Increased Flower Production of *Calibrachoa x hybrida* by the Soil Fungus *Mortierella elongata*[1]

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Abstract

Calibrachoa (*Calibrachoa x hybrida*) is a highly valued solanaceous flowering ornamental plant, characterized by its drought-hardiness, abundant flowering, and diverse flower colors. Recently, the saprobic soil fungus *Mortierella elongata* was isolated as a root endophyte from eastern cottonwood (*Populus deltoides*) and identified as a potential biological amendment for bioenergy and food crops. Two greenhouse experiments were conducted by transplanting rooted cuttings of Calibrachoa cv. 'Kabloom Deep Blue' into a potting media mixture amended with 1 or 2% volume mix ratio of millet seed colonized with one of four isolates of *M. elongata*. Plants were assessed weekly for flower production and 86 days post inoculation for leaf/stem, root, and total dry weight. *M. elongata* strain 624- significantly increased flower production compared to the non-inoculated millet seed controls at 6 and 7 wk post amendment in both experiments. Above and below ground vegetative dry weight for plants grown in potting media mixture amended with *M. elongata* isolates varied across isolates, fungal amendment concentrations, and experiments. Our results suggest that potting media mixture amended with *M. elongata* strain 624- can increase flower production of Calibrachoa during peak marketable periods.

Index words: Calibrachoa, plant growth promotion, plant reproduction, biological amendment.

Species used in this study: Calibrachoa (*Calibrachoa x hybrida*); *Mortierella elongata* Linnem.

Significance to the Horticulture Industry

Growers prize Calibrachoa for their abundant production of aesthetically pleasing and colorful flowers, an aspect known to be influenced by fungal endophytes in other plants. Our research suggests that the soil fungus *Mortierella elongata* can increase the number of flowers produced by Calibrachoa during peak marketable periods. Leaf/stem and root growth varied in response to amendment of the fungus to potting media. The addition of a biological-based amendment that conveys a beneficial and desirable horticultural characteristic has the potential to become a valuable component for increasing marketability of Calibrachoa while also potentially decreasing synthetic fertilizer costs for growers.

Introduction

The flowering ornamental plant Calibrachoa (*Calibrachoa x hybrida*) is commonly known as million bells. The genus *Calibrachoa* Llave and Lex. is an annual or perennial shrub-like groundcover plant with woody stems, exhibiting numerous small trumpet-shaped flowers. Calibrachoa was commercialized due to its desirable horticultural characteristics, including attractive flowers, range of flower colors, drought tolerance, and plant habit. Calibrachoa belongs to the Solanaceae family, is a close relative of petunia and likely originated in southern South America (Olmstead et al. 2008). As of 2014, annual sales of Calibrachoa in the United States were valued at 45 million US dollars (USDA 2014). Calibrachoa are primarily produced as vegetative cuttings in the US with production located in South America and Africa. Growers prize Calibrachoa for its abundant and prolonged flower production. Fungal endophytes have recently been implicated in shortening time to flowering and promoting proliferation of flowers (Das et al. 2012, Ghanem et al. 2014). Fungal endophytes of plants are defined as asymptomatic fungal infections that reside within host tissue for at least a portion of their life cycle without causing apparent harm and commonly occur in most species of plants (Petrini 1991, Strobel & Daisy 2003, Carroll 1988). Beneficial fungal endophytes can increase plant biomass and aid in nutrient acquisition (Khalmuratova et al. 2015, Usuki & Narisawa 2017, Newsham 2011). Endophytic fungi of roots are emerging as a potential alternative to chemical-based amendments and inputs, due to their promotion of plant growth and reduced environmental impact (Kauppinen et al. 2016).

