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Phosphorus mobilizing consortium Mammoth P enhances plant growth

Peter Baas, Colin Bell, Lauren M Mancini, Melanie N Lee, Richard T Conant, Matthew D Wallenstein

Global agricultural productivity may be constrained by the finite and limited supply of phosphorus (P), adding to the challenges in meeting the projected needs of a growing human population in the coming decades. In addition, when P fertilizers are added to soils, they can become bound to soils resulting in low fertilizer efficiency. However, P-mobilizing bacteria could potentially liberate soil-bound P, resulting in a higher plant P uptake and increased yield. Bacteria can mobilize P through several mechanisms, suggesting that consortia of P-bacteria may be more effective than single species. Species diversity can have a synergistic, or non-additive, effect on ecosystem functioning (“the whole is more than the sum of its parts”) but rarely is the microbial community structure intentionally managed to improve plant nutrient uptake. We investigated whether inoculation of soils with a four-species bacterial community developed to mobilize soil P could increase plant productivity. In wheat and turf trials, we found that Mammoth P was able to deliver yields equivalent to those achieved using conventional fertilizer applications. Herbs and fruits showed that the combination of fertilizer with Mammoth P significantly increased productivity - in some cases productivity doubled. Metabolites produced by the Mammoth P consortium led to increased yields in some cases, suggesting that microbial products (produced in the absence of plants) played a role in enhancing plant productivity. Results from these trials indicate substantial potential of Mammoth P to enhance P supply to plants, improving P fertilizer use-efficiency and increasing agricultural productivity.

AUTHOR COVER PAGE

Title: Mammoth P: A phosphorus mobilizing microbial consortium that enhances plant growth

Authors: Peter Baas¹, Colin Bell², Lauren Mancini¹, Melanie Lee¹, Richard T. Conant¹ and Matthew D. Wallenstein¹

Affiliations: ¹Natural Resource Ecology Laboratory, Colorado State University, 1231 E Drive, Fort Collins, CO 80523, USA; ²Growcentia Inc., 1552 Riverside Ave, Fort Collins, CO 80524, USA

Corresponding author: Peter Baas, 1231 E. Drive, Colorado State University, Fort Collins, CO 80523, USA, pbaas83@gmail.com

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ABSTRACT

Agricultural productivity may be constrained in the 21st Century by the finite supply of global a phosphorus (P), adding to the challenges in meeting the projected needs of a growing human population in the coming decades. In addition, when P fertilizers are added to soils, they can become bound to soils resulting in low fertilizer efficiency. However, bacteria have the ability to mobilize soil bound P through several mechanisms potentially resulting in a higher plant P uptake and increased yield. Furthermore, species diversity can have a synergistic effect on ecosystem functioning (“the whole is more than the sum of its parts”) suggesting that bacterial communities, or consortia, may be more effective than single species. However, in agriculture management practices, rarely is the soil microbial community structure effectively manipulated to improve plant nutrient uptake. We investigated whether inoculation of soils with a bacterial consortium developed to mobilize soil P named Mammoth P could increase plant productivity. In turf, herbs and fruits, we showed that the combination of conventional inorganic fertilizer combined with Mammoth P increased productivity up to twofold compared to the fertilizer treatments without the Mammoth P inoculant. In wheat trials, we found that Mammoth P by itself was able to deliver yields equivalent to those achieved with conventional inorganic fertilizer applications. The metabolites produced by the consortium in Mammoth P likely played a role in enhancing plant productivity. Results from this study indicate the substantial potential of Mammoth P to enhance P supply to plants, improving P fertilizer use-efficiency and increasing agricultural productivity.

INTRODUCTION

In the 1960s the “Green Revolution” averted a potentially catastrophic lack of food production in the developing world (Khush 2001; Lam 2011) allowing the global population to double while still meeting food demands (Lam 2011; Tilman 1998). Most of the agricultural gains over the last fifty years have been accomplished by a combination of changed management such as irrigation practices, plant genetic manipulation and breeding efforts, and a surge in fertilizer usage such as nitrogen and phosphorus (Conant et al. 2013; Lam 2011). However, current agriculture production is insufficient to provide enough food for the growing global population through the mid-21st century (Abelson 1999; Ray et al. 2013). One challenge facing continued agriculture production are nutrient constraints. Phosphorus (P) is a finite resource with substantial resources found in only 10 countries (Cordell 2010; Jasinski 2013); and peak global phosphorus availability could occur in less than three decades (Craswell et al. 2010; Steen 1998). Phosphorus is a critical nutrient used to maximize plant growth and yield. Reductions in available P fertilizer can severely diminish crop yields. One solution for mitigating the threat of this diminishing resource is to develop sustainable technologies to improve P use efficiently for plant uptake.

