Phosphite and nutrient applications as explorative tools to identify possible factors associated with *Eucalyptus gomphocephala* decline in South-Western Australia

P. M. Scott[1,2,5], B. Dell[1,2], B. L. Shearer[1,3], P. A. Barber[1,2,4], G. E. St. J. Hardy[1,2]

[1]Centre for Phytophthora Science and Management, School of Biological Sciences and BiotechnologyMurdoch UniversityMurdochAustralia

[2]Centre of Excellence for Climate Change, Woodland and Forest Health, School of Biological Sciences and BiotechnologyMurdoch UniversityMurdochAustralia

[3]Science DivisionDepartment of Environment and ConservationPerthAustralia

[4]Arbor Carbon Pty. Ltd.PerthAustralia

[5]Forest ProtectionNew Zealand Forest Research Institute Ltd. ScionRotoruaNew Zealand

Abstract

Tree declines are increasingly being reported around the world. Since the 1990's *Eucalyptus gomphocephala* (tuart) has suffered a significant decline in the Yalgorup region, approximately 100 km south of Perth Western Australia. The complexity of many tree declines makes diagnosis difficult. Robust tools are needed to help identify factors contributing to tree declines. Two experiments tested the effect of trunk applied phosphite, nutrients and combined phosphite and nutrient treatments on wild declining *E. gomphocephala*. Treatment efficacy was tested as a management option to mitigate crown decline and as an explorative tool to help determine disease causality. Experiment 1 assessed the efficacy of combined treatments of trunk injections of different phosphite concentrations, trunk nutrient implants of different compositions and combined phosphite

and nutrient treatments. Experiment 2 assessed the efficacy of different phosphite concentrations. In Experiment 1, phosphite, together with nutrient treatments, increased the average crown condition by 21 %, as measured using a crown health score (CHS) averaged over 4 years, with the greatest improvements evident 6 months after application. Injection of 25 g phosphite/L combined with 0.3 g zinc sulphide gave the greatest increase. In Experiment 2, application of 75 to 375 g phosphite/L increased the CHS compared to the control treatment, with the greatest improvements in trees injected with 150 g phosphite/L. Foliar analysis for Experiment 2 confirmed a significant uptake of phosphite for all phosphite treatments. The increase in the CHS and significant flushes in new growth resulting from phosphite and nutrient treatments highlight the possible involvement of *Phytophthora* species in the decline, as *Phytophthora* species are known to be controlled by phosphite application. Further work on combined phosphite and nutrient applications, with a particular emphasis on zinc, is required to help understand and potentially mitigate the *E. gomphocephala* decline.

Keywords: Phosphite implants; Tuart; Micronutrient implants; *Phytophthora multivora*

Introduction

Tree declines are malfunctions in stands of trees, often leading to protracted death due to the persistent action of damaging factors (Podger 1981; Jurskis 2005). Tree declines significantly affect natural ecosystems, plantations and urban environments, worldwide. Examples include oak declines throughout Europe (Thomas et al. 2002), *Eucalyptus* declines within Australia (Jurskis 2005) and declines of many other species (Ciesla and Donaubauer 1994; Jurskis 2005). Significant urban tree declines have been associated with a range of factors including insect damage (Poland and McCullough 2006) and climate stress (Yang 2009). Internationally the recent impacts of climate change including abnormal drought, flooding and heat stress have contributed to increased tree mortality, especially in association with other stress factors that historically may not have individually attributed to decline (Allen et al. 2010).

The *Eucalyptus gomphocephala* woodland estate has been reduced from 111 600 ha in 1829 to 38 800 ha in 2003, predominantly from urbanization and agriculture (Tuart Response Group 2003). Since the early 1990s, *Eucalyptus gomphocephala* (tuart) health has been steadily declining in the Yalgorup woodlands of the Swan Coastal Plain south of Perth, Western Australia (Longman and Keighery 2002). The Yalgorup *E. gomphocephala* decline is expressed through a range of disease symptoms that include branch deaths and limb falls; however, the most consistent decline symptoms involves a progressive, often uniform thinning and dieback of foliage, followed by a dominance of epicormic growth, and eventual tree death. Disturbances such as grazing, timber harvesting and limestone quarrying (McArthur and Bartle 1980; Keighery 2002) have impacted on ecosystem function and may have contributed to *E. gomphocephala* decline. A range of biotic and abiotic factors including boring insects (Fox and Curry 1980; Fox 1981), foliar and canker pathogens (Taylor et al. 2009), changes in the soil bacterial functional diversity (Cai et al. 2010), changes to fire regimes (Archibald et al. 2005; Archibald 2006; Close et al. 2009; Archibald et al. 2010) and hydrology (Drake 2008), have been associated with *E. gomphocephala* decline, but causal relationships have not been established. The progressive canopy thinning and diebacks typical of some *Phytophthora*-associated tree declines (Shearer and Tippett 1989; Hansen and Delatour 1999; Brasier et al. 2003) suggest that *Phytophthora* species may also contribute to *E. gomphocephala* decline. There is no record in the literature of phosphite (= phosphonate) being applied to *E. gomphocephala*; however, the response of declining *E. gomphocephala* to phosphite treatment may indicate the role of Oomycetes, including *Phytophthora* pathogens, in the decline. Phosphite treatment may be used to help determine if Oomycetes, which include *Phytophthora* species, are involved in plant declines, because phosphite: (a) is very effective in controlling Oomycetes; (b) controls *Phytophthora* organisms *in planta* at concentrations that only partially inhibit pathogen growth in vitro (Guest and Bompeix 1984; Smille et al. 1989; Guest and Grant 1991; Hardy et al. 2001; Wilkinson et al. 2001), (c) has extremely low toxicity to invertebrates, aquatic organisms, or animals at effective application rates (Hardy et al. 2001), and (d) is not directly metabolized by plants and does not have any direct beneficial or deleterious fertilizer effects on plant growth, as a source of phosphate (Guest and Grant 1991; Carswell et al. 1996; Thao and Yamakawa 2009). Phosphite may be metabolized and

