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Using AMF inoculum to improve the nutritional status of *Prunella vulgaris* plants in green roof substrate during establishment

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) have been shown to improve the growth, health, nutrient uptake, flowering and drought tolerance of many terrestrial plant species. Green roofs are generally deficient in nutrients, organic matter and water, and therefore AMF could be extremely beneficial in improving green roof plant performance. Despite this there is a lack of empirical research into artificially introducing AMF into green roof substrates.

In this study, a commercial AMF inoculum was applied to *Prunella vulgaris* green roof plugs grown in small modules on a flat roof in Sheffield, UK. The modules were filled with commercial green roof substrate (80% small particle sized crushed brick, 20% green waste compost) to a depth of 100 mm. AMF inoculum was applied as four treatments: (i) directly with plug, (ii) mixed evenly into surrounding substrate, (iii) split between plug and substrate, (iv) control treatment with no inoculum added.

Significantly greater levels of AMF colonisation of *P. vulgaris* roots was detected in all AMF treatments compared to the control. Low levels of AMF colonisation of *P. vulgaris* roots were also observed in the control treatment, confirming that low levels of AMF inoculum were present in this commercial substrate. Shoot phosphorous (P) concentration was improved in all AMF treatments, however there was no significant effect of any AMF treatment on *P. vulgaris* growth rate or biomass production. The highest AMF colonisation of *P. vulgaris* roots was observed when AMF inoculum was directly added to just the plug. Promisingly, *P. vulgaris* flowering time at the end of the first growing season was also extended in the plug AMF treatment only.

This study has confirmed that commercial AMF inoculum can be used to successfully colonise plants and introduce AMF networks into green roof substrate. Although AMF inoculum was naturally present in the substrate used in this study, levels were extremely low, and unlikely to have any significant effect on plants. This study indicates that care should be taken in the use of AMF inoculum on green roofs, as the growth and health benefits of AMF are not always immediately apparent for green roof plants. In addition much more research is required in order to fully assess the extent of the benefits of AMF on green roof plants and to determine if their use can be financially viable.

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1. Introduction

Green roofs are intentionally vegetated areas of roof. In the last ten years they have become much more common in urban areas due to the numerous benefits (green roof services) they offer (Getter and Rowe, 2006; Oberndorfer et al., 2007). These include: increased stormwater retention (Berndtsson, 2010), reduced urban heat island effect (Bowler et al., 2010; Santamouris, 2014), moderated building temperature (Jaffal et al., 2012), air pollutant retention (Speak et al., 2012) and urban wildlife habitat creation (Getter and Rowe, 2006; Oberndorfer et al., 2007; Dunnett and Kingsbury, 2010). The most common type of green roof, known as an extensive green roof, has a relatively shallow layer of substrate (50–120 mm) in which hardy plants are established (Oberndorfer et al., 2007). Due to their exposed roof location and shallow depth, plants are often exposed to extremes of temperature, moisture, sunlight levels and wind shear (Oberndorfer et al., 2007). In addition, substrates used on green roofs also have low organic matter levels (5–20%) to discourage excessive plant growth and weed invasion (Ampin et al., 2010; Nagase and Dunnett, 2011). For this reason, plant choice on extensive green roofs has been predominately limited to hardy succulents such as *Sedum* spp.

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(Snodgrass and Snodgrass, 2006; Dunnett and Kingsbury, 2010). In recent years greater emphasis has been placed on improving the range of plants used on extensive green roofs (MacIvor and Lundholm, 2011; Cook-Patton and Bauerle, 2012; Nagase and Dunnett, 2013). Slow release fertiliser is often added to green roofs (especially extensive) in order to compensate for low nutrient levels (Ampin et al., 2010). However excessive nutrient availability in substrate can often lead to unsustainable plant growth on a green roof (leading to plant dependence on fertiliser) and nutrient leaching in runoff (Berndtsson, 2010; Nagase and Dunnett, 2011).

A potentially sustainable alternative to slow release fertilisers is the application of arbuscular mycorrhizal fungi (AMF) (Jeffries et al., 2003; Smith and Read, 2008; Brundrett, 2009). AMF are proposed to mitigate nutrient stress commonly found on green roofs and may improve the establishment success of less hardy plant species (John et al., 2014; Molineux et al., 2014). The majority of land plants can form symbiotic relationships with AMF (Smith and Read, 2008) which can lead to increased phosphorus (P) (Van der Heijden et al., 1998) and in some situations, nitrogen (N) uptake by plants, as well as enhanced chlorophyll production (Tsang and Maun, 1999; Zuccarini, 2007; Hodge et al., 2010; Hodge and Storer, 2014). AMF could therefore improve nutrient uptake in the nutrient deficient environment of a green roof, and thus reduce the need for slow release fertiliser application (John et al., 2014; Molineux et al., 2014). Furthermore, AMF has been shown to increase plant resistance to drought (Augé, 2001). The proposed mechanism for this involves a combination of higher stomatal conductance, reduced hydraulic resistance in roots and increased root growth (Augé, 2001). AMF can also improve soil structure by increasing the amount and stability of soil aggregates which subsequently improves the movement of water through the soil (Rillig and Mummey, 2006). This could improve substrate water holding capacity and also reduce nutrient leaching which occurs frequently on green roofs (Berndtsson, 2010; Aitkenhead-Peterson et al., 2011). AMF is also capable of immobilising non-essential and toxic metals (lead, cadmium, zinc, iron), which subsequently prevents their uptake by plants (Meharg and Cairney, 1999). High concentrations of toxic metals have been found in green roof substrate and leachate, although it is not clear how this affected green roof plant health (Speak et al., 2014).