Another challenge to farmers is that P precipitates and strongly binds with soil mineral surfaces. Up to 90% of P fertilizer applied to soils is made unavailable to plants because it binds to Ca, Al and Fe-bearing soil mineral surfaces or is lost from the ecosystem by leaching (Doolette & Smernik 2011; Randriamanantsoa et al. 2013). Soils with a large capacity to bind P are especially concerning because P delivery to plants is inefficient, thus, requiring more inputs relative to the P outputs in harvested crops (MacDonald et al. 2011). Further, plants are only able to utilize soil P when it is dissolved in the form of orthophosphate (Schachtman et al. 1998), thus, total soil P content is not a strong determinant of plant-available P.

Soil bacteria can strongly influence the amount of soil P that is plant-available by solubilizing the mineral-associated P (Malboobi et al. 2009; Osorio & Habte 2014; Tawaraya et al. 2006). They achieve this by releasing organic acids and high-affinity iron chelating siderophores which solubilize mineral-bound P (Richardson et al. 2009), thus, mobilizing P and making it accessible for plant uptake.

Furthermore, microbial nutrient cycling activities are most beneficial for plants effective when they occur within the plant-rooting zone, known as the rhizosphere. In the rhizosphere, microbial communities have been shown to mobilize orthophosphate (De Freitas et al. 1997; Turan et al. 2012) and further increase plant P uptake by stimulating plant root growth (Bal et al. 2013; Penrose & Glick 2003; Rashid et al. 2012). Since there are multiple mechanisms by which microbes can mobilize soil P, microbial consortia (several species) may be more effective than single - species isolates because no single strain can be optimal for all of the various mechanisms that drive this process. Previous studies have shown that a consortium of P-mobilizing bacteria are more effective at making P available than the microbial isolates (Baas et al. in prep) and a few other studies have provided evidence for synergistic effect between multiple microbial species (Kim et al. 1997; Tarafdar & Marschner 1995). Can a consortium of P-mobilizing bacteria improve (phosphorus-intensive) processes like plant emergence, blooming, and productivity?

The emerging number of microbial biostimulant products on the market offer promise of improved microorganism activities to enhance plant growth. One of these products, Mammoth P™ (Growcentia, Fort Collins, CO, USA), is a microbial biostimulant comprised of a novel microbial consortium selected for its superior capacity to solubilize soil P. In this study, we tested the effect of Mammoth P to increase plant emergence, blooming, and productivity for a variety of plant species. Our objective was to test the efficacy of this microbial biostimulant to enhance plant growth across a wide variety of crops, including: wheat, herbs, fruits and turf grass. We predicted that the addition of a consortia of microbes found in Mammoth P would increase plant productivity, whereas the greatest effect would occur when the plant is both fertilized and inoculated with Mammoth P. We further predicted that secondary metabolites produced by the microbes - but in the absence of active microbes - could have a positive effect on plant performance.

METHODS

The inoculum

We tested the effect of the Mammoth P consortium consisting of four bacterial taxa (*Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas putida* and *Comamonas testosteroni*; **Table 1**) on plant productivity. Bacterial cultures were grown in a proprietary, P-limiting media (Growcentia Inc., Fort Collins, CO, USA) for three days reaching at least 10^8 colony forming units (CFU) ml⁻¹ (Black 2008).

Experimental design

Plant productivity was assessed in multiple greenhouse trials for hard red winter wheat (*Triticum aestivum*), fescue turf grass (*Festuca arundinacea*; Kentucky 31 variety), jalapeño (*Capsicum annuum*; early jalapeño variety), cherry tomato (*Solanum lycopersicum*; Sweetie variety) and basil (*Ocimum basilicum*; Italian Genovese variety). In addition to species listed above, we also tested plant emergence on brandywine tomatoes (*Solanum lycopersicum*), marigold flowers (*Tagetes patula nana*; Petite variety) and broccoli raab (*Brassica rapa ruvo*). We tested plant performance with fertilizer, Mammoth P, metabolites produced by the Mammoth P consortium, and interactions among the treatments. Treatments varied and included some or all of the following seven: 1) P-limiting media (media control); 2) inoculation with Mammoth P; 3) Mammoth P metabolites; 4) fertilizer only; 5) Mammoth P plus fertilizer; 6) Mammoth P metabolites plus fertilizer and 7) water. Mammoth P metabolites were collected by filtering culture through a 0.2 micron filter. Fertilization rates were conducted following manufacturer's recommendations. Plants in the greenhouse were watered daily and the temperature was $22 \pm 2.5^\circ\text{C}$ with supplemental growth lights running a total of 16 hours a day.