converted to plant available phosphate via soil bacteria (White and Metcalf 2007), although the conversion to plant available phosphate is slow (Adams and Conrad 1953; McDonald et al. 2001), and would therefore not negate the use of phosphite as a diagnostic tool. Phosphite may, however, exacerbate symptoms of phosphate deficiency, by suppressing the typical molecular and developmental responses of plants to phosphate deficiency (Carswell et al. 1996; Carswell et al. 1997; McDonald et al. 2001; Ticconi et al. 2001; Abel et al. 2002; Varadarjan et al. 2002). Fungicides with known targets and/or modes of action can be used in studies of indigenous communities to help quantify disease (Paul et al. 1989; Newsham et al. 1994). Stem injections of other chemicals with known biological effects, such as mineral nutrients and insecticides, may help to clarify the involvement of some abiotic and biotic factors in tree decline.

Eslick (2005) and Close et al. (2009; 2011) have identified zinc deficiency as a factor associated with *E. gomphocephala* decline. South-western Australia is an ancient, semi-arid land with a diverse native flora long adapted to the nutrient poor soils. About 40 % of these soils are known to be deficient in zinc (Zn) for crop and pasture production when the land is converted to agriculture. Furthermore, Zn deficiency is readily induced in eucalypt seedlings when fertiliser lacking Zn is applied (Wallace et al. 1986; Dell and Wilson 1989). Thus, it is possible that use of fertiliser in agricultural pursuits adjacent to the *E. gomphocephala* trees may contribute to nutrient stress in mature *E. gomphocephala* trees. It is also possible that a loss of fine roots due to the presence of *Phytophthora* species may reduce the uptake of nutrients such as Zn because micronutrients tend to be concentrated in the shallow topsoil where mycorrhizas proliferate.

Systemic nutrient implants and injections have effectively been used to correct nutrient deficiencies in ornamental and horticultural plants including *Quercus* species (Smith 1978; Harrell et al. 1984; Markham 1987), *Pinus* species, *Liquidambar* species (sweet gum), *Magnolia* species, *Photinia villosa* (oriental photinia) (Smith 1978), *Prunus avium* (flowering cherry), *Acer* species (maple) (Kielbaso 1978; Smith 1978; Harrell et al. 1984) and *Carya illinoinensis* (pecan) (Worley and Littrell 1978; Worley et al. 1980). The application of systemic nutrients via the main stem, in contrast

to soil-applied fertilisers, enables us to ensure the successful uptake of nutrients and investigate the effect of particular nutrients on individual trees by 'bypassing' the root system.

The aim of this study was to determine if *Phytophthora* species are associated with *E. gomphocephala* decline and if nutrient implants, including zinc, reverses decline symptoms. As there is no record in the literature of phosphite being applied to *E. gomphocephala*, an objective of Experiments 1 and 2 was to determine the sensitivity of *E. gomphocephala* to phosphite.

Methods

Two experiments were conducted on mature stands of *E. gomphocephala* in declining woodland at site 1 and 2. Trees treated in Experiment 1 and 2 were surrounded by dead trees and had poor crown health with crown health scores (CHS) of less than 3 out of a total of 5.4 (Grimes 1978).

Experiment 1- explorative trial to determine tree responses to different treatments

Experiment 1 was established as an initial explorative trial in April 2005, near Yalgorup National Park at site 1. *Eucalyptus gomphocephala* trees with circumference over bark ranging from 91 - 399 cm [mean (±SEM) of 221.4 ± 12.7 cm] and heights from 9.6 to 35 m [mean (±SEM) of 19.3 ± 0.9 m] and with access for clear crown assessment and foliar sampling were selected at random. Single trees were systemically treated with phosphite injections and nutrient implants either alone or in combination, in a cross-classified design with three replicates for each treatment interaction (Table 1). Phosphite treatments included three concentrations (25, 50 and 75 g phosphite/L) diluted with deionised water from a Foli-R-Fos 400 (UIM Agrochemicals Pty Ltd, Rocklea, Queensland, Australia), a 40 % (400 g/L) solution of mono-di potassium phosphite neutralised to pH 6.7 and a phosphite control treatment comprised of deionized H_2O. Nutrient treatments included implants of zinc (MEDICAP ZN®), iron (MEDICAP FE®), a combined treatment with N, P, K, Fe, Mn, Zn (MEDICAP MD®, Complete) and a control nutrient application with no nutrients (Table 2). Nutrient implants used within this study were selected based on nutrient

deficiency symptoms (Eslick 2005) and the availability of systemic nutrient implants. Treatment injections were allocated randomly across the site.

Treatment with a combination of phosphite liquid injections and nutrient implants on the same tree were spaced 20 cm apart vertically to ensure different treatments did not interfere with each other. Each tree was given a phosphite treatment, applied every 20 cm around the circumference of the tree, and a nutrient treatment every 10 cm, giving 1.5 treatments per 10 cm of circumference.

