each plate, from the edge of the

initial inoculum to the extreme outer margin area of fungal mycelial development and growth rates (mm day^{-1}) calculated. Radial growth measurements were taken 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days post inoculation (dpi). Mean values of each of the six replicates were used to calculate MDG and PRG on amended compared to unamended control PDA. PRG was calculated as (radial growth on amended PDA/radial growth on unamended control PDA) × 100, and was used to calculate percent inhibition (calculated as 100-PRG = percent inhibition). The effective concentrations that reduced mycelial growth by 50% (EC_{50}) and 90% (EC_{90}) were determined by probit transforming the PRG and regressing against the Log_{10} of amendment concentrations. This experiment was repeated three times with similar results obtained each time.

Determination of fungistatic properties of phosphite

Experiments were a randomised complete design with six replications. Mycelial plugs, prepared as before, were placed into 10 mL SDW containing 0 (control), 10, 50, 100 and 250 µg/ml of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH (n=6), and incubated in darkness in a growth chamber maintained at 18° +/- 2^0 C for 10 days. The plugs were retrieved, rinsed twice in SDW and transferred onto fresh unamended PDA and grown in darkness at 18° +/- 2^0 C (n=6) for 10 dpi. Growth responses were measured and the presence or absence of growth determined if the concentrations were fungicidal or fungistatic. Colony diameters, as determined above on solid media, were also used to assess the fungistacity of phosphite over 10 dpi. This experiment was repeated twice with similar results each time.

Microscopic analysis of the effect of phosphite on hyphal morphology

Microdochium nivale hyphal morphology was examined by bright field and fluorescence microscopy using a Bresser epifluorescence microscope. Mycelia, sampled from the outer margins of actively growing colonies, growing on PDA amended with 0 (unamended control), 10, 50, 100 and 250 µg/ml of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH were examined. The

fluorescent dye, Calcofluor White, was used to visualise hyphae as in Dubas *et al*. (2010). Images were captured using a Canon D1100 camera and processed by Adobe Photoshop version 5.0 LE (Adobe Systems, Inc., San Jose, CA).

Effects of phosphite on conidial germination

Experiments were a randomised complete design with six replications. *Microdochium nivale* conidial suspensions were filtered through sterile cheesecloth, to remove mycelium, and 50 µl aliquots were transferred to 1.5 ml tubes and mixed with 1 ml solutions of 0 (control), 10, 50, 100 and 250 µg/ml concentrations of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH. Aliquots (50 µl) of the mixtures were pipetted onto depressions in cavity microscope slides and immediately placed on moist tissue paper in 9 cm Petri dishes and sealed (n=6). Following incubation in darkness in a growth chamber maintained at 18° +/- 20 C for 48 h, the samples were agitated using an orbital shaker for 1 h then 20 µl pipetted onto fresh slides. The number of germinating conidia was counted and percent germination calculated (conidia germinated/total conidia x 100). Conidia were considered to be germinated when the germ tube extended to at least twice the length of the conidium (Mills *et al.*, 2004). This experiment was repeated twice with similar results each time.

Data analysis

Data were analysed using the statistical programme SPSS Statistics 21. Anova assessed for significant differences among the four isolates of *M. nivale* used. Data were assessed prior to analyses to ensure they met the requirements for the relevant statistical methods used. Residual analyses were performed to test for the assumptions of the two-way Anova, outliers assessed by inspection of boxplots, normality assessed using Shapiro-Wilk's normality test and homogeneity of variances was assessed by Levene's test. Two-way Anova, assessed significant effects and interactions on MDG, percent inhibition, the fungicidal or fungistatic properties of phosphite, colony diameters and on the percent germination of conidia. Where there were

significant effects or interactions, one-way Anova, followed by Tukey HSD post hoc tests, at a significance level of $p = 0.05$, were used to determine and separate statistical differences. For calculation of EC_{50} and EC_{90} values, probit analysis was used to transform percent inhibition from sigmoid to linear data and then regress against the Log10 of amendment concentrations. One-way Anova was then assessed for significant differences among compounds. Where required, data were suitably transformed prior to analyses and back-transformed for presentation of charts.

Results

Effects of phosphite on *in vitro* mycelial growth of *M. nivale* on solid media

Measurement of mycelial growth of *M. nivale* isolates grown on amended PDA were carried out from 1 to 10 dpi. Anova determined no significant ($p > 0.05$) differences in responses among the four isolates used and therefore the data were pooled to produce mean daily growth rates (MDG). Percent relative growth (PRG) rates of *M. nivale* grown on amended PDA were used to determine the percent inhibition. The analyses determined a significant ($p < 0.05$) difference in growth inhibition among compounds and rates of concentrations used, (Fig.1). Both H_3PO_3 and KH_2PO_3 caused significant inhibition of mycelial growth compared to all other compounds. EC_{50} and EC_{90} values, calculated at 5 dpi, were 40.99 and 80.90 μg/ml for the H_3PO_3 and 35.95 and 77.68 μg/ml for the KH_2PO_3, respectively. In contrast, there was no significant ($p > 0.05$) growth inhibition with H_3PO_4, KH_2PO_4 and KOH amendments. Statistical analysis determined the KH_2PO_3 PRG growth values were significantly ($p < 0.05$) lower than the H_3PO_3. Mycelial growth of *M. nivale* was suppressed by PO_3^{3-} presence when compared to plates amended with H_3PO_4, KH_2PO_4 and KOH (Fig. 2).