It has been well established that AMF can improve the health of host plants in conventional ecosystems (Smith and Read, 2008), however to date few empirical trials has been conducted into the benefits of artificially introducing AMF inoculum into green roof substrate (John et al., 2014; Molineux et al., 2014). Previous research has indicated that AMF inoculum is present in some established green roof substrate (McGuire et al., 2013; Rumble and Gange, 2013; John et al., 2014; Young, 2014) and can survive when introduced to a green roof either intentionally (Molineux et al., 2014) or as part of the planting process (John et al., 2014). However to date only three studies have been published on the effect of intentionally introduced AMF inoculum on green roof plant growth and health. All of these studies showed increased plant growth with AMF inoculum, but the effect upon plant health or nutrient uptake was not clear (Busch and Lelley, 1997; Meyer, 2004; Sutton, 2008).

AMF networks have previously been found in low levels in commercial green roof substrate planted with a selection of grassland and prairie plants (McGuire et al., 2013; John et al., 2014) and on established extensive green roofs (Rumble and Gange, 2013; Young, 2014). The mineral content of newly mixed green roof substrate (80–90%) is unlikely to contain significant amounts of AMF inoculum (spores, colonised root fragments or AMF hyphae) due to its non-biological origin. AMF inoculum may be found in some types of organic matter used in green roof substrate (for example green waste compost is likely to contain a certain amount of AMF inoculum due to its wide range of biological source material). However, as green waste compost is sometimes heat treated to denature weed seeds (WRAP, 2008), any AMF spores, root fragments or hyphae may also be denatured. Some AMF inoculum could also be transported into substrate via the wind or more likely by water or animal vectors (Smith and Read, 2008), however this process is inconsistent and dependent on local sources on AMF inoculum and the conditions in which the substrate is stored. The presence of an active and healthy AMF network will lead to the presence of colonised root fragments and AMF hyphae in growing media. The main means for a plant to become colonised by AMF is coming into contact with these root fragments or AMF hyphae opposed to AMF spores present in the growing media (Jasper et al., 1991; Merryweather and Fitter, 1998). Colonised root fragments and AMF hyphae are relatively immobile (although can be moved by animal vectors or water) compared to AMF spores and therefore are unlikely to be transported onto a green roof. In addition the vast majority of plants which colonise green roof substrate during its storage or once installed are likely to be wind-blown seeds (Nagase et al., 2013). These seeds will be 'sterile' in the sense that they have not come into contact with AMF hyphae or colonised roots and therefore are unlikely to significantly affect the AMF network of the substrate. Therefore commercial green roof substrate is unlikely to consistently contain high levels of naturally occurring AMF inoculum regardless of how long and where it has been stored. Even if AMF is present in commercial substrate at low levels, it will then take a long time to build up to biologically significant levels after installation on a roof (John et al., 2014; Molineux et al., 2014). A number of green roof substrate companies currently sell substrate and seed mixes with AMF inoculum incorporated into them (Bauder, 2012; Mycorrhizal Applications Inc., 2013) and there are a number of case studies in which AMF inoculum has been incorporated into a green roof (Living Roofs, 2003; Grothe and Trichie, 2006). However it is still not clear if the use of AMF inoculum on green roofs has a beneficial impact on green roof plants, and if so what is the most effective and cost efficient method of applying AMF inoculum to large areas of green roof substrate.

This paper aims to explore this major gap in green roof literature by examining the effect of artificially introducing AMF inoculum on green roof plant growth and physiological health. The plant species (*Prunella vulgaris*) was selected as it is increasingly used on green roofs as part of wildflower plant mixes (Bauder, 2012; Boningale, 2015), and has previously been shown to be highly responsive to AMF inoculation in calcareous grassland (Streitwolf-Engel et al., 1997; Van Der Heijden, 2004). In order to do this a roof top experiment was set up to examine the effect of AMF inoculum on *P. vulgaris* plugs in green roof substrate over one year. In order to assess the most efficient method of applying AMF inoculum to the plugs four treatments were used (a) AMF inoculum added to plug substrate, (b) AMF inoculum added to surrounding substrate, (c) AMF inoculum added to plug and substrate, and (d) no AMF inoculum added.

It was hypothesised that the addition of AMF inoculum to a green roof substrate/plug would aid the establishment of *P. vulgaris*. In addition it was hypothesised that applying AMF inoculum directly to the plugs as opposed to the substrate would result in a much higher rate of AMF colonisation and therefore would provide greater benefits to the host plant.

2. Methods

2.1. Location and timing

The roof used for this trial was located in Sheffield, UK (53.23° N, 1.28° W) a city with a temperate seasonal climate. A flat asphalt roof (80 m^2) enclosed by a 1.2 m high wall and located on the 9th floor of

Fig. 1. (a) View of the test site before module installation on the 9th storey of the Education Building, University of Sheffield. (b) Installed green roof modules with *Prunella vulgaris* plugs planted. Aspect in both figures is facing north.

a University of Sheffield building was used as the study site (Fig. 1). The trial was conducted from June 2013 to August 2014.