Treatment efficacy was assessed using the CHS, foliar nutrient status and phytotoxicity expressed as chlorotic and necrotic tissue in the foliage. The CHS was assessed at the time of injection and at 6 months, 1, 2 and 4 years after injection. Leaf nutrient status and phytotoxicity were measured at the time of injection and after 6 months. At the start of Experiment 1 the crown height, diameter measured at a height of 1.5 m and the original CHS were assessed and used as covariates in the analysis.

Experiment 2 – to confirm the ability of phosphite to improve tree canopy health

Experiment 2 was established to clarify the specific role of phosphite, demonstrated in Experiment 1, in March 2007 near Yalgorup National Park at site 2. *Eucalyptus gomphocephala* trees of similar size with trunk circumference between 29 - 127 cm [mean (±SEM) of 72.1 ± 4.1 cm] and height from 9.6 – 35 m [mean (± SEM) of 18.8 ± 1.0 m], and with access for clear crown assessment and foliar sampling were selected at random. Individual trees were injected with one of six concentrations of phosphite (0, 75, 150, 225, 300 and 375 g phosphite/L). The control treatment of 0 g phosphite/L was comprised of deionized H_2O injections. Ten replicate trees for each treatment were randomly distributed across 10 blocks in a replicated blocks design, selected at random among available trees. Replicate blocks were spread north to south over 1.9 km.

Treatment efficacy was measured using the CHS as per Experiment 1, phosphite uptake and phytotoxicity. The CHS was measured at the time of treatment injection and 1 year after injection. Phosphite uptake and phytotoxicity was measured 3 months after injection. At the start of Experiment

2 the crown height, diameter measured at a height of 1.5 m, and the original CHS, were assessed and used as covariates in the analysis.

Sites

The vegetation community type for both experiments was southern *E. gomphocephala – A. flexuosa* woodland (Donald and Prescott 1975), dominated by *E. gomphocephala* exhibiting typical decline symptoms observed throughout the Yalgorup region.

Experiment 1, site 1, was located 88.5 km south of Perth, Western Australia, 32.708143ºS and 115.637193ºE, covering approximately 0.96 ha, adjacent to Yalgorup National Park. Experiment 1 was located on a Vasse complex soil type, which is derived from marine, lagoonal and estuarine deposits, associated with low lying topography, susceptible to water logging and associated with wetland species including *Melaleuca* species (McArthur and Bartle 1980).

Experiment 2, site 2, was located approximately 100 km south of Perth Western Australia, 33.8711ºS and 115.7149ºE, in state forest covering approximately 10 ha. Experiment 2 was located on the eastern margin of the Spearwood complex dune system, associated with hilly ridges and deep sands, resulting from the leaching of older dunes and through wind accumulation (McArthur and Bettenay 1960; Seddon 1972; McArthur and Bartle 1980; McArthur 1991).

Sampling and *Phytophthora* isolation

To determine the presence of *Phytophthora* species, rhizosphere soil was sampled at the start of Experiment 1 in April 2005, and at the start of Experiment 2, in March 2007 and processed according to the methods of Stukely et al. (1997). Experiments 1 and 2 were then resampled in September 2007 using the same sampling methodology and the harvested rhizosphere material was processed for isolation of *Phytophthora* species as described in Scott et al. (2009). For Experiment 1, five randomly selected trees were sampled, while for Experiment 2, each treated tree including the control trees were sampled. Four soil root samples of about 10 × 10 × 10 cm were collected around the base of each tree, mixed and a 1 L subsample was collected.

Phosphite injection

Phosphite was applied at a rate of 1 mL/cm trunk circumference with injections of 20 mL given at every 20 cm of circumference around the trunk 1.5 m above ground level (Shearer et al. 2006). Holes were drilled through the outer bark layer into the sapwood at 20 cm intervals with a 6.5 mm drill bit and the phosphite solutions were injected using 20 mL spring-loaded tree syringes that lock tightly into the trees (Chemjet Pty Ltd, Bongaree, Queensland, Australia) (Shearer et al. 2006). Where required, the thick outer bark layers were removed using a 20 mm diameter spade drill bit.

Nutrient application

Three nutrient treatments were included in Experiment 1: zinc, iron, and combined nutrient (iron, manganese, zinc, nitrogen, phosphorus and potassium) MEDICAP® implants (Creative Sales, Inc., Fremont, Nebraska, United States of America) (Table 2). The three nutrient treatments (Table 2) were applied as capsule implants in accordance with the manufacturer's instructions following protocols outlined on the MEDICAP® Material Safety Data Sheets. Capsules were applied around the trunk circumference, at a height of between 0.5 and 1 m. Capsule diameters were 0.95 cm (3/8 in.) and were applied as close as possible to the manufacturer's recommended spacing's of 10 cm apart around the circumference. Holes were drilled with a 0.95 cm bit approximately 3.2 cm into the sapwood, drill shavings were removed and the capsules were manually inserted with a 6 mm diameter section of wooden dowel, until the capsule plug was flush with the cambium.