Fungistatic properties of phosphite

Colony diameters of the *M. nivale* isolates, which had been immersed in a range of compound concentrations for 10 days, were grown on and recorded at 5 (Fig. 3) and 10 dpi. Mean colony

diameters with concentrations of 0 (control) and 10 µg/ml had no significant ($p > 0.05$) effect. While there were significant ($p < 0.05$) differences in growth determined following immersion in the 50, 100, 250 and 500 µg/ml concentrations, with some suppression of growth, there was no complete inhibition. Further evidence of the fungistatic rather than fungicidal properties of phosphite was determined by measurement of colony diameters growing on H_3PO_3 and KH_2PO_3 amended PDA at 10 dpi. Evidence that phosphite reduces rather than fully inhibits growth can be seen in Fig 4, which show that colonies continued to grow to the end of the 10 dpi experimental period.

Effects of phosphite on hyphal morphology

Microdochium nivale hyphae, viewed using brightfield microscopy at 100x magnification in unamended control PDA (Fig. 5 A) showed normal morphology, as evidenced by the smooth hyphal outlines. Hyphae grown on H_3PO_4 (Fig. 5 B) and KOH (Fig. 5 C) amended PDA, appeared similar to those on unamended controls. *M. nivale* hyphae grown on H_3PO_3 at concentrations of 75 and 100 µg/ml amended PDA, displayed an altered hyphal morphology (Figs 5 D and 5 E). In the presence of phosphite, *M. nivale* hyphae appeared swollen, short-branched and stunted, compared to hyphae grown on PO_4^{3-} and KOH amended plates.

Effects of phosphite on conidial germination

Microdochium nivale conidia in amended solutions were incubated in darkness and conidial germination assessed. Conidia in all the 0 µg/ ml^{-1} unamended controls did not achieve 100% germination, with the highest rate of 85.6% determined in one of the sets of 6 replicates. Whilst there were only minor differences in germination rates in the 10 µg/ ml^{-1} concentrations of all compounds, at the 50, 100 and 250 µg/ml concentrations, germination rates in the H_3PO_3 and KH_2PO_3 amended plates were significantly ($p < 0.05$) less than with all other compounds (Fig. 6).

Discussion

The majority of research with phosphite for controlling plant pathogens has been with oomycetes (Coffey and Bower, 1984; Smillie *et al.*, 1989; Cook *et al.*, 2005; Garbelotto *et al.*, 2008). In contrast, relatively few studies have focused on phosphite suppressing the *in vitro* growth of ascomycetes (Reuveni *et al.*, 2003; Burpee, 2005). Numerous assessments of *M. nivale* mycelial growth on amended PDA were conducted, and bright field and fluorescence microscopy was used to assess effects on individual hyphae and conidial structures. These studies have shown that phosphite reduces mycelial growth, interferes with morphological development and reduces spore germination. Whilst the effects of phosphite on *M. majus* were investigated by Hofgaard *et al.* (2010), the present study is the first to provide equivalent data for *M. nivale*, the more significant pathogen of turf grasses. Significant growth suppression of *M. nivale* was shown in the presence of phosphite with no statistical ($p > 0.05$) difference between the four *M. nivale* isolates, despite being sourced from different geographical locations. Replication of these studies using a wider pathogen population would be of value as it would verify the findings here that all isolates are affected to similar levels.

Phosphite significantly suppressed *in vitro* mycelial growth of *M. nivale.* This inhibitory effect was also reflected in the disruption of hyphal morphology and the reduction in percent conidial germination. This sensitivity of *M. nivale* to phosphite was further evident from EC_{50} and EC_{90} values of 40.99 and 80.90 µg/ml for the H_3PO_3 and 35.95 and 77.68 µg/ml for the KH_2PO_3, respectively, at 5 dpi.

While both H_3PO_3 and KH_2PO_3 inhibited growth, the EC values highlight significant ($p < 0.05$) differences between these compounds. The differences in EC values could be attributed to combinations of compounds used, where there were significant ($p < 0.05$) differences between the inhibitory effects of both compounds at all concentrations used, with the exception of the 250 µg/ml. Bucking and Heyser (1999) stated that the presence of K facilitates the uptake of

mobile polyphosphate into fungal cells, maintaining that it helps retain the charge balance and pH of the fungal cell and is the counter ion to the transport of polyphosphates into the vacuole. Darakis *et al*. (1997) concluded the presence of K facilitated phosphite uptake into *Phytophthora capsici* hyphae. If mycelial growth suppression is used as an indicator of increased phosphite assimilation, then this enhanced assimilation of phosphite in the presence of K may have occurred, as statistically KH_2PO_3 produced significantly ($p < 0.05$) greatly inhibition compared to H_3PO_3. Compared to phosphite amendments, concentrations of H_3PO_4, KH_2PO_4 and KOH induced no similar significant inhibitory effects. The inhibitory effects of phosphate, at concentrations of 50 μg/ml and above, while significantly ($p < 0.05$) less than that of phosphite, were not unexpected. Reuveni *et al*. (1996) studying the infection of cucumber (*Cucumis sativus* L.) by the ascomycete pathogen *Sphaerotheca fuliginea* (Schlecht.:Fr.), demonstrated that disease symptoms were suppressed by a foliar spray treatment of KH_2PO_4. Howard (2001) confirmed that phosphate had fungicidal properties against a number of fungal species *in vitro*.

The effect of KOH on mycelial growth inhibition is an area of particular interest. Levels of K, currently recommended for management of cool-season amenity turfgrasses, appeared to increase susceptibility to *M. nivale*, when compared to lower K inputs (Soldat and Koch, 2016). As phosphite is most commonly pH adjusted with KOH, the results here (Fig. 1) showed that KOH concentrations of 100 and 250 μg/ml significantly inhibited mycelial growth compared to similar concentrations of H_3PO_4 and KH_2PO_4. This inhibitory effect possibly due to the increased pH of KOH amendments.