We used two varieties of red hard winter wheat (Byrd and Hatcher) and two types of soil from 1) the Agricultural Research Development & Education Center (ARDEC) and 2) Waverly, a 130 ha Colorado State University managed property located north of Fort Collins, Colorado ($40^\circ 42' 54''\text{N}$, $105^\circ 50' 53''\text{W}$). The wheat was vernalized for 6 weeks at 7°C and the seedlings were subsequently

planted into planter trays. After two weeks the plants were transplanted to a one gallon pot containing the same Waverly and sand mixture or ARDEC and sand mixture. The pots were filled with 2 mm sieved Waverly soil or 4 mm sieved ARDEC soil mixed (1:1) with washed sand. Plants were treated with either Mammoth P, Mammoth P metabolites, Hoagland's solution or water at the initial planting of the seedlings (1 mL pot⁻¹), at transplant (5 mL pot⁻¹) and a third time 2 weeks after transplanting (5 mL pot⁻¹). After two months the plants were harvested for aboveground biomass. Plant material was dried at 65°C until at constant weight to determine total plant dry biomass.

Fescue turf grass was planted in a Waverly-sand mixture in planting trays (0.7 L & 1.4 L). We tested the seven treatments described above excluding the P-limiting media treatment. Mammoth P inoculation was done at planting with 30 mL per .56g of seed. The fertilizer treatment was done at planting using the slow-release fertilizer formulation Scotts Turf Starter following the manufacturer's recommendations (The Scotts Company LLC, Marysville, OH, USA). After two months the aboveground biomass was collected, dried at 65°C and weighted for aboveground productivity.

The herb and fruit experiments were conducted using 4 mm sieved soil from the Agricultural Research Development & Education Center (ARDEC) mixed 1:1 with Fafard 4P potting soil (Sun Gro Horticulture Inc., Agawam, MA, USA). First we planted cherry tomato, basil, and jalapeño seeds in 30 mL of soil mixture using a planter tray. After 7 weeks from planting the all seedlings were transplanted to a 6 inch pot (1 L) and after 3 months from planting the cherry tomatoes were transplanted to a 2 gallon pot. To provide a baseline level of nutrient availability all plants were fertilized weekly for the first month after transplanting using half the recommended level of fertilization with Jack's classic 20:20:20 fertilizer (125 ppm) and weekly (i.e. tomato) or twice monthly (i.e. basil and jalapeño) with 100 ppm N 15-5-15 Technigro (Sun Gro Horticulture Inc., Agawam, MA, USA). These fertilization regimes represent a minimal basal level of fertilization (Heeb et al. 2005). The jalapeño and basil plants designated to receive inoculum were inoculated at planting, after 1, 1.5, 2, and 3 months in addition to immediately after transplanting. The cherry tomato plants were inoculated with Mammoth P after planting, transplanting and 1, 1.5, and 3 months. The treatment volume was 2 mL pot⁻¹ (planter) and 5 mL pot⁻¹ (6 inch and

gallon pot). The fertilized treatments received the slow release formulation Miracle Gro Shake 'n Feed® (The Scotts Company LLC, Marysville, OH, USA), as recommended, at planting and after 5 months. Basil plants were cut back to the top four starter leaves to prevent flowering and, thus, maximize leaf productivity. Fresh weight of basil leaves collected was used to determine yield. Jalapeno buds and blooms were counted twice monthly and jalapeno peppers were harvested when 5cm was reached or the pepper had turned red. Fresh weight of peppers was used to determine yield. After 6 months we harvested the remaining peppers and basil leaves and determined a terminal yield by fresh weight of harvest. For cherry tomato plants, we determined the number of buds, blooms and red tomatoes twice monthly for 4 months after planting.

Statistics and calculations

We determined the treatment difference using analysis of variance analyses (ANOVA) with multiple comparisons using Tukey tests. The vegetable data were analyzed using a repeated measures approach with orthogonal contrasts to determine treatment differences. Data was tested for normality using Q-Q plots and if proven non-normal, were log transformed. Significant differences indicate $p < 0.05$ unless stated otherwise. All statistics were conducted in SAS JMP 11.0.

RESULTS

Plant emergence and bloom development

The addition of a slow release fertilizer significantly reduced plant emergence by 55-50% compared to the Mammoth P treatment, metabolite, media, and water treatment (**Figure 1**). The proportional increase in emergence of the fertilized treatment compared to the Mammoth P ($+89 \pm 44\%$) and the metabolite ($+108 \pm 70\%$) treatment were significantly greater than zero ($p < 0.001$).

Jalapeno plants showed the time to develop blooms were significantly lower for the Mammoth P (9%), metabolite (14%), and Mammoth P plus fertilizer (16%) treatments compared to the fertilizer only

treatment (**Figure 2**). Additionally, the fertilized treatment showed no accelerated bloom development compared to the control or media control treatments. Combining Mammoth P with a fertilizer significantly reduced the time to first bloom while the metabolite treatment with fertilizer did not show a reduced time to first bloom.