The fungus *Mortierella elongata* Linnem. is commonly found in soil throughout temperate regions of the world and typically obtains nutrients for growth and development from non-living and decaying organic matter as a saprobe (Wagner et al. 2013, Tedersoo et al. 2014). More recently, *M. elongata* has been isolated as an endophyte from actively growing roots of field mustard (*Brassica campestris* L.), common reed [*Phragmites australis* (Cav.) Trin. ex Seud.] and eastern cottonwood (*Populus deltoides* W. Bartram ex Marshall) (Khalmuratova et al. 2015, Narisawa et al. 1998, Bonito et al. 2016). Liao et al. (2019) observed an increase in total plant dry weight of black cottonwood (*Populus trichocarpa* Torr. & Gray) inoculated with *M. elongata* isolate 93. *Mortierella elongata* is also known to host bacterial endosymbionts, including *Mycoavidus cysteinexigens* Ohshima, that have been hypothesized to reduce fungal radial growth rates (Ohshima et al. 2016, Uehling et al. 2017). Other species within the genus

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Mortierella have been investigated for plant growth promotion properties. For example, *M. alpina* Peyronel is linked to increased dry weight and number of apical buds in *Crocus sativus* L. (Wani et al. 2017). Additionally, *M. hyalina* (Harz) W. Gams was shown to increase above-ground dry weight of *Arabidopsis thaliana* (L.) Heynh. seedlings (Johnson et al. 2019).

The benefit of a root fungal endophyte such as *M. elongata* for increasing flowering and plant weight in ornamental plants remains understudied. In this study, we investigated the interaction between the root endophytic fungus *M. elongata* and Calibrachoa. The primary objective of this study was to determine the effect of *M. elongata* isolates and fungal amendment concentration on above and below ground plant weight, and flower production of Calibrachoa. We hypothesized that *M. elongata* will increase plant weight and flowering when amended to potting media mixtures.

Materials and Methods

Fungal isolates. Two isolates of *M. elongata* (93+ and 624+) sampled from *P. deltoides* roots along the Yadkin River in North Carolina by Rytas Vilgalys (Duke University, Durham NC) were used in this study (Bonito et al. 2014). These isolates were derived from internal root tissue and confirmed to harbor endosymbiotic bacteria (Bonito et al. 2016, Uehling et al. 2017). Two additional strains (93- and 624-) generated by treating *M. elongata* isolates 93+ and 624+ with antibiotics were also used in this study (Uehling et al., 2017). Pure cultures of each isolate and strain of *M. elongata* were grown on malt extract agar (Becton, Dickinson and Company, Sparks, MD) for approximately 2 wk at 24 C (75.2 F) under photoperiod with a 12 h light/dark cycle (48 μE m^{-2} s^{-1}). Isolates and strains of *M. elongata* were maintained in long-term storage in sterilized distilled water at 23 C (73.4 F) and -80 C (-112 F) in a sterile 25% glycerol solution.

Production of M. elongata for greenhouse experiments. Millet seed [300 g (10.6 oz)] was mixed with an equal volume of distilled H_2O [300 ml (10.1 oz)] in a 3.79 L (1 gal) plastic jug, plugged with a foam stopper capped with aluminum foil and autoclaved for 45 min at 121 C (249.8 F) (124 kPa) for three consecutive days. After plastic jugs with millet seed reached room temperature, half of a 9-cm (3.5 in) diameter petri dish of each isolate or strain grown on malt extract agar was cut into small plugs and aseptically added to each jug. Plastic jugs were incubated at room temperature 21 C ± 2 C (69.8 F ± 4 F) under fluorescent light for 4 wk. Millet seed amended with one half of a 9-cm (3.5 in) diameter petri dish of malt extract agar without fungus served as a control.

Greenhouse experiments. Tip cuttings 4-6 cm (1.6-2.4 in) in length and less than one week old of *Calibrachoa* cv. 'Kabloom Deep Blue' were obtained from Ball Seed Co. (Chicago IL). Tip cuttings were dipped in a rooting hormone powder (Root Boost with 0.1% active ingredient indole-3 butyric acid, Tech Pac, LLC. Lexington KY). Cuttings were placed in soilless peat-based potting media (Farfard 2P, Sun Gro Horticulture, Agawam, MA) in 128 cell flats for 3 wk. Plants were misted intermittently to promote root formation in a greenhouse at North Carolina State University, Raleigh. Rooted cuttings of Calibrachoa were transplanted individually into 10-cm (4 in) square pots, with a 2:1:1 ratio of Farfard 2P potting media, pasteurized soil (50 C/122 F for 30 min), and pasteurized sand. Twenty-four hours prior to transplanting, the potting media mixture was amended with millet seed colonized by individual isolates (93+ or 624+), or strains (624- or 93-) of *M. elongata* at a 1% or 2% volume mix ratio (v/v). The millet seed and potting media mix was thoroughly homogenized by agitation. Controls consisted of non-inoculated millet seed added at 1 or 2% v/v and a non-amended control. All plants were fertilized with one teaspoon of Osmocote (14N–6.2P–11.6K) to provide minimal fertilization. There were three replicates of five plants per each treatment arranged in a randomized complete block design. The experiment was conducted twice. Experiment 1 was initiated on March 18 and harvested June 11, 2017. Experiment 2 was initiated on July 9 and harvested October 2, 2017. Minimum and maximum greenhouse temperatures were recorded daily throughout the duration of the experiments.