To date, there have been no published data specifically on the growth suppression of *M. nivale*, by phosphite *in vitro*. The results here, however, reflect the findings of Cook *et al.* (2009), who carried out a series of *in vitro* studies using KH_2PO_3 and KH_2PO_4 amended growth

medium, inoculated with the oomycete pathogen *Pythium aphanidermatum.* Whilst KH_2PO_3 inhibited growth of mycelia, KH_2PO_4 had no effect on growth, comparable to the results found here with *M. nivale* suppression. The closest related research to the present study was by Hofgaard *et al*. (2010), who examined the *in vitro* mycelial growth of *M. majus* on PDA amended with a range of concentrations of a foliar fertiliser containing 731 g/l of a 50% KH_2PO_3 solution. At 10 μg/ml, mycelial growth was reduced by more than 90% and at concentrations above 50 μg/ml, growth was inhibited fully. Their results appear to show phosphite as having significantly lower EC_{50} values than those reported here, either perhaps because *M. majus* is more susceptible to phosphite than *M. nivale*, or possibly due to differences in experimental methods.

The mode of action by which phosphite inhibits mycelial growth has been the subject of a number of studies. Most conclude that inhibition involves disruption of phosphorus metabolism and inhibition of enzymes involved in the glycolytic and phosphogluconate pathways (Grant *et al.*, 1990; Niere *et al.*, 1994; Stehmann, 2000; Mcdonald *et al.*, 2001). Barchietto *et al.* (1992) demonstrated that phosphite interacts with phosphate for the catalytic site of phosphorylating enzymes, and concluded that in *Phytophthora* spp. the activity of phosphite produced a physiological state similar to that produced as a result of P limitation. The disruption to hyphal morphology in *M. nivale* may be due to P deficiency in the presence of phosphite. This malformation of hyphae induced by phosphite/phosphate antagonism was also seen by Wong (2006), who studied the effect of phosphite on the hyphal morphology of *Phytophthora* spp. In the presence of phosphite, hyphae were stunted and swollen, again in a manner similar to those of *M. nivale.* This P deficiency view is supported by the findings of Niere *et al*. (1994), who concluded that phosphite inhibition in *Phytophthora* spp. was due to interference with phosphate metabolism, as the presence of phosphite led to increases in both

298 pyrophosphate and polyphosphate. They concluded that increased accumulation of phosphite
299 interfered with phosphate metabolism and diverted ATP from other pathways of metabolism,
300 resulting in decreased mycelial growth rates. Furthermore, they suggest that accumulation of
301 pyrophosphate and polyphosphate also alters the ion balance concentrations of potassium,
302 magnesium, calcium and iron, influencing the activity of enzymes catalysing essential steps
303 in metabolism.

304 An important aspect of this study was to determine if phosphite acted as a fungicide and killed
305 the pathogen or was fungistatic, reducing or slowing hyphal growth. Evidence of the
306 fungistatic properties of phosphite were clearly demonstrated when, after being immersed in
307 a range of phosphite concentrations for 10 days, *M. nivale* recommenced growth after transfer
308 to un-amended PDA, without displaying any major malformation and in a manner similar to
309 the samples immersed in phosphate and KOH. Complimenting these data, and supporting the
310 fungistatic rather than fungicidal properties of phosphite, are that when plated on phosphite
311 amended PDA, *M. nivale* growth, while significantly reduced, was not fully suppressed, but
312 continued to grow at a reduced rate over 10 dpi.

313 The ability of oomycetes and fungi to tolerate the presence of phosphite and maintain a
314 suppressed growth rate can be explained by Dunstan *et al.* (1990), who found that *P. palmivora*
315 was able to remove phosphite from its mycelium. Similarly, Smillie *et al.* (1989) found that
316 phosphite accumulated in *P. palmivora* during the first 5 days of growth, but showed a
317 subsequent decrease in cellular phosphite. Results of a metabolite profile study of
318 *Phytophthora* spp. by Grant *et al*. (1990) led them to conclude that phosphite accumulation in
319 mycelium was transient, as within 9 days phosphite had completely disappeared from the
320 mycelium. This supports the findings in this present study, were we found full suppression of
321 growth 5 dpi in PDA amended with phosphite at 250 µg/ml. However, from 6 to 10 dpi growth
322 in the 250 µg/ml amendments commenced and increased toward the end of the 10 dpi period.

This area merits further research as to the means by which this occurs. It may be that as phosphite is assimilated by the fungus phosphite to phosphate ratio in the media is altered and as Smillie et al. (1989) concluded phosphate significantly influences the take up of phosphite This determination of phosphite as a fungistat rather than a fungicide has significant relevance to disease control programmes and to the marketing of phosphite products. Depending on the active ingredient and its biochemical mode of action, a fungicide can be applied either as a preventative measure or as a curative to control disease infection. With a fungistatic compound, which slows the growth rather than kills the pathogen, the control programme usually requires treatment as a preventative measure, therefore requiring continuous sequential applications. The sequential application programme would ensure the phosphite was always present *in planta,* in order to continually suppress pathogen growth.