Crown assessment

Crown assessment for each tree was from the north aspect of each tree at approximately the same distance as tree height, and from the same position for all assessments. Crown condition was determined by ranking characteristics of the crown condition, against stylised images and a written description of characteristics of crown decline, or dieback (Grimes 1978). Characteristics of crown dieback included the relative crown position, crown size, crown density, proportion of dead branches, and proportion of epicormic growth. The scale for each crown characteristic was originally determined using *Corymbia maculata* (synonym *E. maculata*), *E. fibrosa* and *E.*

drepanophylla (Grimes 1978). The crown condition for each tree was determined by adding the scores for each characteristic for each tree to give the CHS. The CHS for each tree was rated on site and verified against digital images captured with a Fujifilm FinePix S500® digital camera, at each assessment. To reduce variation between repeated assessments of each tree, the assessments were done by the same person and in the same order and where possible, assessments were only performed on cloudless days. For Experiment 1, the reliability of the crown assessment method was tested by the same observer assessing the treated trees on a separate occasion within two weeks of the first assessment without reference to the original results. The two assessments were compared using regression analysis and were significantly correlated ($r = 0.70$).

Foliar nutrient assessment

Leaf nutrient status was determined for trees in Experiment 1 from twenty youngest fully expanded leaves (YFEL) per tree, selected at random throughout the upper 40 % of the crown to ensure leaves were not shaded and had approximately uniform light exposure. Leaves were collected 6 months after treatment using a Bigshot® Slingshot (Sherrill, Greensboro, North Carolina, United States of America) to propel a weighted bag with an attached rope over desired branches which were then pulled to the ground. The leaves were handled with powder-free latex gloves, bulked for each tree and transferred into a fan forced oven at 70 °C for 48 h, to reach a constant mass. The dried leaves were ground in a stainless steel mill and a 4 g subsample was analysed by CSBP Limited (Bibra Lake, Western Australia, Australia). The samples were ground and microwave digested in concentrated HNO_3. The concentrations of Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn were determined using an inductively coupled plasma analyser (Perkin Elmer, Connecticut, United States of America). To determine the concentration of N, finely ground plant material was combusted at 950 ºC in oxygen using a Leco FP-428 Nitrogen Analyser, measuring the released nitrogen from the sample as it passed through a thermal conductivity cell.

Phosphite uptake

For Experiment 2, the phosphite concentrations within the leaves were determined for each trunk injected tree, from 10 randomly selected YFEL harvested 3 months after treatment, at the same time as leaves used for foliar nutrient analysis. Leaves were handled with powder free latex gloves and bulked for each tree. Leaves were then washed in 1 % Deconex® 15E phosphate free detergent (In vitro Technologies, Noble Park North, Victoria) solution and rinsed twice in DI water. All samples were dried at 60 °C for 4 days, ground to 1 mm and sent to the WA Chemistry Centre (Perth, Western Australia, Australia) for phosphite analysis. To each 0.5 g ground sample, 5 mL of 0.1 M sulphuric acid was added and extractions occurred overnight on a roller-shaker. Following 20 min centrifugation at 6970 g, 100 µL of the clear acid extract was added to 1 mL of 50 µg/mL methyl phosphonic acid in methanol (internal standard solution). A phosphite standard curve was prepared by adding 100 µL of solutions containing from 0.05 to 100 µg/mL phosphite to 11 tubes containing 1 mL of internal standard solution. The solutions were mixed and diazomethane was added to 400 µL of the samples in excess until a persistent yellow colour was observed. Excess diazomethane was neutralised with a few drops of 2 % acetic acid, then the dimethyl phosphite content was determined by gas chromatography. A split less injection with a D.B-Wax column (J & W Scientific, Salsam, California, United States of America) and a phosphorous-specific flame photometric detector (Hewlett Packard, United States of America) were used. The limit of quantitation was 0.5 µg/g dry weights. A replicate sample was taken every 10 samples to provide a control during the analysis. Two control samples of known phosphite content were included in each batch of 40 samples analysed.

Phytotoxicity

Phytotoxicity symptoms were determined on 20 leaves from each tree. Leaves were randomly collected from around the tree, from representative branches removed from the upper 40 % of the crown, 4 weeks after phosphite injection. Phytotoxicity was measured as the percentage area of harvested leaves exhibiting chlorosis or necrosis (Barrett 2001; Hardy et al. 2001; Barrett et al. 2004) on both the abaxial and adaxial surfaces. Symptoms of phytotoxicity were compared to images by Barrett (2001).

Statistical analysis

Analyses were carried out in Statistica software package Version 5 (Statsoft 1999). Assumptions of normality were checked by plotting residuals (Clarke and Warwick 2001). For both experiments the CHS for each time in the analysis is expressed as a ratio of CHS at that time to the CHS at time 0 (when the trial began). Changes in the CHS were transformed to square root arcsin values to homogenize the variance. Where appropriate, the Pearson correlation coefficient was calculated for a measure of association between variables. For both Experiments 1 and 2, the crown height, diameter at 1.5 m height, and CHS at the time of treatment application were tested as covariates in the analysis. Significance was determined for both experiments at $P \leq 0.05$.

In Experiment 1, the dependent variable CHS was analysed using a cross classified (factorial) multivariate analysis of variance (MANOVA) with fixed factors of phosphite (concentrations 25, 50 and 75 g/L and phosphite control), nutrients (zinc, iron, complete nutrients and nutrient control) and time (6 months, 1 year, 2 years and 4 years) as the repeat measure factor, and the CHS as the dependent variable. The effect of injection treatments on foliar nutrient concentrations were analysed individually for each measured nutrient as a factorial repeated measures MANOVA with fixed factors of applied phosphite and nutrients injections, and time (at application 0 months and 6 months) as the repeat measure factor. The Bonferroni correction was not applied, in accordance to Moran (2003).