2.2. Green roof modules

Green roof modules were created with plastic trays of 400 mm × 300 mm × 120 mm. Drainage holes were drilled at regular intervals into the base of each tray in order to mimic a conventional installation and allow free draining substrate. A root proof membrane was also fitted inside the tray to prevent loss of substrates throughout the trial. Each module was filled to a depth of 100 mm with commercial green roof substrate sourced from Boningale GreenSky Ltd., composed of 80% crushed recycled brick (2–5 mm particle size) and 20% green waste compost (Table 1). Green waste compost (Green Estate, Sheffield, UK) was composed of composted garden waste collected in Sheffield. The modules were located in a randomised block design and raised off the roof surface in order to prevent water logging. The outside of each module was painted white in order to reduce the amount of heat absorbed from direct sunlight as conventional green roofs do not have exposed sides.

2.3. Planting

2.3.1. P. vulgaris

In June 2013 four *P. vulgaris* plug plants (sourced as SkyPlugs™ from Boningale Nurseries Ltd.) were planted into each module at equal distances from one another which translates to a planting density of 45 plugs m^{-2}. Due to an especially dry summer each module was given supplementary watering twice a week in order to aid establishment throughout July 2013 of 4.8 L $month^{-1}$ which translates into 40 mm rainfall. Additional watering was also given twice a week during early August 2014 of 2.5 L per module (21 mm rainfall) due to a prolonged period of low rainfall. AMF inoculum (sourced from Plantworks Ltd. as Rootgrow Professional containing spores, mycelium, dried plant root containing mycelium and attapulgite clay colonised by mycelium of several different species) was applied to plug plants and substrate as a powder in five treatments according to manufacturer's specifications (Table 2). Each treatment had five replications making a total of forty modules.

2.3.2. Plantago lanceolata *bait plants*

Three seedlings of *P. lanceolata* bait plants were planted in the middle of each module and grown for two months between August and October 2013 in order to obtain a 'live' update on AMF colonisation of the substrate. Seeds were surface sterilised with sodium hypochlorite for 3 min and thoroughly rinsed with autoclaved water, transplanted to autoclaved sand and grown in a controlled growth cabinet for four weeks prior to planting.

2.4. Growth and flowering

P. vulgaris was measured throughout the growing season to assess maximum plant vertical and horizontal growth. Plants were measured every 7–14 days from June 2013 to October 2013, and every 30 days from March 2014 to August 2014. Plants were not measured between October 2013 and March 2014 due to lack of growth during winter months. The number of flowers produced per module was also recorded throughout the trial.

2.5. Biomass

P. vulgaris was harvested in August 2014 (Day 403) and *P. lanceolata* bait plants in October 2013 (Day 70). For both species all above ground biomass was harvested, dried at 80 °C for two days and weighed to obtain dry weight. Roots were washed in water to remove all traces of brick and compost before root biomass was measured. A sample selection of root for AMF colonisation

Table 1
Physical and chemical characteristics of the commercial substrate available from Boningale Ltd. used in this trial. All physical values were calculated according to FLL standards (FLL 2008). For methods see online supporting information. Data available from Boningale Ltd.

Measurement	Characteristic	Value
Physical	Organic matter (%)	11.00
	Permeability (mm/min)	71.66
	Water holding capacity (%)	34.99
	Oven dried density (g/cm^3)	1.10
	Saturated density (g/cm^3)	1.39
	Pore volume (%)	36.78
	Air content at water content max (%)	1.8
Chemical	Plant available P ($\mu g\,P\,g^{-1}$ substrate)	11.14
	Plant available N ($\mu g\,N\,g^{-1}$ substrate)	11.26
	Total P ($\mu g\,P\,g^{-1}$ substrate)	88.26
	Total N ($\mu g\,N\,g^{-1}$ substrate)	208.87

Table 2
AMF treatments for *P. vulgaris* plug plants grown in green roof modules. Per Plug application rate refers to amount of AMF inoculum placed at the bottom of the plug hole during planting. Substrate application rate refers to the amount of inoculum mixed homogenously into the substrate before planting. AMF inoculum was applied as a powder using the manufacture's measuring device which measured in ml, 1 ml = 0.92 g.

Treatment number	AMF inoculum application rate		
	Per plug	Substrate	Total (module)
1	0 ml	0 ml	0 ml
2	20 ml	0 ml	80 ml
3	0 ml	80 ml	80 ml
4	10 ml	40 ml	80 ml
5 (no plug plants)	0 ml	0 ml	0 ml

analysis was removed with a scalpel, dried with a paper towel and weighed. The remaining root material was dried with a paper towel and weighed to obtain fresh weight, and then dried at 80 °C for two days and weighed again to obtain dry weight.

2.6. Chlorophyll content

The chlorophyll content of *P. vulgaris* was measured 61 and 281 days after planting with a chlorophyll meter (Minolta Chlorophyll Meter SPAD-502). The youngest four leaves from each plant that were large enough to be measured were assessed and the mean calculated for each green roof module.

2.7. Leaf P and N concentrations

Leaf tissue P and N content was determined on oven-dried (70 °C for 48 h) ground samples from the final biomass harvest, following Kjeldahl digestion (Allen et al., 1974). Approximately 50 mg dry plant biomass was digested in 1 ml concentrated sulphuric acid with 1 microspatular of catalyst (1:10 $CuSO_4$:$LiSO_4$) for 7 h at 375 °C. After a dilution (1:50 dH_2O), total P was determined via colorimetric determination by using a Cecil Ce 1020 spectrophotometer (Leake, 1988). After a dilution (1:100 in distilled water) total N was determined by Flow Injection Analysis (Burkard FIA Flo2, Burkard Scientific, Uxbridge, UK).