Conidial production is vital in the spread of inoculum, therefore any reduction would have a significant impact on disease spread and incidence. The results here show that the inclusion of phosphite in the propagating solution led to a significant reduction in conidial germination. This inhibition of spore germination by phosphite has been well documented in oomycetes, but less so in ascomycetes (Reuveni *et al.*, 2003; Mills *et al.*, 2004). Wong (2006) for example, showed that phosphite retarded spore germination in *Phytophthora* spp., and also provided evidence that phosphite caused distortion and lysis of the spores. Although phosphite inhibited spore germination in *M. nivale,* no conidial distortion or lysis was observed. While there are no published data on the effect phosphite has on *M. nivale* conidial germination, Hofgaard *et al.* (2010) demonstrated that increased phosphite concentrations correlated directly with delayed sporulation of *M. majus* on detached wheat leaves. Based on *in vitro* and detached leaf experiments, they concluded phosphite can suppress fungal reproduction and slow pathogenic growth, allowing a host plant's defence system time to react, reducing the severity of infection.

This study has produced significant and novel data which is relevant to methods of turfgrass disease prevention and control. The main conclusions are that phosphite suppressed *M. nivale* mycelial growth, disrupted hyphal morphology and reduced conidial germination. Both hyphae and conidia are infective propagules, providing inoculum for the diseases caused by *M. nivale.* It is clearly demonstrated here that the incorporation of phosphite into growth media significantly suppresses the growth and development of these infective propagules *in vitro* and therefore supports the findings of Dempsey *et al.* (2012) where it was demonstrated that phosphite significantly reduced *M. nivale* infection in the field. Further work in this area should assess the possible effect on turfgrass phosphate metabolism in the presence of phosphite and determine any effects on turfgrass growth.

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Figure legends

Figure 1 Inhibition of *Microdochium nivale* mycelial growth on phosphorous acid (H_3PO_3), phosphoric acid (H_3PO_4), dihydrogen potassium phosphite (KH_2PO_3), dihydrogen potassium phosphate (KH_2PO_4), and potassium hydroxide (KOH) amended PDA.

Inhibition of *M. nivale* mycelial growth on PDA amended with a: 10 µg/ml; b: 50 µg/ml; c: 100 µg/ml; d: 250 µg/ml of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH, presented as % inhibition of growth on unamended PDA. Growth rates calculated from pooled data of each of the four *M. nivale* isolates, n=6, by measuring the colony radii at four points on each plate, 4 dpi. Bars are 95% confidence intervals. Letters indicate significant differences among compounds, as determined by Tukey HSD at p = 0.05.

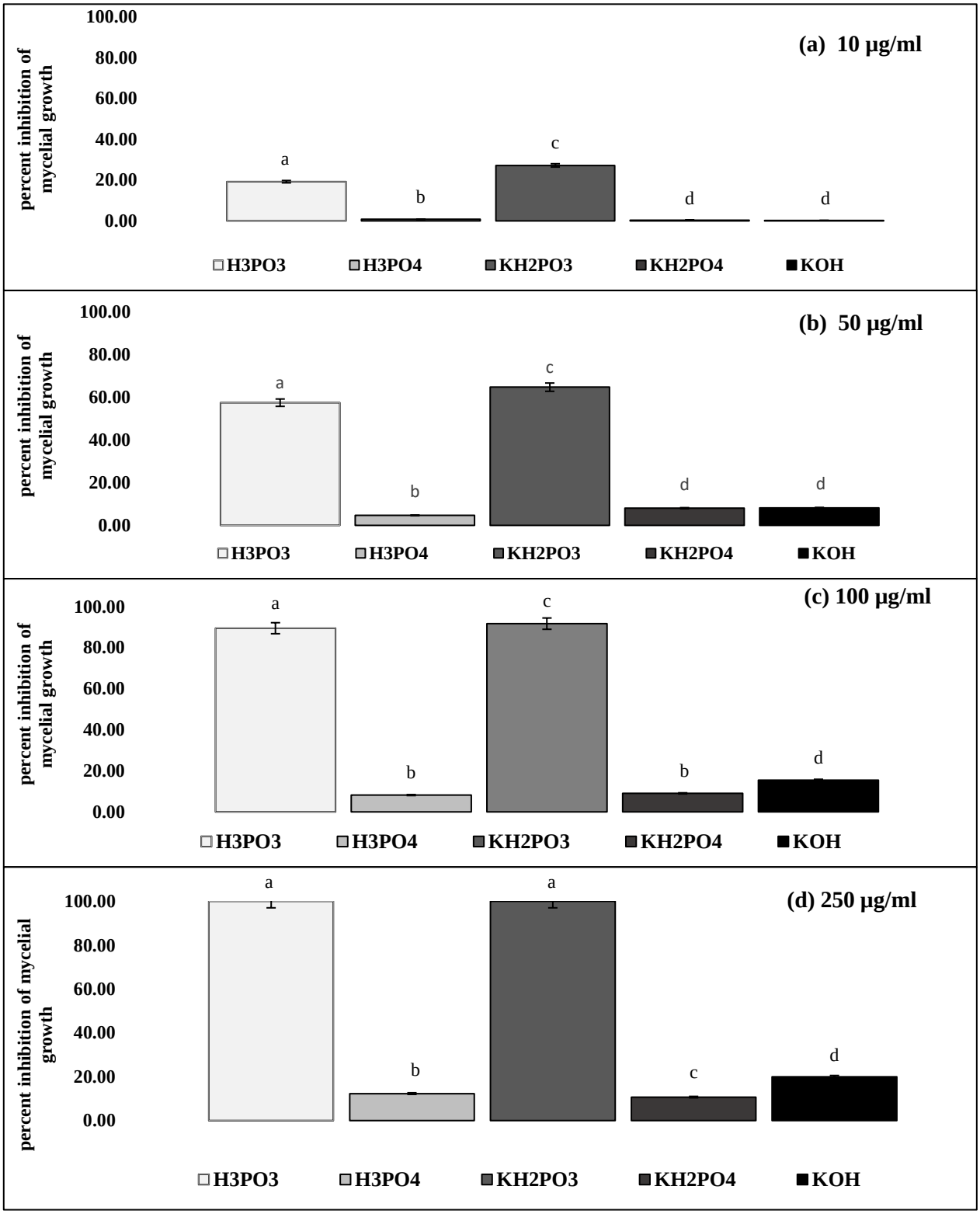
Figure 2 *Microdochium nivale* colonies on amended PDA at 5 days post inoculation.
A: unamended control; B: phosphorous acid (H_3PO_3), 100 µg/ml; C: phosphoric acid (H_3PO_4), 100 µg/ml; D: dihydrogen potassium phosphite (KH_2PO_3), 100 µg/ml; E: dihydrogen potassium phosphate (KH_2PO_4), 100 µg/ml F: potassium hydroxide (KOH), 100 µg/ml.