Plant growth assessment. Since flowering is an important horticultural production trait for Calibrachoa, the number of fully emerged flowers per plant was counted weekly following flower emergence from wk 2 to 11. Twelve weeks after transplanting, leaves, stems and flowers were harvested by clipping each plant stem at the surface of the potting media mixture and placing plant tissue into a paper bag. Roots from each plant were gently rinsed under running tap water to remove excess potting media mixture. Leaves/stems (foliage) and roots were dried separately in paper bags for 8 h at 50 to 60 C (122 to 140 F) in a Thelco drying oven (Precision Scientific Company, Chicago IL). Dried foliage and roots were weighed separately using a Metler Toledo ME4002E scale (Columbus, OH). Plant tissue analysis of dried leaves from individual plants collected from experiments post-harvest were analyzed at the North Carolina Department of Agriculture (NCDA, Raleigh NC). 2 plants were selected from each treatment group for individual analysis.

Statistical analysis. All statistical analyses were performed using SAS (version 9.4, SAS Institute, Cary NC). Mean plant dry weight, flower metrics, and plant tissue analysis data from experiment 1 and 2 were subject to analysis of variance (ANOVA) using the PROC GLM procedure of SAS. A generalized linear model was utilized for continuous and count data for leaves/stems, root weight, and flowering assuming a normal distribution for continuous data and Poisson distribution for count data (Stroup, 2015). When ANOVA was significant for the isolate by amendment volume interaction, simple effects were examined to compare isolates by individual amendment volumes. Post hoc analysis of data was performed with Tukey's honestly significant difference test to examine significant differences among means (alpha= 0.05).

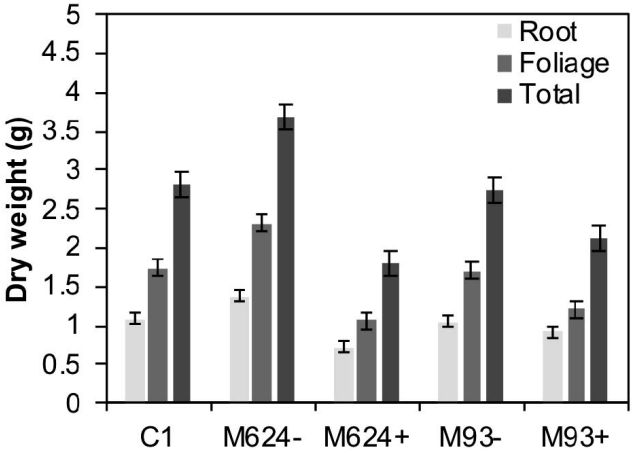

Fig. 1. Root, foliage (leaf/stem), and total dry weight of Calibrachoa cultivar 'Kabloom Deep Blue' grown in potting media mixture amended with a single isolate (624+ or 93+) or strain (624- or 93-) of *M. elongata* at 1% millet seed amendment volume for experiment 1. Plants were harvested at 12 weeks post inoculation. Dried foliage and roots were weighed separately. C1 represents the non-inoculated millet seed control at 1% v/v.