2.8. Root colonisation

After harvesting *P. lanceolata* and *P. vulgaris*, roots were carefully washed with distilled water and a small sample taken for staining. Root staining (according to Brundrett and Bougher, 1996) was used to highlight AMF colonisation. A sample of root was cleared in KOH (10%, w/v) for 120 min and then placed in HCl (10%, v/v) for 15 min. Roots were then stained with Trypan Blue for 15 min and stored in 50% glycerol until needed.

AMF colonisation rates were quantified using the modified grid line intersection method (Giovannetti and Mosse, 1980). Stained roots and a small amount of 50% glycerol were randomly dispersed in a 9 cm petri dish with gridlines marked on at 12.7 mm (0.5 in.) intervals. Any roots intersecting a gridline were assessed for AMF colonisation in order to give a % colonisation rate. For each replicate 100 intersections were observed.

2.9. Statistical analysis

To determine the effect of AMF treatments on *P. vulgaris* shoot biomass, root biomass, root:shoot ratios, AMF colonisation, flower production and *P. lanceolata* AMF colonisation, one way ANOVAs were performed on linear models. Any dataset not meeting the assumptions of the model were $\log_{10}$ transformed. Any dataset not meeting the assumptions of the model with values less than 1 were $\log_{10}$ transformed after the addition of 1 to every value. To determine the effect of AMF treatments on *P. vulgaris* growth rates a Generalised Linear Model with Poisson distribution was used with day number as a random factor.

All analyses were carried out in R Studio version 2.15.1 (22.6.2012) (The R Foundation for Statistical Computing).

3. Results

3.1. P. vulgaris *AMF colonisation*

All three AMF treatments had significantly higher AMF colonisation rates than the control (one way ANOVA, $F = 21.31$, $p < 0.05$) (Fig. 2). However when all the AMF inoculum was added to just the plug, colonisation rate was significantly higher than when the inoculum was split between plug and substrate (Tukey HSD, $p < 0.05$) (Fig. 2).

3.2. P. vulgaris *growth*

P. vulgaris growth (vertical or horizontal) during both growing seasons was not significantly affected by AMF treatment compared to the control (GLM, $p > 0.05$, $F = 0.26$ and 0.81) (Fig. 3a, b). All *P. vulgaris* plants, regardless of AMF treatment, showed little vertical growth in the first growing season (Days 0–100), with all plants showing some horizontal growth (Fig. 3a). All *P. vulgaris* plants regardless of AMF treatment subsequently showed large amounts of vertical in the second growing season (Fig. 3a), but little horizontal growth (Days 300–400) (Fig. 3b).

P. vulgaris final shoot and root biomass was not significantly affected by AMF treatment compared to the control (one way ANOVA, $F = 1.27$ and 1.02, $p > 0.05$) (Table 3). However *P. vulgaris* root:shoot ratio was significantly higher in all AMF treatments compared to the control (one way ANOVA, $F = 2.97$, $p < 0.05$) (Table 3).

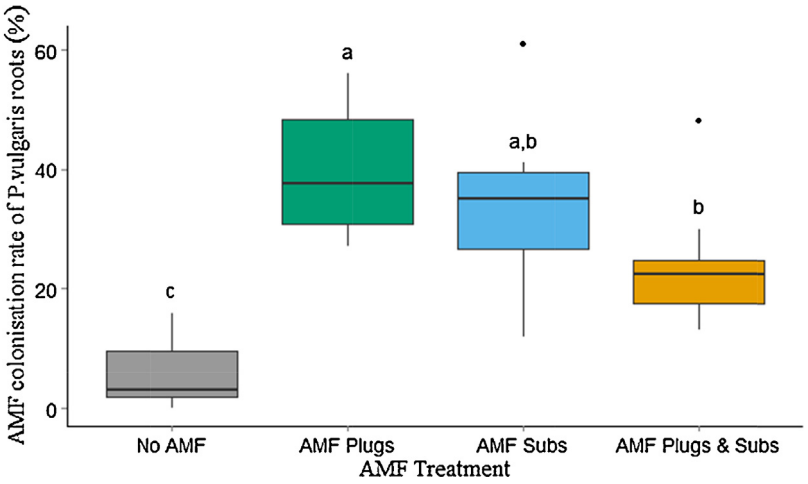

Fig. 2. Percentage AMF colonisation of *P. vulgaris* roots grown from June 2013 to August 2014. No AMF, only plug plants; AMF Plugs, AMF inoculum added to plugs; AMF Subs, AMF inoculum added to substrate; AMF Plug and Subs, AMF inoculum added to plugs and substrate. The middle bar represents treatment mean, the upper and lower box hinges represent the first and third quartiles, the thin black line represents the complete spread of data and black dots represent any outlying data points. Treatments with the same letter do not differ significantly from one another (Tukey HSD, $p < 0.05$).

Table 3
Shoot biomass, root biomass and root:shoot ratios of *P. vulgaris* grown from June 2013 to August 2014. AMF Plug, AMF inoculum added to plugs, AMF Plug and Subs, AMF inoculum added to plugs and substrate; AMF Subs, AMF inoculum added to substrate; No AMF, only plug plants. Statistical significances of *p*-values: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Statistical significances were calculated from one-way ANOVA and Tukey HSD post hoc test, Df = 28. Letters indicate statistical differences between means.