Figure 3 Effect of immersion of *Microdochium nivale* mycelium in solutions of phosphorous acid (H_3PO_3), phosphoric acid (H_3PO_4), dihydrogen potassium phosphite (KH_2PO_3), dihydrogen potassium phosphate (KH_2PO_4), and potassium hydroxide (KOH).
Microdochium nivale colony diameters (mm) 5 days after transfer to unamended PDA, following immersion for 10 days in a: 50 µg/ml; b: 100 µg/ml; c: 250 µg/ml; d: 500 µg/ml solutions of H_3PO_3, H_2PO_4, KH_2PO_3, KH_2PO_4 and KOH. Data are mean values, n=6, pooled from four *M. nivale* isolates. Bars are 95% confidence intervals. Letters indicate significant differences between colony diameters at each compound concentration used, as determined by Tukey HSD at p = 0.05.

Figure 4 Radial growth of *Microdochium nivale* mycelium 10 days post inoculation on phosphorous acid (H_3PO_3) and dihydrogen potassium phosphite (KH_2PO_3) amended PDA.
Microdochium nivale colony diameters in mm, 10 days post inoculation, growing on PDA amended with 0 (control), 10, 50 100 and 250 µg/ml of H_3PO_3 and KH_2PO_3.Colony diameters were determined by measuring the radii at four points on each plate. Bars are 95% confidence intervals. Letters indicate significant differences between compounds at each amendment concentration, as determined by Tukey HSD at p = 0.05.

Figure 5 Brightfield micrographs of *Microdochium nivale* hyphal growth in amended PDA.
a: unamended control; b: phosphoric acid (H_3PO_4), 100 µg/ml; c: potassium hydroxide (KOH), 100 µg/ml; d: phosphorous acid (H_3PO_3), 75 µg/ml; e: phosphorous acid (H_3PO_3), 100 µg/ml.

Figure 6 Effect of phosphite on germination of *Microdochium nivale* conidia.
Germination of M. nivale conidia following immersion in solutions of a: 10 µg/ml; b: 50 µg/ml; c: 100 µg/ml; d: 250 µg/ml µg/ml concentrations of phosphorous acid (H_3PO_3), phosphoric acid (H_3PO_4), dihydrogen potassium phosphite (KH_2PO_3), dihydrogen potassium phosphate (KH_2PO_4), and potassium hydroxide (KOH) after incubation at 18° +/- 2^0 C for 48 h. Data were arcsine transformed prior to analysis and back-transformed for this graph. Bars are 95% confidence intervals. Letters indicate significant differences between compounds as determined by Tukey HSD at p = 0.05.