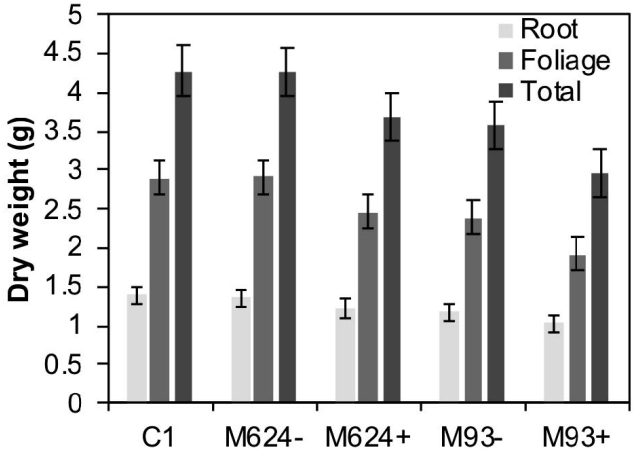

Fig. 2. Root, foliage (leaf/stem), and total dry weight of Calibrachoa cultivar 'Kabloom Deep Blue' grown in potting media mixture amended with a single isolate (624+ or 93+) or strain (624- or 93-) of *M. elongata* at 2% millet seed amendment volume for experiment 1. Plants were harvested at 12 weeks post inoculation. Dried foliage and roots were weighed separately. C1 represents the non-inoculated millet seed control at 2% v/v.

Results and Discussion

Greenhouse experiment 1. Leaf/stem, root, and total dry weight differed significantly by isolate and there was a significant amendment volume by isolate interaction for each plant growth parameter assessed ($P<0.002$). Therefore, treatments were analyzed for each amendment volume separately to examine simple effects. This resulted in comparison of fungal treatments to non-inoculated controls of sterile millet seed of the same amendment volume. Rooted cuttings grown in the potting media mixture amended with *M. elongata* strain 624- exhibited higher leaf/stem ($P<0.0001$), root ($P=0.0005$), and total ($P<0.0001$) dry weight compared to the non-inoculated millet seed control at 1% v/v (Fig. 1). In contrast, cuttings grown in potting media mixture amended with *M. elongata* isolate 624+ exhibited significantly lower dry weight ($P< 0.0001$) for all three dependent variables (leaf/stem, root and total dry weight) at 1% v/v. Rooted cuttings amended with *M. elongata* isolate 93+ at a 2% inoculum volume exhibited significantly lower leaf/stem, root, and total dry weight compared to the non-amended 2% v/v controls ($P< 0.0001$, $= 0.0096$, and $=0.0001$, respectively) (Fig. 2). Significant differences among isolates were observed for flower production for wk 5, 6 and 7. The isolate by amendment volume interaction was significant ($P<0.018$) for flower production in wk 5, 6 and 7, hence experiments were analyzed separately for each amendment volume to determine simple effects. Rooted cuttings grown in potting media mixture amended with *M. elongata* strain 624- at 1% v/v produced significantly more flowers than the 1% v/v non-inoculated millet seed control at 5 ($P<0.0001$), 6 ($P=0.0007$) and 7 ($P< 0.0001$) wk (Fig. 3). Rooted cuttings planted in potting media mixture amended with *M. elongata* isolate 624+ and 93+ at 1% v/v produced significantly less flowers than the 1% v/v non-inoculated millet seed control at weeks 6 and 7 ($P= <0.04$) (Fig. 3). Calibrachoa cuttings produced in potting media mixture amended with *M. elongata* strain 624- at 2% v/v produced significantly more flowers than the 2% v/v non-inoculated millet seed control at weeks 5 and 6 post inoculation ($P= 0.0001$) (Fig. 3).