	Treatment (±SE)					
	AMF Plugs	AMF Plugs and Subs	AMF Subs	No AMF	*p*-Value	*F*-value
Shoot (g)	44.48[a] (±2.09)	39.90[a] (±1.67)	44.1[a] (±2.37)	46.56[a] (±3.42)	0.399	1.02
Root (g)	28.47[a] (±0.72)	29.39[a] (±1.22)	28.19[a] (±1.03)	26.41[a] (±1.75)	0.113	2.67
Root:shoot	0.65[a,b] (±0.03)	0.75[a] (±0.07)	0.66[a,b] (±0.05)	0.57[b] (±0.01)	*	2.97

3.3. P. vulgaris *flowering*

During the first growing season none of the AMF treatments had a significant effect on *P. vulgaris* flowering compared to the control until after 112 days after planting (one way ANOVA, $p > 0.05$) (Table 4a). The direct addition of AMF inoculum to *P. vulgaris* plugs significantly increased the number of flowers on each *P. vulgaris* plant 123 days after planting compared to the control and the other two AMF treatments (one way ANOVA, $F = 2.94$, $p < 0.05$). However this effect was no longer significant 162 days after planting (Table 4a).

At the start of the second growing season, untreated *P. vulgaris* plants showed significantly earlier flower emergence 347 days after planting compared to all three AMF treatments (one way ANOVA, $F = 2.95$, $p < 0.05$). However this effect was no longer significant 351 days after planting (one way ANOVA, $F = 2.31$, $p > 0.05$) (Table 4b). All plants produced more flowers compared to the previous growing season, however all showed a large decline in late July (Day 370) due to a prolonged drought (Table 4b). During this drought, flower numbers of *P. vulgaris* plants with AMF inoculum applied directly to the plugs did not decline at the same rate to control plants (one way ANOVA, $F = 4.39$, $p < 0.05$), however this effect was longer significant 392 days after planting (Table 4b).

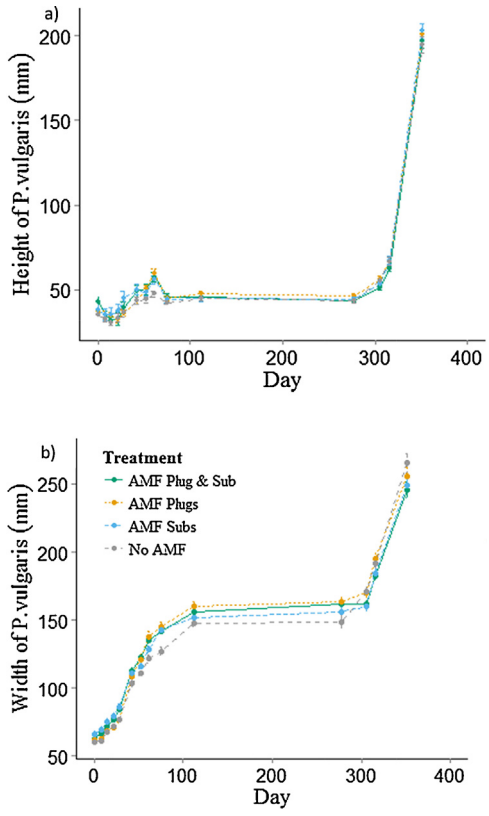

Fig. 3. Mean (a) height and (b) width of *P. vulgaris* during two growing seasons. Day 1 = 26.6.13, Day 400 = 2.8.14. Error bars represent one standard error. No AMF, only plug plants; AMF Plugs, AMF inoculum added to plugs; AMF Subs, AMF inoculum added to substrate; AMF Plug and Subs, AMF inoculum added to plugs and substrate.

3.4. P. vulgaris *nutrient status/chlorophyll*

Living *P. vulgaris* leaves grown in all three AMF treatments had significantly higher concentrations of P compared to the control (one way ANOVA, $F = 7.32$, $p > 0.05$) (Table 5). None of the AMF treatments had a significant effect on the N concentration of live *P. vulgaris* leaves compared to the control (one way ANOVA, $F = 1.48$, $p < 0.05$) (Table 5).

P. vulgaris leaf chlorophyll concentration was not significantly affected by any of the AMF treatments 61 and 281 days after planting compared to the control (one way ANOVA, $F = 1.54$ and 1.87, $p < 0.05$) (data not shown).

3.5. P. lanceolata *AMF colonisation*

All three AMF treatments had significantly higher AMF colonisation rates of *P. lanceolata* (20–30%) than the two non-AMF treatments (0–2%) (one way ANOVA, $F = 47.54$, $p < 0.05$) (Fig. 4). The majority of the two non-AMF treated modules did not experience any AMF colonisation of *P. lanceolata*, with AMF only present in a low number of modules (Fig. 4). The three AMF treatments did not significantly differ from one another (Tukey HSD, $p < 0.05$) (Fig. 4).

4. Discussion

4.1. *Effect of AMF on* P. vulgaris

This study has shown that AMF networks can be successfully introduced to green roof systems, which supports the conclusions of previous studies (Meyer, 2004; Sutton, 2008; John et al., 2014; Molineux et al., 2014). Furthermore, this study is the first to show the significant benefits of AMF colonisation on the nutritional status (leaf P content) of a green roof plant species.