In Experiment 2, the dependent variable CHS was analysed as an ANOVA, measured 1 year after treatment application with different phosphite concentrations 0 (control), 75, 150, 225, 300 and 375 g/L. Phosphite uptake and phytotoxicity were analysed as an ANOVA, measured 3 months after treatment application for all injection concentrations. Foliar nutrient concentration was analysed as an ANOVA, measured 3 months after treatment application for phosphite at 150 g/L and the control trees without phosphite injection.

Results

No *Phytophthora* species was isolated at the start of Experiment 1 in April 2005 or at the start of Experiment 2 in March 2007. However, in September 2007, *P. multivora* was isolated throughout both sites for Experiments 1 and 2.

The covariates of crown height, diameter measured at a height of 1.5 m, and CHS at treatment application, were not significantly different between treatment variables for Experiments 1 ($P \leq 0.24$) and 2 ($P \leq 0.26$).

Experiment 1- explorative trial to determine tree responses to different treatments

The treatment interaction of phosphite, nutrients and time was not significant ($P = 0.13$): however, the treatment interaction of phosphite with nutrients had a significant ($P = 0.05$) influence on the CHS (Fig. 1). The greatest improvement of a 56.4 % increase in the CHS resulted from phosphite at 25 g/L combined with MEDICAP ZN® treatments.

In the treatments without nutrient injections, trees injected with phosphite at 25 and 50 g/L did not significantly vary from the control; however, treatments with phosphite at 75 g/L significantly improved CHS by 40.5 % compared to the control. MEDICAP FE® treatments did not result in a significant improvement in CHS, compared to the control; however, the average CHS of iron-phosphite treatments did increase when phosphite was increased from 25 g/L to 75 g/L.

In the treatments without phosphite injections MEDICAP ZN® and MEDICAP MD treatments did result in a significant improvement of CHS by 13.0 and 37.4 %, respectively. Analysis of the combined treatments showed that all MEDICAP ZN® phosphite treatments resulted in a significant improvement in CHS compared with the control; however, the improvement decreased with increasing phosphite concentration. MEDICAP MD together with phosphite at 25 and 75 g/L, significantly improved the CHS by 42.6 and 21.4 % respectively, compared with the control. The treatments of MEDICAP MD and phosphite at 50 g/L did not significantly vary from the control. No individual treatment resulted in a significant decrease in the CHS compared to the control (Fig. 1).

All measured foliar nutrient concentrations were not significantly ($P > 0.07$) influenced by the main treatments or interaction of phosphite and nutrient treatments 6 months after injection.

Phytotoxicity was predominantly observed as regions of localized patchy necrosis although some non-necrotic leaf patches were chlorotic. No treatment within Experiment 1 resulted in phytotoxicity symptoms covering more than 5 % of the sampled leaf surface. Phytotoxicity was only clearly exhibited on trees treated with 75 g phosphite/L combined with the complete nutrient treatment.

Experiment 2 – to confirm the ability of phosphite to improve tree canopy health

Phosphite treatments (Fig. 2) did not significantly ($P = 0.13$) affect CHS, and phosphite concentration within harvested leaves was not correlated ($r^2 = 0.01$) with CHS. However, a priori analysis indicates that the combined CHS of all trees trunk injected with different phosphite treatments was significantly ($P = 0.05$) higher than the control treatments.

By 1 year after phosphite injection there was a significant nonlinear relationship between injected phosphite concentration and change in CHS, with greatest improvement of CHS for trees injected with 150 g phosphite/L and least for trees not injected with phosphite: Percentage change CHS = -29.10 + 25.32 Phosphite concentration - 5.29 Phosphite concentration^2 + 0.30 Phosphite concentration^3 ($r^2 = 0.90$).

Phosphite concentrations in leaves from phosphite trunk injected trees were significantly ($P < 0.01$) greater for all phosphite treatments compared to the control. Phosphite concentration in leaves was significantly positively correlated with injected phosphite concentration: Foliar phosphite concentration = $3.51e^{0.87 \text{ Treatment phosphite concentration}}$ ($r^2 = 0.82$) (Fig. 3).

Foliar phytotoxicity occurred for phosphite concentrations greater than 225 g/L with the highest measured phytotoxicity of 5 % of the foliage exhibiting burn symptoms for 375 g/L. The expressions of phytotoxicity on harvested leaves were similar in both experiments. Phosphite treatment did not have a significant ($P = 0.99$) effect on the foliar nutrient concentration.