Plant productivity

Plant productivity was generally significantly greater if Mammoth P was applied compared to the fertilizer only treatments and the greatest improvements in productivity compared to the fertilized treatment were found for the Mammoth P plus fertilizer treatments, with increased productivity of up to 91%. Additionally, Mammoth P metabolites plus fertilizer treatments often had positive effects on productivity similar to the Mammoth P plus fertilizer treatment.

Hard red winter wheat productivity (**Figure 3**) for the fertilized, metabolites, and Mammoth P treatments ranged from 0.77 – 1.4 g plant⁻¹ and the increase in productivity from the mean water control was greatest in the Mammoth P treatment (41 ± 6%) followed by the fertilized (23 ± 9%) and the metabolite treatment (16 ± 7%). Overall, the Mammoth P treatment was significantly greater than the water control and in the ARDEC soil type the metabolite treatment exhibited greater productivity.

Fescue turf grass productivity (**Figure 4**) was greatest when Mammoth P or its metabolites were combined with fertilization with productivity being 74-91% greater than traditional fertilization practices. The fertilizer plus Mammoth P and fertilizer plus metabolite treatments were significantly greater than both the water control ($p = 0.9$ for metabolite & fertilizer) and the fertilized treatment.

Herb and fruit productivity (i.e. basil, jalapeño and tomato) showed significant treatment effects over time. After 6 months of growth the jalapeño productivity (**Figure 5**) for the Mammoth P plus fertilizer treatment were significantly different (41 ± 16%) compared to the fertilizer treatment. Mammoth P inoculation plus fertilizer was not significantly different from the fertilizer treatment for jalapeño and basil while marginally significant for tomato ($p < 0.1$). Basil productivity in the fertilized treatments with or without Mammoth P or metabolites were significantly greater than the remaining treatments. Jalapeño

productivity was significantly greater with Mammoth P plus fertilizer than the water control treatments. Tomato productivity was greater for the Mammoth P plus fertilizer ($p < 0.09$) and the metabolite plus fertilizer ($p < 0.001$) treatments.

DISCUSSION

In this study we showed that the consortia of microbes found in Mammoth P has the potential to dramatically improve plant emergence and productivity across a wide variety of plant species and soil types. These results suggest a crucial role for the value in developing sustainable technologies such as this microbial biostimulant and microbial metabolites in enhancing plant-microbial feedbacks to stimulate bloom and improve plant production.

Emergence and bloom enhancement

Microbial inoculation with Mammoth P or its metabolites were able to enhance plant emergence across a wide variety of plants. Shortening time-to-bloom by 16% in jalapeño plants for both the metabolite and Mammoth P treatments suggest that microbial products may produce a more advantages environment for plant development. We argue that the usage of a multi-species inoculum allows the consortium to adapt to a wide variety of environmental conditions while maintaining efficacy. We hypothesize that the interactions among the inoculum's microbial constituent species resulted in the formation of metabolites capable of enhancing the plant investment in fruit development. Previous studies have found positive effects of inoculation on the rate of emergence (Kropp et al. 1996; Lucy et al. 2004) which been linked to increased root growth (Chanway et al. 1991). But to our knowledge, this phenomenon has never been observed in such a wide variety of plants.

Microbial metabolites could increase plant allocation to bloom development by being rich in compounds capable of solubilizing P and micronutrients by reducing the soil pH (Khan et al. 2009), by producing extracellular enzymes capable of liberating nutrients (Baas et al. in prep; Khan et al. 2009), or by acting as a plant hormone. Indeed, rhizosphere microbial production of metabolites such as salicylic acid,

ethylene, glutamate and auxins have been linked to increased plant disease resistance (Van Wees et al. 2008), growth promotion (Spaepen 2015) and stimulating the induction of flowering (Raskin 1992). Basil showed no effect from Mammoth P or its metabolites and it is likely the plants were already functioning at maximum capacity with generally having lower nutrient requirements than fruits (Sharafzadeh & Alizadeh 2011; Tesi et al. 1994).

Plant productivity

We found inoculation with Mammoth P to generally result in greater levels of productivity compared to traditional fertilizer treatments. We also found Mammoth P to enhance productivity when combined with a slow release fertilizer treatment. Previous studies have shown that up to 50% of ecosystem productivity is due to plant-microbial relationships with a variety of bacterial, archaeal, and mycorrhizal species (Adesemoye & Kloepper 2009; Hassan et al. 2013; Van Der Heijden et al. 2008). We found that the inoculation of the four-species consortia, Mammoth P, improved productivity by up to 91%, suggesting that the potential of plant-microbial interactions in enhancing productivity can be even greater than 50%. Our findings also confirm the conceptual framework proposed by Van Der Heijden et al. (2008) suggesting that the lower nutrient availability would improve any effect of microbial inoculations. Product efficacy reports of similar products reported flowering to increase by 26-40% (sunflower) and 2-14% (*chrysanthemum*) with productivity increases between 5-20% (Savov et al. 2011; Savov et al. 2012). Increased productivity with Mammoth P was not only similar but consistent among a wider variety of crop species.