All three AMF treatments in this trial showed significantly greater *P. vulgaris* root AMF colonisation and leaf P. This result was expected as AMF colonisation of plant roots often leads to greater levels of plant P due to the greater foraging capability of AMF mycelium and its greater ability to access immobile forms of P (Smith and Read, 2008). Increased accessibility to P is important for early season plant growth (Grant et al., 2001), crop/seed production (Grant et al., 2005), flower production (Fenner, 1986; Petraglia et al., 2013) and photosynthesis, respiration and metabolism (Vance et al., 2003). Thus, the observed increase in P levels in the plant leaf tissue should be beneficial to plant health. The uptake of N by *P. vulgaris* was not improved by any AMF treatment compared to the controls in this trial. This was not unexpected as there is still debate of the importance of AMF colonisation for N uptake by host plants, as it is now widely regarded that AMF only provides host plants with N under certain conditions or when there is very little available N (Hodge et al., 2010; Hodge and Storer, 2014).

Previous studies have shown the effect of AMF on green roof plant growth (Meyer, 2004; Sutton, 2008), and measured

Table 4a
Mean number of flowers per *P. vulgaris* plant during the first growing season when grown in green roof modules and treated with a number of AMF treatments. AMF Plug, AMF inoculum added to plugs; AMF Plug and Subs, AMF inoculum added to plugs and substrate; AMF Subs, AMF inoculum added to substrate; No AMF, only plug plants. Statistical significances of *p*-values: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Statistical significances were calculated from one-way ANOVA. Letters indicate statistical differences between means. Df = 28.

Days post-installation	June 13 1	July 12	July 18	July 25	July 33	Aug 39	Aug 46	Aug 61	Sept 75	Sept 84	Oct 104	Oct 112	Oct 123	Dec 162	Jan 14 195	Jan 214	March 256
AMF Plug	0.00	0.00	0.00	0.09	0.13	0.09	0.06	0.28	0.19	0.22	0.06	0.13	0.16[a]	0.06	0.03	0.03	0.03
AMF Plug and Subs	0.00	0.03	0.03	0.06	0.16	0.22	0.13	0.28	0.19	0.13	0.00	0.00	0.00[b]	0.03	0.00	0.00	0.00
AMF Subs	0.00	0.13	0.09	0.22	0.38	0.31	0.19	0.16	0.00	0.03	0.00	0.09	0.00[b]	0.00	0.00	0.00	0.00
No AMF	0.00	0.03	0.09	0.16	0.25	0.09	0.13	0.19	0.19	0.03	0.00	0.00	0.00[b]	0.06	0.03	0.00	0.00
p	NA	0.05	0.14	0.18	0.26	0.12	0.56	0.18	0.39	0.21	0.4	0.26	*	0.66	0.57	0.4	0.4
F	NA	2.41	1.74	1.59	1.34	1.86	0.75	1.58	1.03	1.53	1	1.35	2.94	0.53	0.67	1	1

Table 4b
Mean number of flowers per *P. vulgaris* plant during the second growing season when grown in green roof modules and treated with a number of AMF treatments. AMF Plug, AMF inoculum added to plugs; AMF Plug and Subs, AMF inoculum added to plugs and substrate; AMF Subs, AMF inoculum added to substrate; No AMF, only plug plants. Statistical significances of *p*-values: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Statistical significances were calculated from one-way ANOVA. Letters indicate statistical differences between means. Df = 28.

Days post-installation	April 14 278	May 337	June 347	June 351	June 354	June 357	June 361	June 365	July 375	July 383	July 392	July 398	Aug 14 403
AMF Plug	0.00	0.00	0.00[b]	0.03	0.22	0.56	4.16	10.47	7.16	1.66[a]	0.38	0.00	0.16
AMF Plug and Subs	0.00	0.00	0.00[b]	0.00	0.00	0.22	4.13	9.97	6.75	1.13[ab]	0.25	0.16	0.59
AMF Subs	0.00	0.00	0.00[b]	0.00	0.00	0.09	3.41	9.41	7.06	1.00[ab]	0.56	0.06	0.38
No AMF	0.00	0.00	0.16[a]	0.19	0.28	0.94	6.09	12.06	6.38	0.47[b]	0.06	0.13	0.63
p	NA	NA	*	0.08	0.21	0.15	0.08	0.21	0.83	**	0.12	0.66	0.36
F	NA	NA	2.95	2.31	1.52	1.8	2.33	1.54	0.3	4.39	1.97	0.54	1.09

Table 5
P and N content of living *P. vulgaris* leaves in August 2014. AMF Plug, AMF inoculum added to plugs; AMF Plug and Subs, AMF inoculum added to plugs and substrate; AMF Subs, AMF inoculum added to substrate; No AMF, only plug plants. Statistical significances of *p*-values: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Statistical significances were calculated from one-way ANOVA and Tukey HSD post hoc test, Df = 28. Letters indicate statistical differences between means.

	Treatment (±SE)					
	AMF Plugs	AMF Subs	AMF Plugs and Subs	No AMF	*p*-Value	*F*-value
P content (μg P mg^{-1} dry leaf)	1.8[b] (±0.1)	1.6[b] (±0.1)	1.5[b] (±0.2)	0.9[a] (±0.2)	***	7.32
N content (μg N mg^{-1} dry leaf)	14.6[a] (±0.7)	11.9[a] (±0.9)	11.5[a] (±1.5)	13.1[a] (±1.4)	0.24	1.48

AMF colonisation and fungal populations in green roof substrate (McGuire et al., 2013; John et al., 2014; Molineux et al., 2014). AMF inoculum in the form of prairie top soil has been shown to colonise the roots and increase the growth of prairie grasses grown in 9 cm deep substrate (95% inorganic, 5% compost) when added with a polyacrylamide water absorbent gel (Sutton, 2008). When added by itself the inoculum had no significant effect on plant growth, suggesting that the water absorbent gel was needed to facilitate AMF benefits (Sutton, 2008). Similarly the biomass production of alpine grasses and herbs germinated in substrate (95% inorganic, 5% compost) from seed was initially increased by the use of AMF inoculum after 10 weeks, although this effect was no longer significant after 25 weeks of growth (Meyer, 2004).