Discussion

Phosphite injections improved crown health at concentrations which have been shown to be effective in other species, known to be infected with *P. cinnamomi*, including *Persea americana* (avocado) (Whiley et al. 1991) and *B. grandis* and *E. marginata* (Shearer and Fairman 2007). The improvement in the crown health of *E. gomphocephala* trees following trunk injection with phosphite at concentrations of 75 g/L or 150 g/L is strong evidence that *Phytophthora* pathogens have contributed to the decline in *E. gomphocephala* CHS. It is likely that *P. multivora* was present at both sites at the start of Experiment 1 in April 2005 and Experiment 2 in March 2007, even though it was not isolated from either site until September 2007. *Phytophthora multivora* may not have been originally isolated during these experiments; because of entrenched dormant resting structures (Bunny 1996), low population density or problems with the isolation technique. *Phytophthora multivora*, originally identified as *P. citricola* using morphological techniques, has been episodically isolated from rehabilitated bauxite mine sites since 1992 in the jarrah forest on the Darling Scarp, usually after major rainfall events (Hardy personal communication). Similarly, Bunny (1996) needed to repeatedly flood natural soil samples to isolate a *Phytophthora* species, identified using morphological techniques as *P. citricola*, that was probably *P. multivora*. Water logging has been shown to imposes additional stress on plants which may contribute to changes in susceptibility to pathogens (McDonald et al. 2002). Bunny (1996) identified rainfall as an important factor influencing the distribution of *P. citricola* (probably *P. multivora*) isolates, with greatest isolation frequency when rainfall was above 800 mm and much lower isolation frequency at rainfall below 500 mm. However, in 2005 when trees in Experiment 1 were originally sampled and no *Phytophthora* species were isolated, the annual rainfall was 915.22 mm (Bureau of Metorology 2011), compared to 649.40 mm of rain in 2007 (Bureau of Metorology 2011) when *P. multivora* was isolated from both experiments.

Phosphite treatments in Experiments 1 and 2 may have resulted in varying improvements in crown condition because of differences between the sites. Trees in Experiment 1 were taller and had larger circumferences than trees in Experiment 2. Differences in tree age between sites, may account for variation in phosphite effectiveness. Increased host age has been associated with increased sporangia

production and disease severity in a range of disease syndromes, including diseases in *Pinus* species caused by *P. cinnamomi* (Newhook 1959, 1970). Increased host age has also been proposed as a significant contributing factor in the susceptibility of *Quercus* species to *P. quercina* and other *Phytophthora* species (Jung et al. 2000; Jönsson 2006). Trees in Experiment 1 may also have been more susceptible to *Phytophthora* species as they were growing on a site that appeared to be intermittently inundated with water. Waterlogging was not observed on this sites used in Experiment 2. Waterlogging has been shown to be associated with increased infection by *P. cinnamomi* (Dawson and Weste 1982; Davison 1994).

Phosphite injections may have improved the CHS by supressing the impact of *Phytophthora* pathogens; however, further work is required to confirm this relationship. Combined treatments of phosphite and MEDICAP ZN® resulted in greater improvements in the CHS than individual treatments with MEDICAP ZN® or phosphite. Synergistic treatment interactions may have resulted from a range of direct and indirect effects. MEDICAP ZN® application may have improved the CHS by amending an underlying nutrient deficiency within the plant and therefore increasing photosynthetic capacity, improving the resistance of the plant to pathogens including *Phytophthora* species, or through a combination of both.

Combined treatments of MEDICAP MD® and phosphite at 25 g/L, resulted in greater improvements in the CHS, than phosphite application at higher concentrations. Phosphite concentrations above 25 g/L, in combination with MEDICAP MD® may therefore negate the effectiveness of individual MEDICAP MD® injections, through mechanisms which may include subclinical stress.

A range of nutrient applications improved crown health; however, the initial nutrient levels in the trees was not determined at the start of the experiments. Nutrient applications may have increased crown health by ameliorating an underlying nutrient deficiency, increasing resistance to a decline pressure or disease, improving symptoms of decline, or combinations of these factors. Further work is required to determine how different nutrient applications affect nutrient concentrations within *E. gomphocephala* and improve crown health.

All nutrient amendments used within this trial have been shown to have specific associations with disease expression. For example, zinc deficiencies predispose *Carya* species to *Mycosphaerella dendroides* and *Cerospora fusca* pathogens (Moznette 1940) and deficiencies in manganese increase disease expression in *Solanum tuberosum* caused by *P. infestans* (Thompson and Huber 2007). Positive responses in CHS of *E. gomphocephala* were also observed in trees treated with MEDICAP MD® implants. These implants contain a range of nutrients in addition to zinc, including nitrogen, phosphorous, potassium, iron and manganese. All of the nutrients are involved in plant growth and defence in various ways.

Many Australian soils are naturally deficient in zinc, for crop and pasture production (Boardman and McGuire 1990; Brennan 1990). Disease-like symptoms of *Pinus* plantations grown on calcareous soils within the *E. gomphocephala* forest north of Ludlow, were reduced with zinc application (Kessell and Stoate 1936, 1938). Results for *E. gomphocephala* suggest that zinc may be specifically associated with disease resistance and/or the ability to tolerate decline; however, further work is required to resolve these relationships.

There are a number of factors that may be contributing to nutrient imbalances in *E. gomphocephala* trees. Close et al. (2011) suggested the pH dependency of micronutrients such as Zn could be problematic for plants growing in leached sandy soils of low clay and CEC which are widespread in *E. gomphocephala* woodlands. Climate change can also affect the available nutrient supply in soils. For example, drought decreased the uptake of mineral nutrients and reduced the available pool of nutrients in Mediterranean soil under *Quercus ilex* (Sardans and Peñuelas 2007).

Arbuscular mycorrhizae have been shown to significantly increase zinc uptake in *Trifolium* species on calcareous soils (Chena et al. 2002). Zinc application has also been associated with significant increases in root growth of *Triticum* species (Dong et al. 1995). A reduction in uptake of zinc by declining trees may result from multiple factors including a loss of fine root function and associated beneficial mycorrhizae that facilitate zinc uptake, caused by *Phytophthora* root pathogens. Further research is required to determine the relationship between zinc, *E. gomphocephala* health, and beneficial soil microbes.