Mechanisms for plant-microbial interactions

The current paradigm is that microbial nutrient immobilization is important in preventing nutrient loss during the inactive plant development stages while microbial turnover functions similar to a “slow-release fertilizer” at later plant developmental stages (Malik et al. 2013; Singh et al. 1989). We are currently starting to appreciate the importance of microbial products on plant growth (Spaepen 2015) and

our results suggest that these mechanisms might be widespread among plant species and that consortia based inoculation is capable of dramatically enhancing plant productivity. The differing ability of plants to control microbial communities by exudation among plant species (Brimecombe et al. 2000) would determine whether the microbial community could engage in a symbiotic, a neutral or competitive relationship. Some plant species may support a beneficial relationship with the inoculum bacterial community (i.e. jalapeño, wheat and turf) while others are mainly responding to microbial metabolites and live microbes might reduce the metabolite effect by competing for resources with the microbial community (i.e. tomato). These results suggest that microbial communities might be more in control of microbial-plant interactions than previously recognized.

CONCLUSIONS

The Mammoth P consortium provided superior plant emergence, faster blooming and, for most plant species tested, plant productivity. Our results suggest that microbial metabolites may play a crucial role in controlling plant growth and that the microbial community might be controlling plant development in a variety of ways. Although the magnitude of the response was dependent on basal fertilization practices and the specific plant species, inoculation with Mammoth P or its metabolite treatments resulted in positive effects in a wide range of crops. These results indicate the vast potential for future development of consortia-centered inocula to transform agriculture.

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361

362

Table 1 (on next page)

The relative proportions (%) of the top four species representing > 95% of all operationally defined units (OTU).

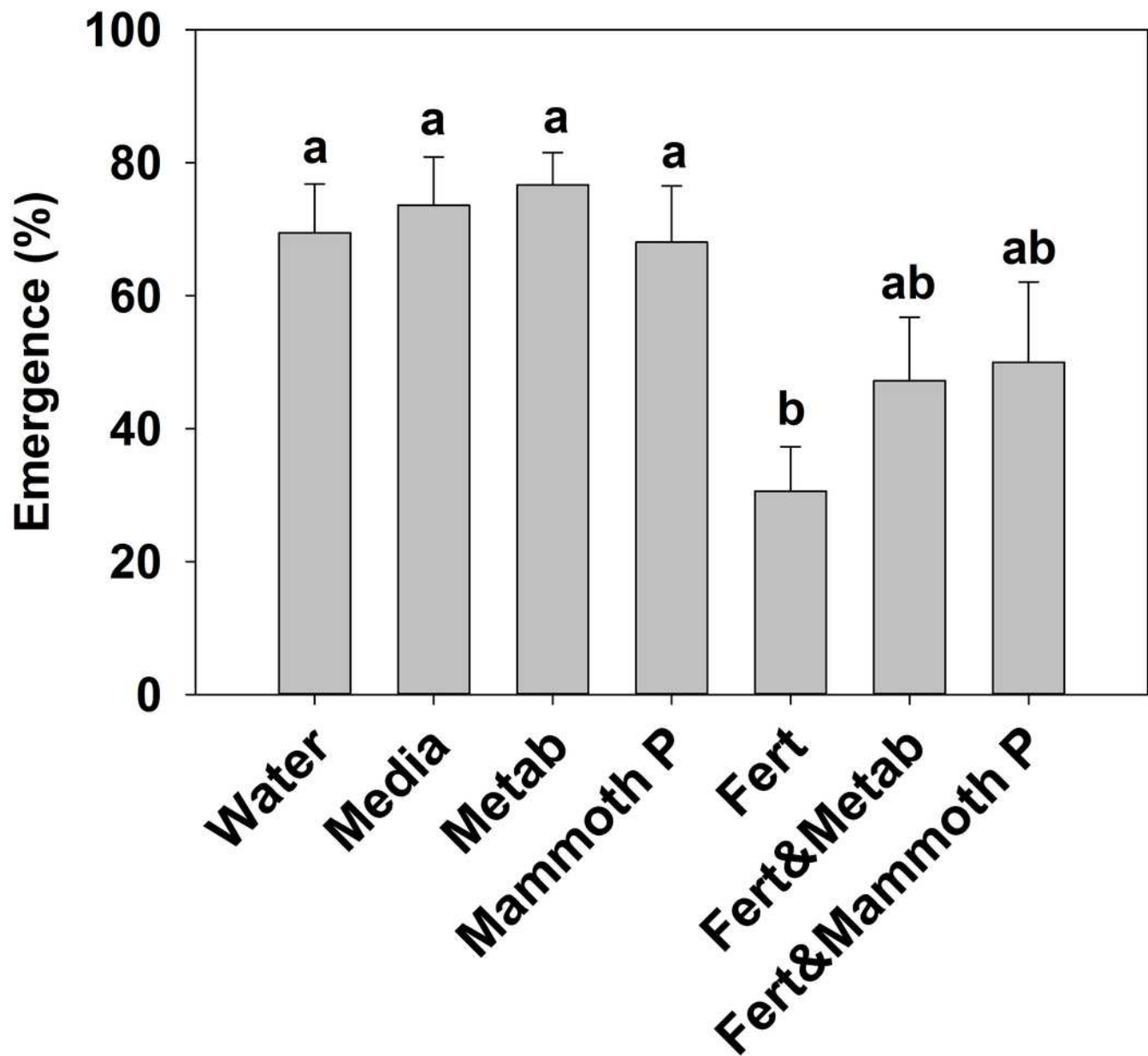
Family	Genus/Species	Abundance (%)
<i>Enterobacteriaceae</i>	<i>Citrobacter freundii</i>	35 ± 4
<i>Enterobacteriaceae</i>	<i>Enterobacter cloacae</i>	17 ± 2
<i>Pseudomonadaceae</i>	<i>Pseudomonas putida</i>	38 ± 6
<i>Comamonaceae</i>	<i>Comamonas testosteroni</i>	6 ± 2
-----Total-----		96 ± 1