In this current trial, the addition of AMF did not have a significant effect on host plant growth at any point. Green roof substrate composition is highly variable, with the amount of organic matter

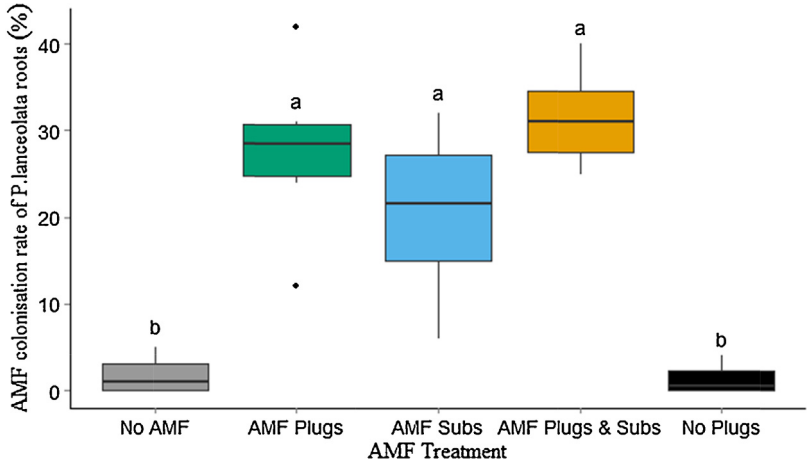

Fig. 4. Percentage AMF colonisation of *P. lanceolata* roots used as bait plants grown from August 2013 to October 2013. No AMF, only plug plants; AMF Plugs, AMF inoculum added to plugs; AMF Subs, AMF inoculum added to substrate; AMF Plug and Subs, AMF inoculum added to plugs and substrate; No Plugs, no plugs or AMF inoculum added. The middle bar represents s treatments mean, the upper and lower box hinges the 1st and 3rd quartiles and the thin black line the complete spread of data. Treatments with the same letter do not differ significantly from one another (Tukey HSD, $p < 0.05$).

used in this trial (20%) significantly higher than previous trials (5%) which showed benefits of AMF on plant growth (Meyer, 2004; Sutton, 2008). AMF has a variety of effects on host plants depending on the nutritional (Menge et al., 1978; Nouri et al., 2014) and physical characteristics of the soil (Van Der Heijden and Sanders, 2002; Escudero and Mendoza, 2005; Posada et al., 2008). This indicates that although AMF may sometimes improve the growth of host plants on green roofs, this improvement may only be significant early in plant establishment (Meyer, 2004), or dependent on plant species and environmental conditions (Van Der Heijden and Sanders, 2002). Additionally, it is now recognised that although AMF colonisation does not always affect plant growth, it can still have an impact upon the amount of P uptake (as demonstrated in this trial), or the mode of P uptake (plant vs AMF) (Smith et al., 2003, 2009).

The increased P levels in the leaves of the AMF inoculated *P. vulgaris* compared to control treatment supports the use of AMF inoculum as an alternative to slow release fertiliser on green roofs. The use of slow release fertiliser significantly contributes to the high levels of nutrient runoff from green roofs (Berndtsson, 2010), reduces the sustainability of a green roof and also acts as an additional long-term financial cost to the building (Peri et al., 2012; Berardi et al., 2014). However more research is needed to assess if AMF can fully replace the use of slow release fertilisers on green roofs (in particular for the provision of N), without compromising plant growth and health, especially on thin extensive roofs with a substrate depth of 40–80 mm.

The addition of AMF inoculum did not significantly affect the survival of *P. vulgaris* plants throughout the trial (data not shown). However, due to the supplementary watering given to plants during establishment and during growing season two, it is difficult to draw conclusions regarding the effect of AMF on plant establishment in terms of drought tolerance. This should be a priority for future research as high plant mortality, especially during establishment, reduces the quality of services provided by a green roof (Snodgrass and Snodgrass, 2006; Dunnett and Kingsbury, 2010; Rowe et al., 2014).

4.2. Natural occurrence of AMF in green roof substrate

AMF has previously been found in substrate from established green roofs composed of 80% crushed brick and 20% commercial compost (Rumble and Gange, 2013; Young, 2014). However, in both studies only the AMF colonisation of host plants was measured, and no quantification of the effect of AMF on plant growth or health was made (Rumble and Gange, 2013; Young, 2014). The present study has shown that AMF inoculum was present in the substrate but only at very low levels which did not have any biological impact upon host plants over the time period assessed. This supports previous evidence that AMF inoculum can be naturally present in commercial substrate, although it is likely that this will be at very low levels (McGuire et al., 2013; John et al., 2014). These low levels of AMF are unlikely to have any significant effect upon plant growth and health, especially in the early stage of green roof establishment. In addition, as green roof substrate is highly variable in its composition, origin and how it is stored, the low level of natural AMF inoculum is also expected to be highly variable between substrates.