None of the treatments in either experiment caused levels of phytotoxicity that significantly reduced the CHS compared to the control trees. Within Australian natural ecosystems, phosphite is typically applied with trunk injections at concentrations between 50 and 200 g/L depending on the sensitivity of the species to phytotoxicity (Hardy et al. 2001). Low phytotoxicity was observed at injected concentrations greater than 225 g phosphite/L, suggesting that *E. gomphocephala* was able to withstand phosphite at high concentrations *in planta* when compared with levels that cause significant toxicity and death in other *Eucalyptus* species (Aberton et al. 1999; Barrett et al. 2004). The low susceptibility of *E. gomphocephala* to phosphite phytotoxicity may be a unique characteristic, as most Myrtaceae are phosphite sensitive (Hardy et al. 2001). It would be useful to determine how the metabolism and translocation of phosphite in *E. gomphocephala* contributes to its low phytotoxicity. Further work is required to determine the exact impact of phosphite phytotoxicity on *E. gomphocephala*. As the improvement in the CHS appears to decrease above phosphite concentrations of 225 g/L; it is likely that the apparent optimal injection rate of 150 g/L will not result in phosphite phytotoxicity that is deleterious to plant growth and health.

The effectiveness of nutrient applications in improving crown health, confirms that declining trees have reached an eco-physiological limit. Further work is required to understand what eco-physiological limits declining *E. gomphocephala* have reached, and how nutrient applications improve crown health. For example, the role of zinc in *E. gomphocephala* decline may be resolved through further injection trials using Zinc alone, or through soil amendment treatments.

Phosphite *in planta* has been shown to suppress *Phytophthora* species at concentrations that only partially inhibit pathogen growth in vitro (Guest and Bompeix 1984; Smille et al. 1989; Guest and Grant 1991; Hardy et al. 2001; Wilkinson et al. 2001). Phosphite itself has no beneficial effect on the growth of healthy plants, and is not a direct P nutrient source (Thao and Yamakawa 2009). The effectiveness of phosphite in controlling *P. multivora* on *E. gomphocephala* needs to be directly measured in situ in both under-bark inoculated stems and on naturally infected trees.

An understanding of the mechanisms of phosphite activity on crown condition is required to help determine the cause of *E. gomphocephala* decline. The use of phosphite as an exploratory tool, to help

determine the role of *Phytophthora* species, is a new method for assessing disease aetiology. Further research is required to explain why phosphite application in Experiments 1 and 2 resulted in varying improvements in crown condition.

Changes in crown health were measured using categorical techniques. Repeat assessments of the same tree may not be entirely consistent as the assessors skills would improve and change over time. To improve the repeatability of assessments, changes in crown health should include non-destructive quantitative physiological measurements of plant water relations and photosynthesis.

Since starting this study Koch's postulates have been satisfied as *P. multivora* has been isolated from the rhizosphere of declining *E. gomphocephala* woodland trees suffering significant fine root loss. The rhizosphere of *E. gomphocephala* seedlings grown under controlled conditions were infested with *P. multivora* and subsequently suffered significant fine feeder root loss compared to controls. *Phytophthora multivora* was then reisolated from the rhizosphere and roots of these artificially infected seedlings (Scott et al. 2012). This study is a novel example of using phosphite injections and nutrient implants, to help determine the cause of a disease, while providing land managers with a tool to mitigate premature decline of valuable, iconic trees.

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Table 1 Factorial design for Experiment 1, showing treatment combinations of phosphite (Phi) at 0, 25, 50, 75 g phosphite/L and Control; and nutrient implants, MEDICAP ZN® (Zinc, Zn), MEDICAP FE® (Iron, Fe), MEDICAP MD® (Complete, Com) and Nutrient Control, applied to 48 trees. Chemical composition of nutrient implants is indicated in Table 2

	Phi 25 g/L	**Phi 50 g/L**	**Phi 75 g/L**	**Phi 0 g/L (Control)**
Zn	Trees 1, 17, 33	Trees 2, 18, 34	Trees 3, 19, 35	Trees 4, 20, 36
Fe	Trees 5, 21, 37	Trees 6, 22, 38	Trees 7, 23, 39	Trees 8, 24, 40
Com	Trees 9, 25, 41	Trees 10, 26, 42	Trees 11, 27, 43	Trees 12, 28, 44
No nutrient (Control)	Trees 13, 29, 45	Trees 14, 30, 46	Trees 15, 31, 47	Trees 16, 32, 48

Table 2 Composition of nutrient capsules

Nutrient treatment	Weight per capsule	Capsule constituents	Dose (mg/10 cm trunk circumference)
Zinc - (MEDICAP ZN®)	1.0 g	Zinc sulphide	300
Iron - (MEDICAP FE®)	1.0 g	Ammonium iron (II) citrate about 28 % Fe	448
Complete 12-4-4 nutrient - (MEDICAP MD®)	0.8 g	Iron	32
		Manganese	32
		Zinc	32
		Total Nitrogen	960
		Ammoniacal Nitrogen	8
		Nitrate Nitrogen	12
		Urea Nitrogen	76
		Available Phosphoric Acid	32
		Soluble Potash	32