1

1

Plant emergence for basil, broccoli, jalapeño, marigold flowers and tomatoes (brandy wine and cherry varieties)

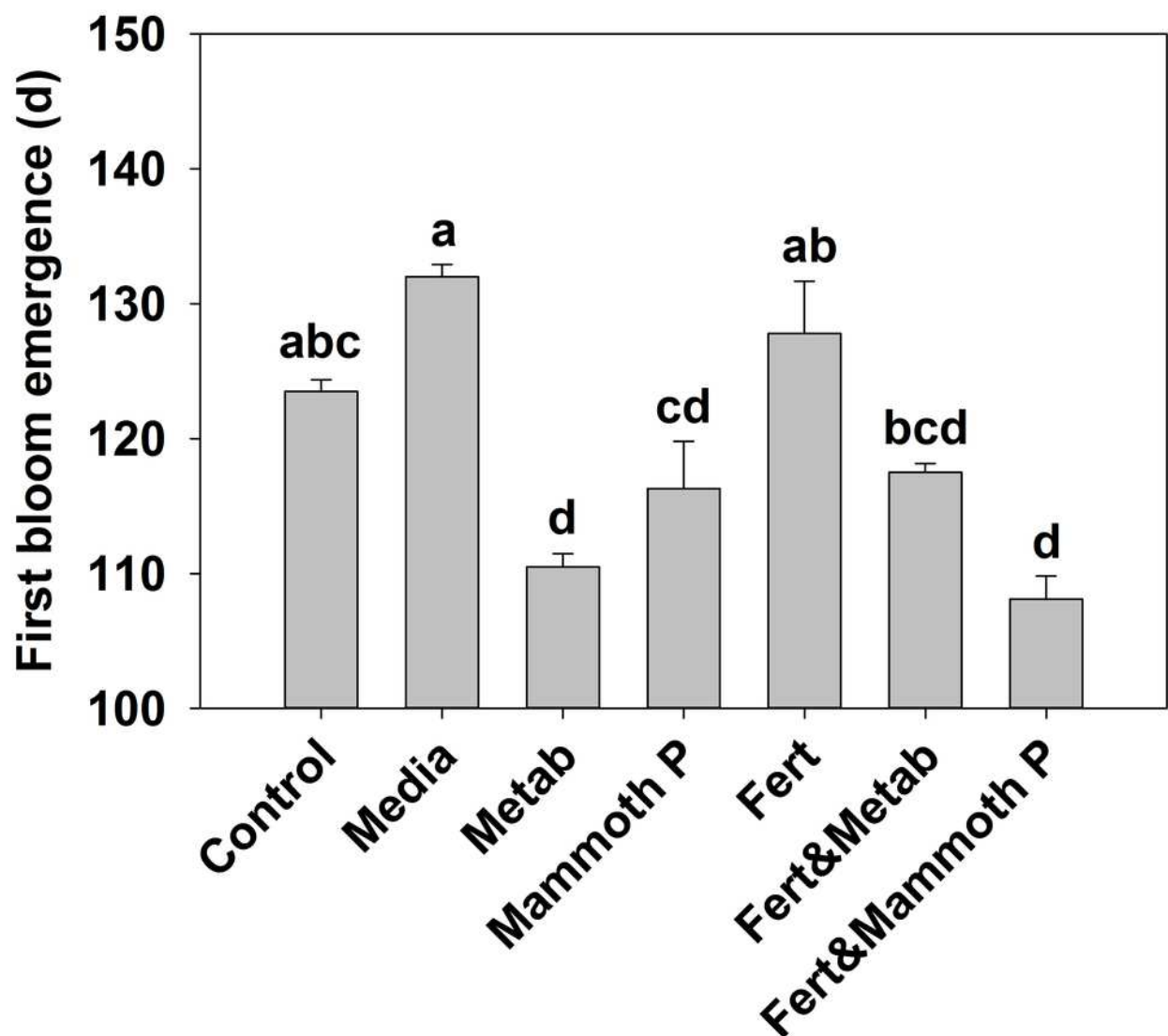
Different letters indicate significant differences. Water = the water control; Metab = culture metabolites; Fert = fertilized with Hoagland's solution and Mammoth P = inoculated with Mammoth P.