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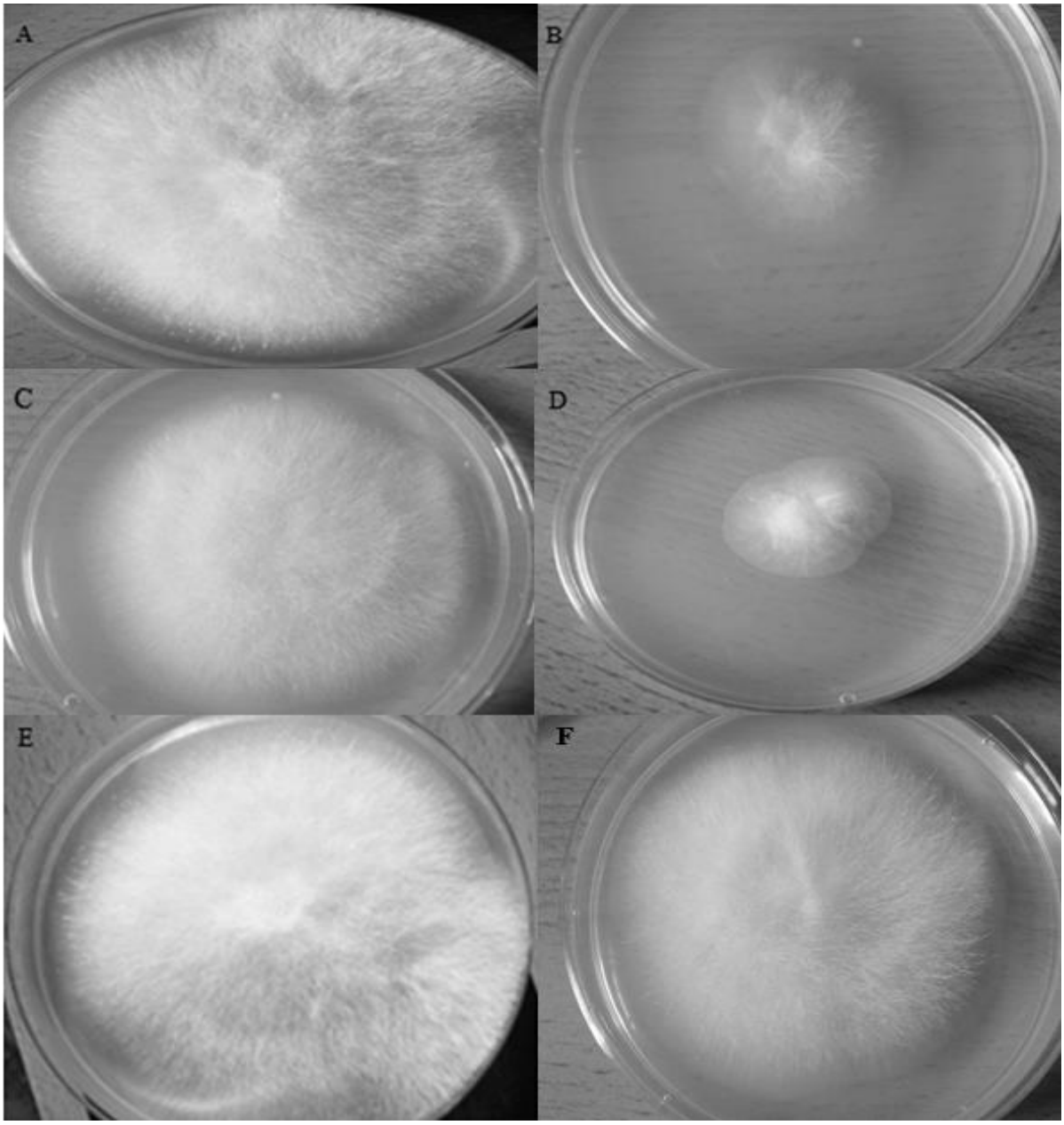

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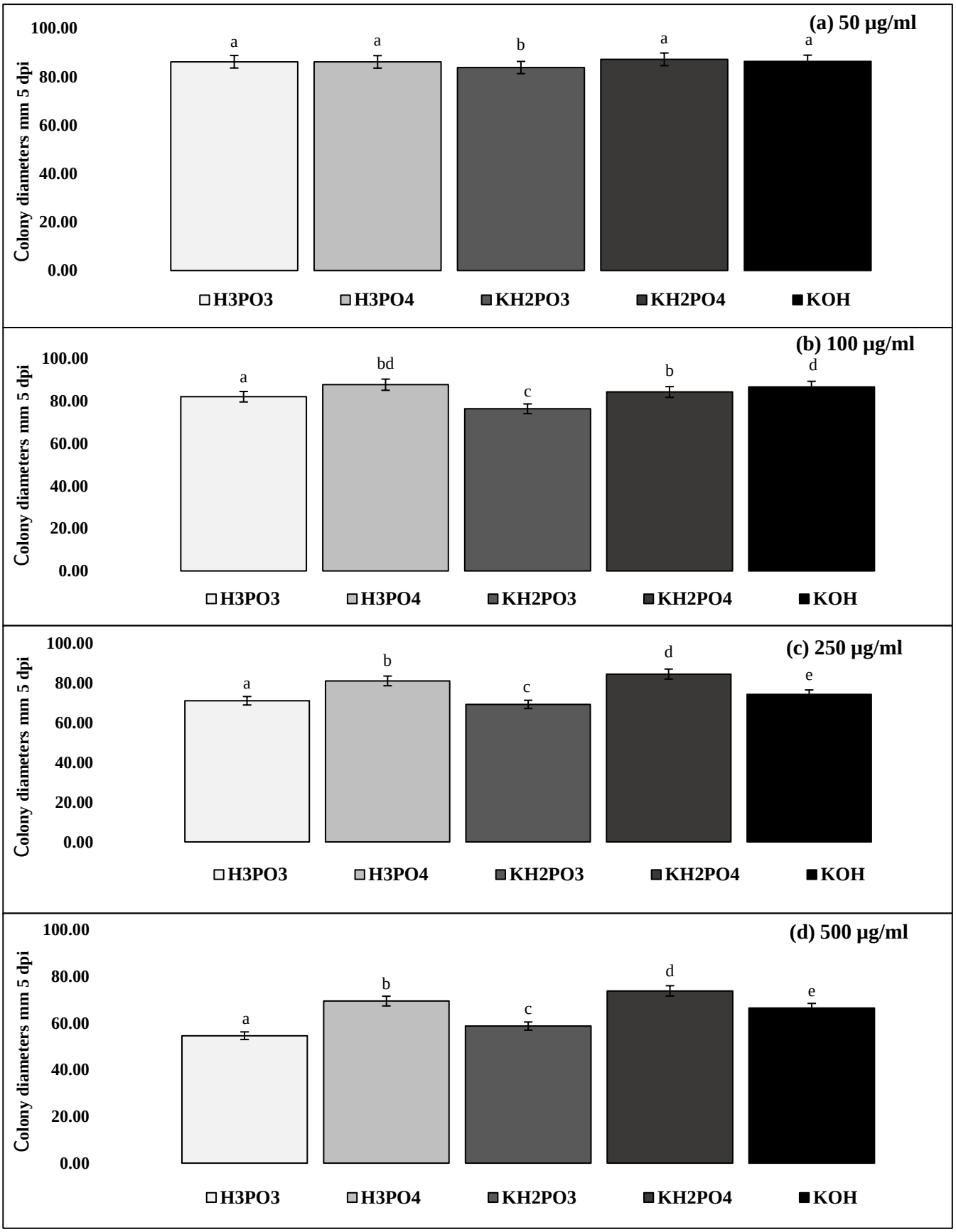