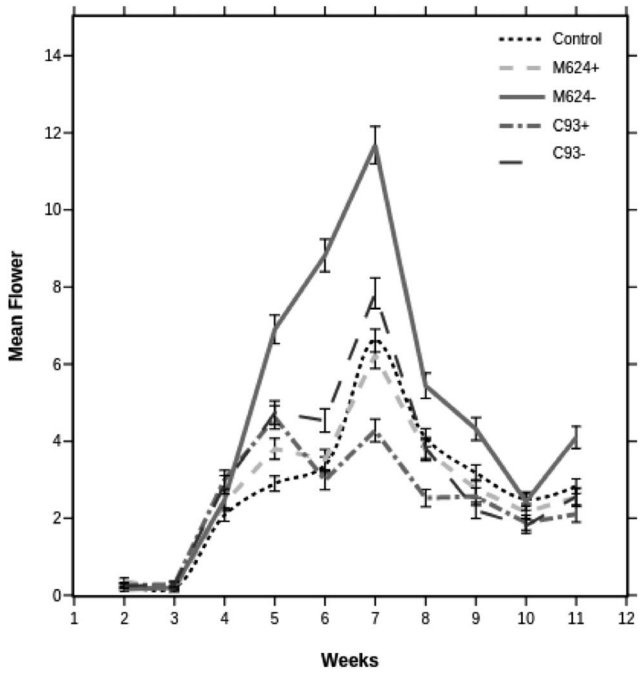

Fig. 3. Means of flower production of Calibrachoa cultivar 'Kabloom Deep Blue' amended with individual isolate (624+ or 93+) or strain (624- or 93-) of *M. elongata* on millet seed and non-inoculated millet seed controls. Data presented for each treatment is an average of 1% and 2% amendment volumes. Flower production assessed weekly at 2 wk to 11 wk post inoculation in experiment 1.

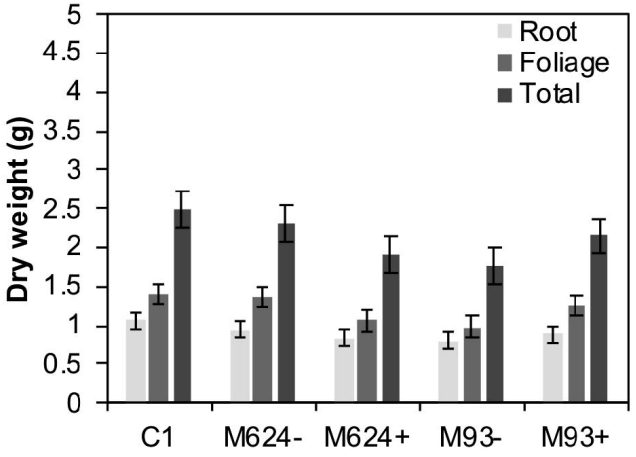

Fig. 4. Root, foliage (leaf/stem), and total dry weight of Calibrachoa cultivar 'Kabloom Deep Blue' grown in potting media mixture amended with a single isolate (624+ or 93+) or strain (624- or 93-) or *M. elongata* at 1% millet seed amendment volume for experiment 2. Plants were harvested at 12 weeks post inoculation. Dried foliage and roots were weighed separately. C1 represents the non-fungus inoculated millet seed control at 1% v/v.

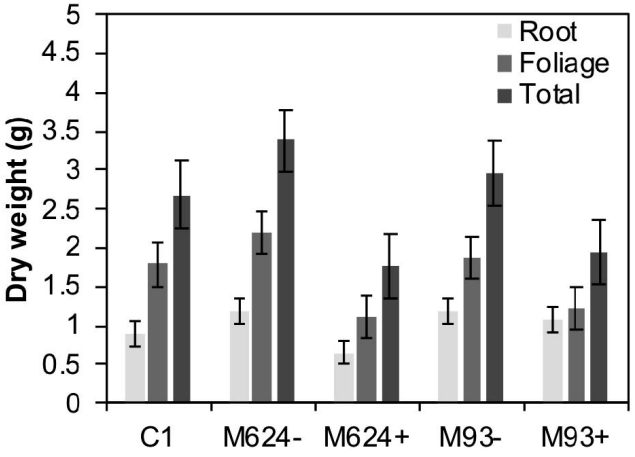

Fig. 5. Root, foliage (leaf/stem), and total dry weight of Calibrachoa cultivar 'Kabloom Deep Blue' grown in potting media mixture amended with single isolate (624+ or 93+) or strain (624- or 93-) of *M. elongata* at a 2% millet seed amendment volume for experiment 2. Plants were harvested at 12 weeks post inoculation. Dried foliage and roots were weighed separately. C1 represents the non-inoculated millet seed control at 2% v/v.