2

Time for the development of the first bloom in jalapeño plants.

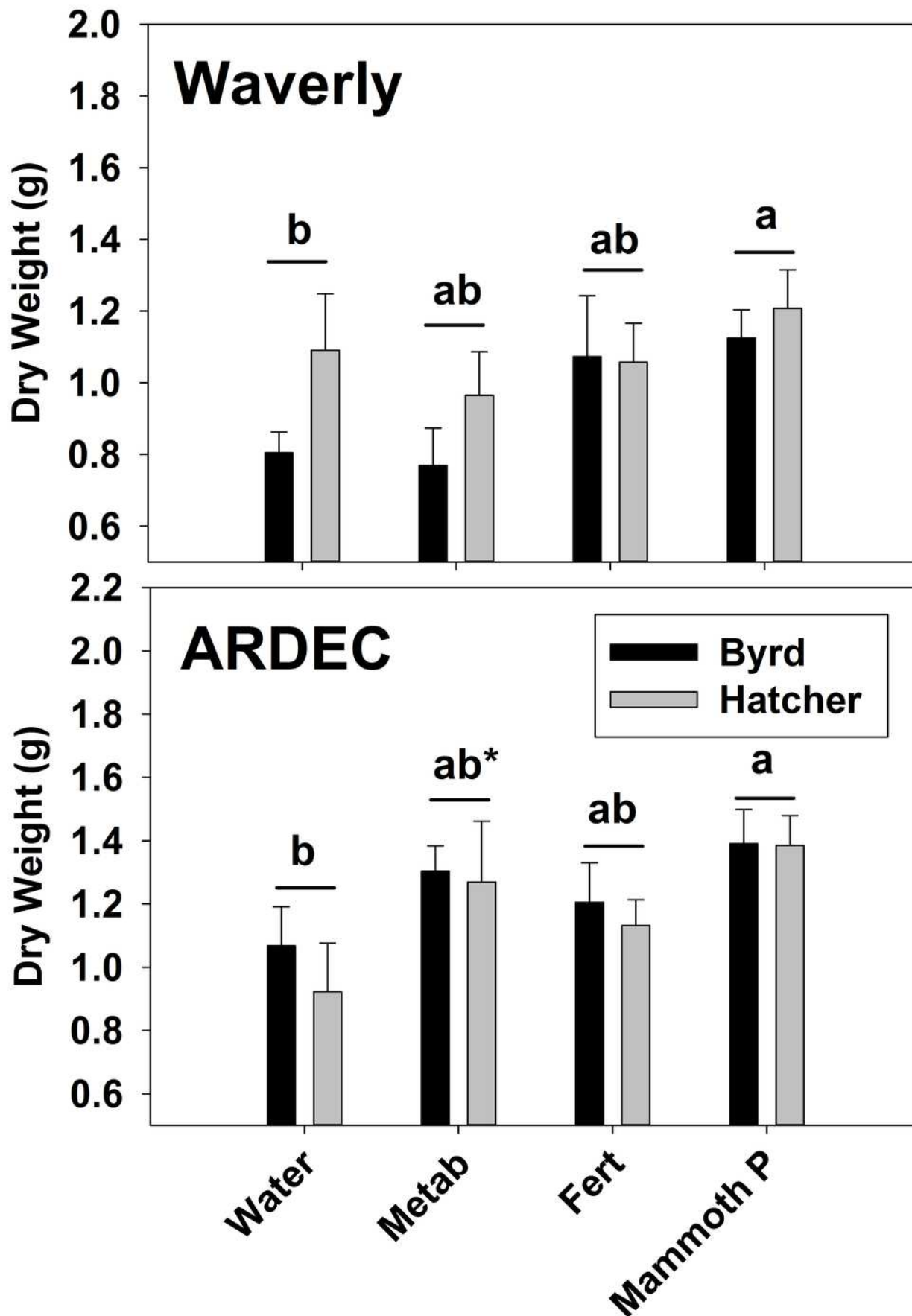
Different letters indicate significant differences. Control = water control; Metab = culture metabolites; Fert = fertilized with Scotts Turf Starter and Mammoth P = inoculated with Mammoth P.



3

Red hard winter wheat aboveground biomass after a two months growth period.