4.3. Different methods of AMF inoculation

The highest rates of AMF colonisation of *P. vulgaris* when treated with AMF inoculum was observed when AMF inoculum was applied directly to the plugs, whilst the lowest when AMF inoculum was applied to both the plugs and substrate. This suggests that applying AMF inoculum directly to plugs at the manufacturer's recommended rate is the most effective way to gain high levels of AMF root colonisation. The total amount of AMF inoculum applied in the plug and substrate treatment was the same as the other two AMF treatments, but was split 50:50 between the plug and substrate. The total amount of inoculum applied in this treatment was less than the manufacturer's recommended amount and therefore was more thinly spread and much less likely to come into contact with *P. vulgaris* roots. The colonisation rate of plant roots by AMF can be increased by the application of AMF inoculum at rates above the manufacturer's recommended limits. However it is unlikely that the added benefits of this increased AMF colonisation will offset the financial cost of applying increased amounts of AMF inoculum (Corkidi et al., 2004; Tarbell and Koske, 2007). The manufacturer's application rates are a good guide to the levels of AMF inoculum needed to achieve good colonisation, but more work is needed to determine optimum rates in green roof substrate. Applying AMF inoculum directly to each plug as it is planted is also more labour intensive than mixing large amounts of AMF inoculum into the substrate offsite, but the improved colonisation rates may justify the added labour whilst the total amount of inoculum used may be less and therefore cheaper.

4.4. Establishment of AMF network

The use of *P. lanceolata* bait plants showed that a viable AMF network was established throughout the whole substrate within 4 months of planting. The presence of widespread and active AMF networks is key for the colonisation of new plants as well as recovery from disturbance (Jasper et al., 1991; Smith and Read, 2008). Importantly, *P. lanceolata* was colonised to the same extent when AMF inoculum was applied either directly to just the plugs or to the substrate, demonstrating that green roofs do not have to have AMF inoculum applied to all of the substrate in order to colonise other plants growing on the roof. By selectively applying AMF inoculum, labour and AMF inoculum costs could decrease, further increasing the efficiency of future AMF inoculum applications.

4.5. Effect of AMF on P. vulgaris flowering

When AMF inoculum was added directly to *P. vulgaris* plugs there was an observed extension of the *P. vulgaris* flowering period in the first flowering season. This was only significant at one time point towards the end of October 2013, and was not measured again until December 2014, by which point the effect on flowering was no longer significant. This extension of flowering may have been caused by the additional P available to the plant, as the plug only AMF treatment showed the highest AMF colonisation. AMF colonisation has previously been shown to increase plant P and the number of bud and flowers in ornamental plants (Perner et al., 2007; Garmendia and Mangas, 2012). However, increased flowering can also be induced by stress conditions, which helps ensure that the chances of plant reproductive success are increased despite potential plant mortality (Obeso, 2002; Yaish et al., 2011). Increased flowering may have a negative effect on the reproductive success of a plant in the short term because of poor seed quality, or in the long term by reducing the amount of resources available to the plant for future growth and reproduction (Obeso, 2002). For example, in this study the prolonged flowering may have been detrimental to the long term health of *P. vulgaris* as plants invested resources producing extra flowers at a time when pollinator activity would be much lower at the end of the growing season. Interestingly the control *P. vulgaris* plants showed the earliest flower emergence at the start of the 2nd growing season. Therefore more research is needed in order to determine if AMF colonisation of green roof plants has any consistent effect on the timing and level of flowering. If this is the case, it is important to know if this flowering provides extra aesthetic

and pollination benefits for the green roof or actually compromises the long term health and survival of plants on the roof.

4.6. Future research

Future research should focus on the effect of AMF inoculum on green roof plants. In particular this should include the effect on plant establishment, long term reproduction success, plant survival during drought conditions and the effect of increased nutrient uptake on plant health. The effect of AMF inoculum on the whole plant community and green roof service provision should also be investigated, for example; effect of AMF altered plant growth and survival on green roof heat reduction and stormwater retention, the effect of increased plant flowering on pollinators and the long term plant diversity of the green roof.

5. Conclusions

This study has confirmed that commercial AMF inoculum can be used to successfully colonise green roof plants and introduce AMF networks into green roof substrate. Although this study did not detect any effect on plant growth or leaf N concentrations, leaf P concentrations were higher in all AMF treatments.

Significantly higher AMF colonisation rates were found when AMF inoculum was applied directly to the plug plants. This suggests that despite being more labour intensive, this method of application is more effective at colonising plants with AMF. In addition, it should also be significantly cheaper as a much smaller amount of AMF inoculum is needed.

Low levels of naturally present AMF inoculum was detected in the commercial substrate used in this trial. This supports previous work suggesting that although AMF may be present in commercial substrate, it is only present in very low amounts which are unlikely to have any significant biological impact in the short to medium term (John et al., 2014). Overall the research in this paper, and the limited previous work suggests artificial introduction of AMF to green roofs via substrate can be successful (Meyer, 2004; Sutton, 2008; Molineux et al., 2014). AMF could potentially replace the use of slow release fertilisers which are commonly used on green roofs and lead to limited benefits for plant growth and physiological performance (Meyer, 2004; Sutton, 2008).

However, care should be taken in the use of AMF on green roofs, with this trial showing that the benefits of AMF are not immediately apparent in a green roof context. Clearly much more work is needed to fully assess if the benefits of AMF, which are often observed in conventional ecosystems, can be replicated on green roofs. This will in turn determine if the financial implications of adding AMF inoculum to green roofs can be justified. In addition, the benefits of AMF should not be expected to compensate for poor green roof design or plant choice but should complement existing green roof species as well as increasing the palette of hardy plants used on green roofs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ufug.2015.08.012.

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