Greenhouse experiment 2. Leaf/stem, root, and total dry weight differed significantly among isolates. There was a significant isolate by amendment volume interaction for each metric ($P < 0.05$). Therefore, isolates were analyzed by amendment volume to examine simple effects. Rooted cuttings amended with *M. elongata* strains 93+, 624+, and 624- exhibited slightly lower leaf/stem, root, and total dry weight compared to the non-inoculated millet seed control at 1% v/v. Cuttings grown in potting media mixture amended with *M. elongata* strain 93- at 1% v/v had significantly lower leaf/stem ($P=0.0103$) and total ($P=0.0143$) dry weight compared to the non-inoculated millet seed control at 1% v/v (Fig. 4). At the 2% inoculum volume, rooted cuttings grown in potting media mixture amended with *M. elongata* isolates did not exhibit significant differences in leaf/stem, root and total dry weight compared to the non-inoculated millet seed control at 2% v/v. Non-significant trends indicated that 93- and 624- exhibited slightly greater foliage, root, and total dry weight compared to the non-inoculated millet seed control, while 93+ and 624+ exhibited slightly lower foliage, root, and total dry weight compared to the non-inoculated millet seed control (Fig. 5). Flower production for all fungal inoculum amendment volumes and isolates varied across weeks 2 through 11 of the experiment (Fig. 6). A significant ($P < 0.025$) isolate by amendment volume interaction for flower production was observed during weeks 4, 6 and 7 and isolates were examined separately for each amendment volume to determine simple effects. Rooted cuttings grown in potting media mixture amended with *M. elongata* strain 624- at 2% v/v produced significantly more flowers than the non-inoculated millet seed control at weeks 6 and 7 ($P < 0.0001$) (Fig. 6).

The application and identification of novel fungal root endophytes with beneficial and desirable plant growth responses represent a growing area of interest for plant scientists (Le Cocq et al. 2016, Lugtenberg et al, 2016). In this study, Calibrachoa plants grown in potting media mixture amended with *M. elongata* strain 624- had a greater total number of flowers during weeks 6 and 7 in both experiments compared to non-inoculated controls. We assessed flower production on a temporal scale rather than a single time point to more accurately reflect the Calibrachoa production system and timeframe of flowering

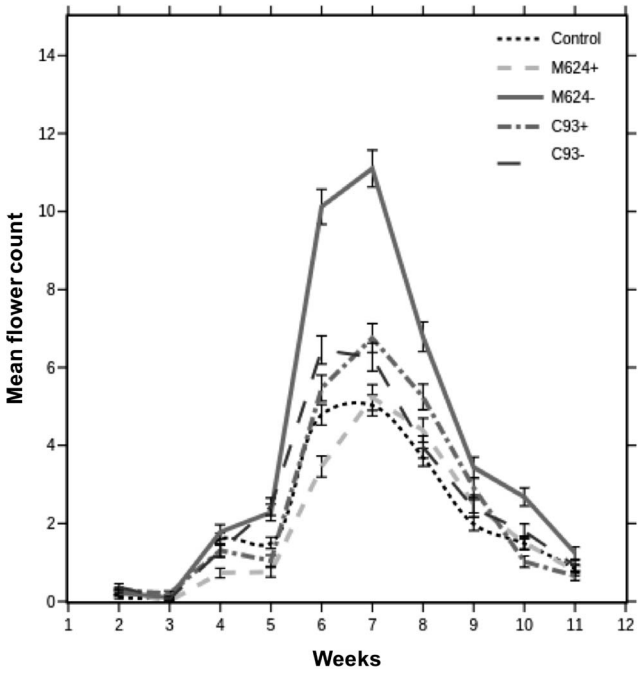

Fig. 6. Means of flower production of Calibrachoa cv. 'Kabloom Deep Blue' amended with individual isolate (624+ or 93+) or strain (624- or 93-) of *M. elongata* on millet seed and non-inoculated millet seed controls. Data presented for each treatment is an average of 1% and 2% amendment volumes. Flower production assessed weekly at 2 wk to 11 wk post inoculation in experiment 2.

that corresponds to grower demands for marketable plants. An association between amendment of the fungal endophyte *Piriformospora indica* Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken and increased flower or reproductive structure production has been previously reported in Indian coleus (Das et al. 2012) and cyclamen (Ghanem et al. 2014).