Fig. 1 The effect of phosphite liquid, nutrients and combined phosphite liquid plus nutrients on *Eucalyptus gomphocephala* crown health score (CHS) (Grimes 1978). Treatment effect is indicated by the mean (± standard error) percentage change trees averaged over 4 years after systemic trunk treatment with: phosphite liquid (Phi) ( ); nutrient implants ( ); and phosphite liquid plus nutrient implant ( ) treatments. Individual treatments are represented by combinations of Phi 25, 50 and 75 corresponding to phosphite concentrations of 25, 50 and 75 g phosphite/L; and nutrient treatments of MEDICAP ZN® (zinc - Zn), MEDICAP FE® (iron - Fe) and MEDICAP MD® (nitrogen, phosphorus, potassium, iron, manganese, zinc - MD). Crown condition was measured as the CHS (Grimes 1978), and is represented as the percentage change in CHS compared to CHS at the time of treatment, averaged over 4 years of assessment. Negative values indicate a decrease in the CHS over time. Statistics are for one-way ANOVA. Treatment interactions analysed with the *post hoc* test (Dunnett) showing a comparison with the control group and * indicating significant differences to the control group at $P \leq 0.05$

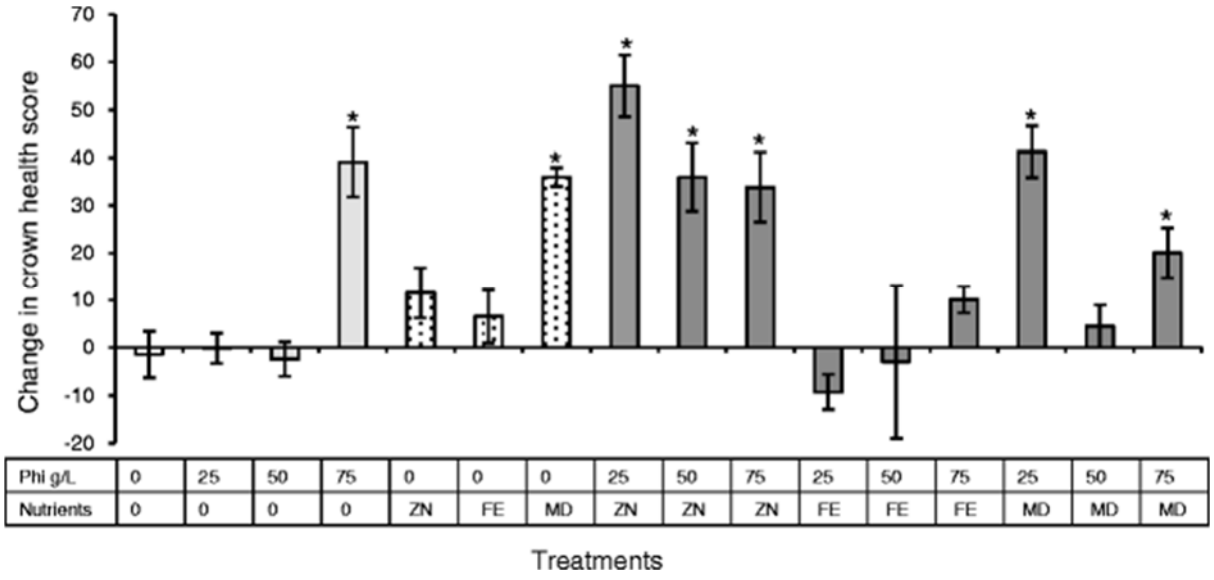

Fig. 2 The effect of phosphite on *Eucalyptus gomphocephala* crown health score (CHS) (Grimes 1978), indicated by the mean (± standard error) percentage change, 1 year after trunk injection with phosphite at concentrations of 0 (control), 75, 150, 225, 300 and 375 g phosphite/L. Negative values indicate a decrease in the CHS over time

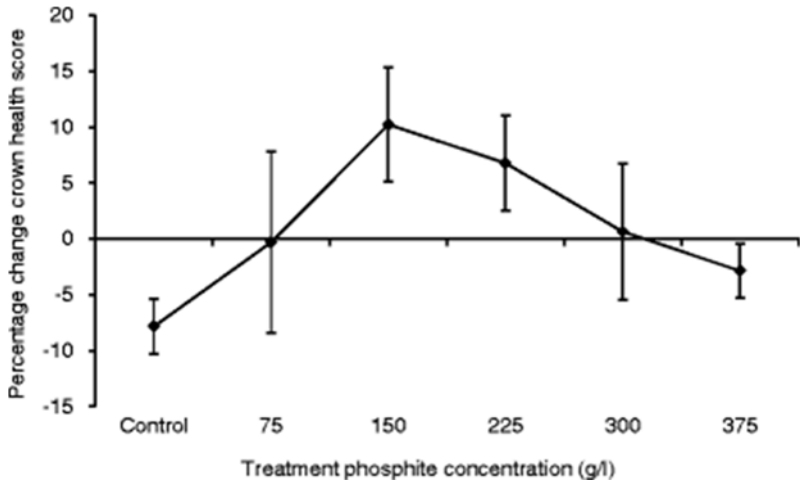

Fig. 3 The effect of phosphite on *Eucalyptus gomphocephala* phosphite concentration, indicated by the mean (± standard error) for phosphite concentrations, measured as phosphorus acid in leaves, 1 year after trunk injection phosphite at concentrations of 0 (control), 75, 150, 225, 300 and 375 g/L phosphite/L