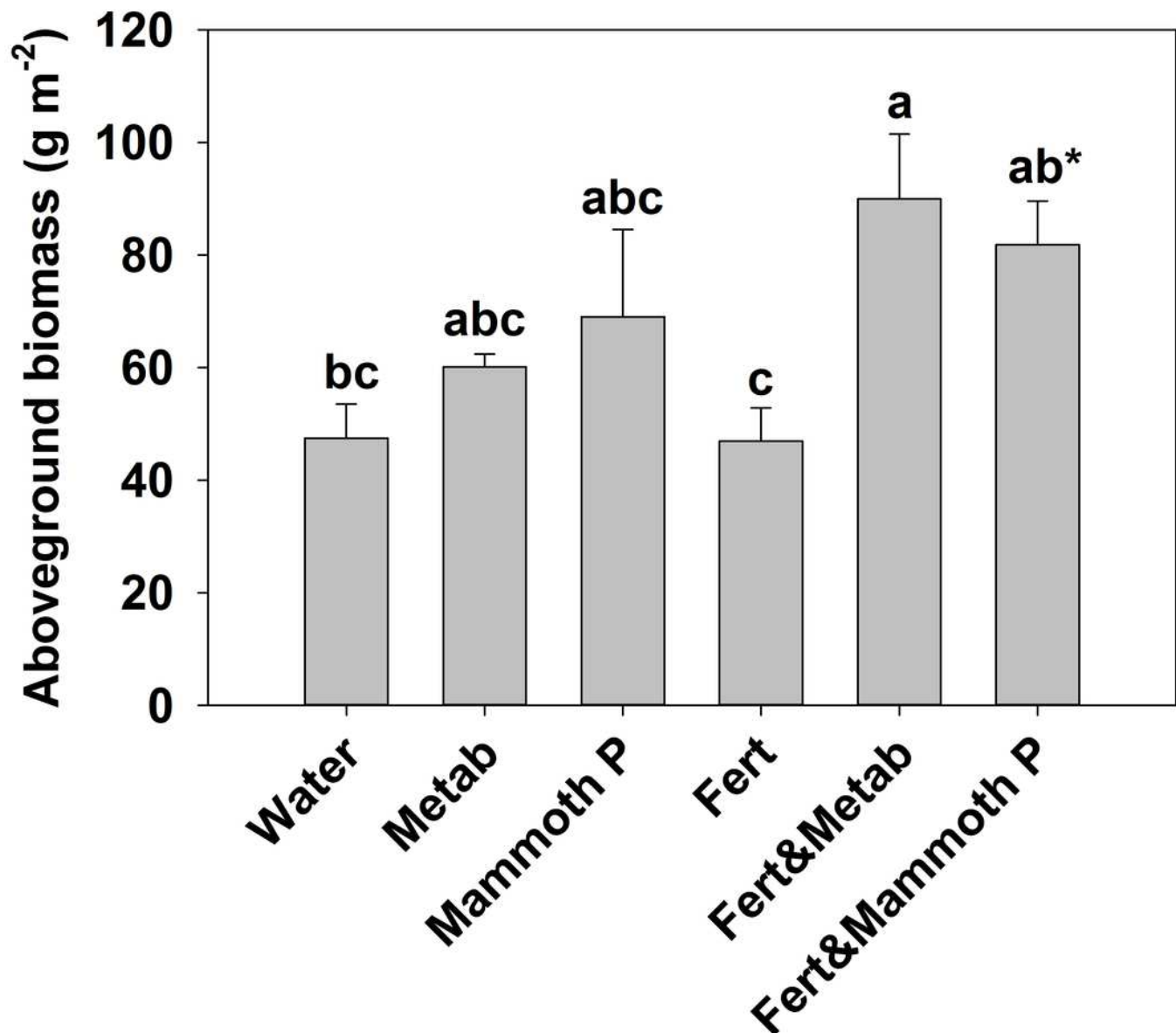
Aboveground biomass data is shown for the Waverly (top) and ARDEC (bottom) soil types. Different letters indicate significant differences. Water = the water control; Metab = culture metabolites; Fert = fertilized with Hoagland's solution and Mammoth P = inoculated with Mammoth P. *different from the water control at $p = 0.06$.



4

Fescue turf grass aboveground biomass two months after seeding.

The bars indicate the mean and the error bars indicate the standard error with different letters indicate significant differences. Water = the water control; Metab = culture metabolites; Fert = fertilized with Hoagland's solution and Mammoth P = inoculated with Mammoth P. *different from the water control at $p = 0.09$.



5

Plant productivity over time for the cumulative basil leaf (a), cumulative jalapeño peppers (b) and total number of cherry tomato fruits (c).

The points indicate the mean and the error bars indicate the standard error. Control = water control; Media = sterile media; Metab = culture metabolites; Fert = fertilized and MP = inoculated with Mammoth P.