The association of a diverse suite of endosymbiotic bacteria within endophytic fungi has been previously documented (Hoffman & Arnold, 2010). Variation of plant growth response to individual isolates of *M. elongata* depended largely on presence or absence of endosymbiotic bacteria. Our research suggests that the presence of endosymbiotic bacteria within *M. elongata* did not enhance plant growth promotion properties of the fungus. Radial hyphal growth of *M. elongata* isolate AG77 significantly decreases ($P<0.05$) when associated with the bacterial endosymbionts compared to an antibiotic cured strain of *M. elongata* AG77, suggesting a metabolic cost of harboring the endosymbiotic bacteria (Uehling et al., 2017). It is not known specifically how the endosymbiotic bacterial community is composed within *M. elongata* isolates 624+ and 93+, or if the community composition changes over time and/or responds to environmental factors. A direct link between the presence and function of endosymbiotic bacteria sourced from endophytic fungi and plant growth has yet to be firmly established.

Promotion of plant weight by root-associated fungal endophytes is well documented (Rodriguez et al., 2009, Mandyam et al., 2013, Schulz et al., 2002, Ansari et al., 2013, Newsham, 2011). In our study, addition of *M. elongata* strain 624- increased plant dry weight in experiment 1, but a similar increase in plant dry weight was not observed in experiment 2 for the 1% fungal amendment volume. In general, Calibrachoa in experiment 1 exhibited higher leaf/stem, root, and total plant dry weight compared to plants in experiment 2. In both experiments, significant differences were observed among cuttings planted in potting media mixture amended with 1% and 2% v/v inoculated or non-inoculated millet seed for leaf/stem, root, and total dry weight. Rooted cuttings amended with 2% v/v inoculated or non-inoculated millet seed exhibited significantly higher dry weight for all three metrics compared to rooted cuttings amended with 1% v/v inoculated or non-inoculated millet seed. Cuttings grown in potting media mixture amended with 1% v/v inoculated or non-inoculated millet seed exhibited significantly higher dry weight for all three metrics compared to non-amended controls. Among the controls, inoculum volume 1% and 2% exhibited significantly higher leaf/stem, root, and total dry weight when compared to control volume 0% ($P<0.0001$ for foliage, root, and total weight).

Plant tissue analysis revealed minor differences among treatments for various nutrients. Significant differences were observed with the element boron, in which adding isolate 93- resulted in significantly greater concentrations compared to the non-amended/non-inoculated controls ($P< 0.05$). The observed variation in plant growth metrics among *M. elongata* isolates and experiments in this study suggests that environmental factors and amendment volume may affect root and leaf/stem dry weight. Greenhouse air temperatures differed between the two experiments, with mean minimum and maximum air temperatures for experiment 1 of 20.7 and 34.2 C (69.3 and 93.6 F) and experiment 2 of 22.8 and 36.5 C (73.0 and 97.7 F), respectively. Gams et al. (1972) demonstrated that growth and survival of *M. elongata* isolates in pure culture are inhibited by temperatures at or above 35 C (95 F). In floriculture plants, higher mean daily air temperatures are inversely related to plant weight (Vaid & Runkle, 2014). The temperatures observed during the second experiment may have decreased fungal growth, although we did not examine or determine optimal fungal growth temperatures *in planta*. Further research is needed to establish optimal environmental conditions for root colonization by *M. elongata*.

The floriculture industry relies on consistent production of aesthetically valuable flowering plants. Biological-based amendments are growing in popularity in greenhouse production systems and therefore increasing in market value (Bailey et al. 2010). Fungal endophytes represent novel biological-based sources for increasing plant growth and health with broad applications across agriculture and horticulture. Our studies suggest that *M. elongata* strain 624- has the potential to increase flowering during marketable periods of Calibrachoa and become a valuable component of biological-based amendments. Future experiments could expand the evaluation of flower promotion effects of *M. elongata* on other Calibrachoa cultivars and additional plant hosts. Experiments to examine the sensitivity of *M. elongata* to commonly applied chemical products under floriculture production conditions are also necessary for effective deployment. Our results represent the first report of the fungal endophyte *M. elongata* increasing flower production in an ornamental plant species.

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