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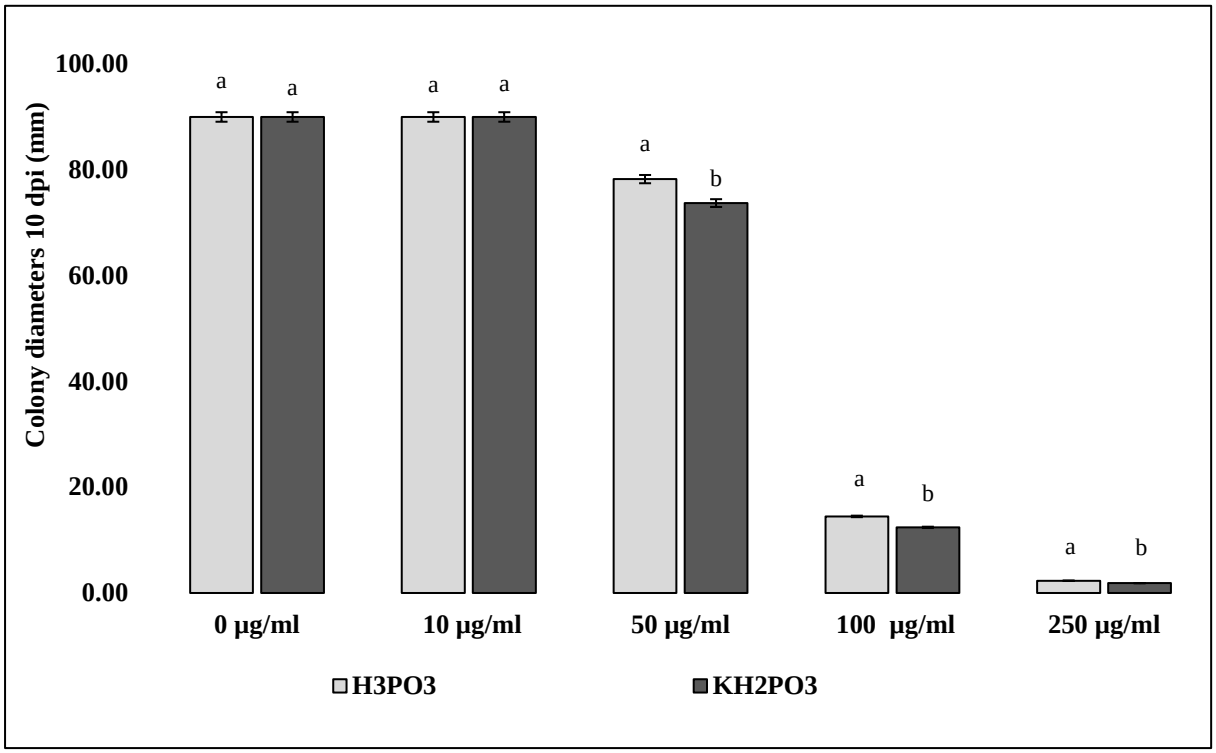

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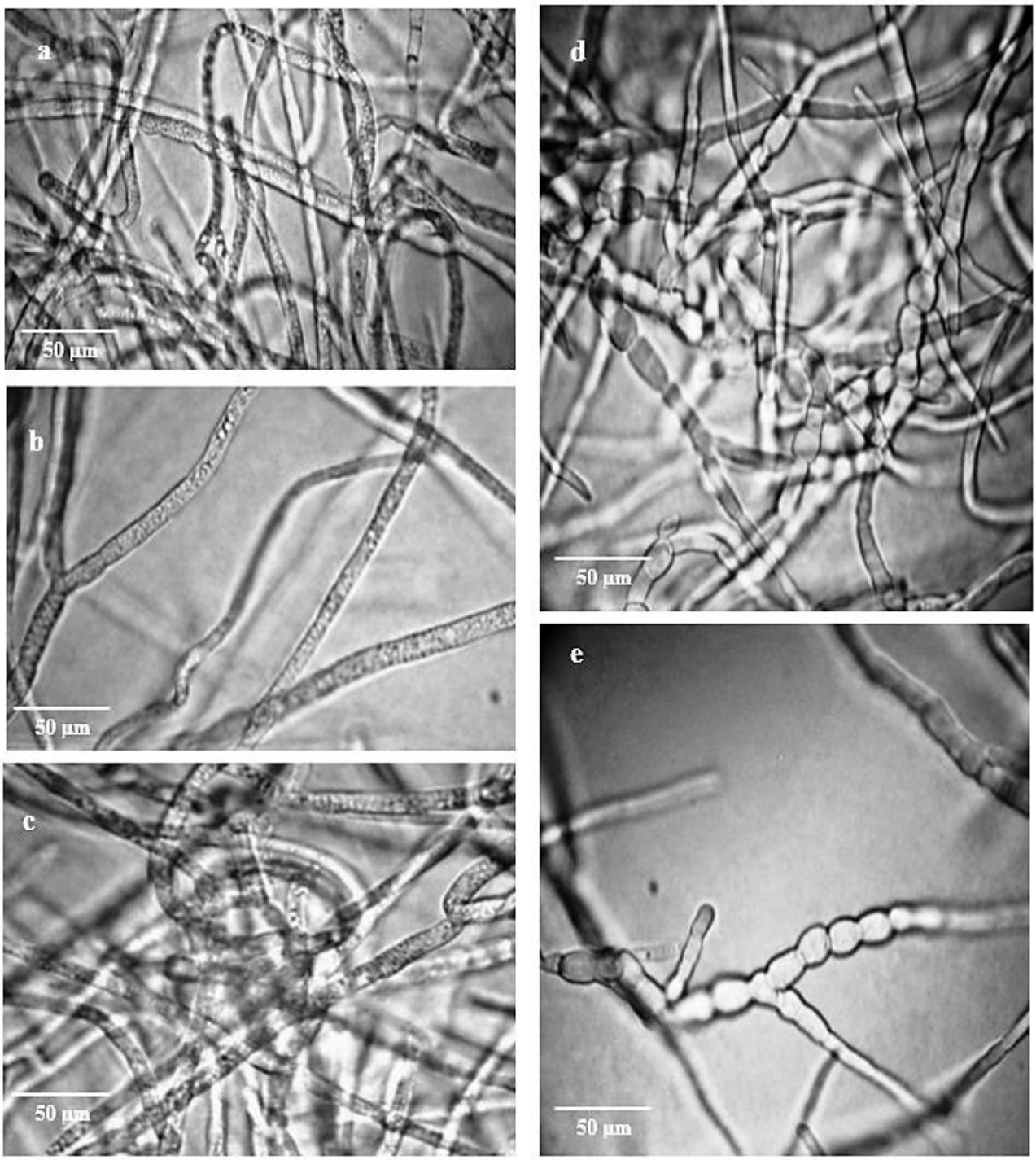

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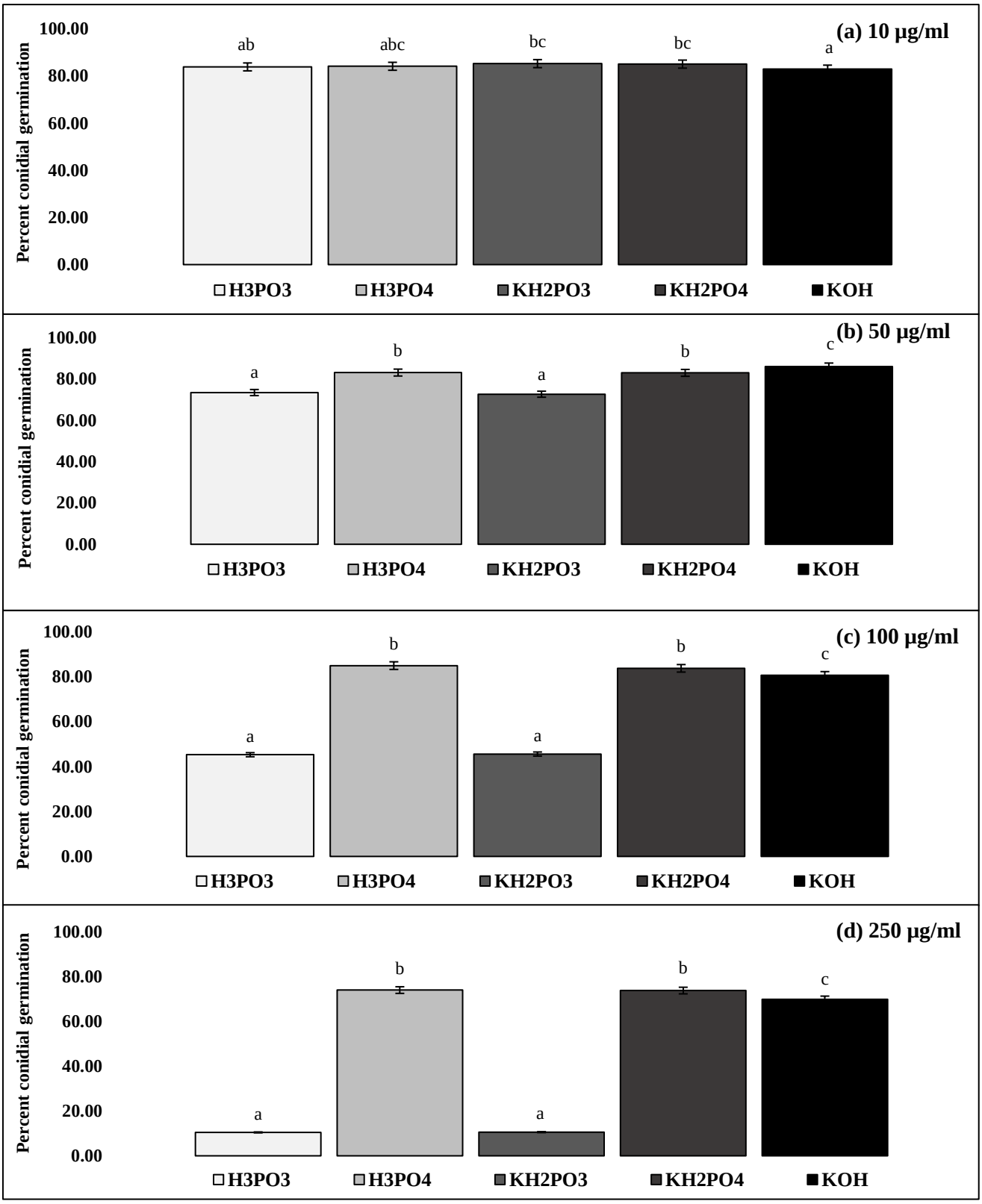

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