Probiotic Bacteria, Anaerobic Soil Disinfestation, and Mustard Cover Crop Biofumigation Suppress Soilborne Disease and Increase Yield of Strawberry in a Perennial Organic Production System

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Abstract

Black root rot complex and crown rot of strawberry caused by soilborne fungi limit sustainable strawberry production in the northeastern United States, especially in perennial systems, including matted row and plasticulture. As pathogen populations build up over time in the rhizosphere and infect the root system, feeder roots are pruned, which diminishes nutrient and water uptake and causes stunted plant growth or death. Alternative management options are needed for many organic and small growers who can't use chemical fumigants due to new regulations and potential health hazards. Strawberry plug plants were grown on beneficial microbe-inoculated or uninoculated planting mix followed by transplanting in fruiting field plots that either was biofumigated with mustard cover crop (MCC), anaerobically disinfested (ASD), or left untreated. Different combinations of plug plants and field plot treatments were used to determine the efficacy of individual treatments or synergistic effects from combination treatment. Plug plants were transplanted in pretreated plastic mulched raised beds and grown following a typical organically recommended production system. Plants grown on TerraGrow (TG)-inoculated planting mix showed enhanced plant vigor in the fruiting field compared with untreated plants. Weeds that grew through planting holes were significantly ($P \leq 0.045$) suppressed in ASD plots compared with untreated plots in the first year. Plants treated with a combination treatment of TG and ASD had significantly higher fruit yield in both years (2019 and 2020), although the difference was greater in the second year. Plant vigor and survival in treated plots except MCC were also significantly higher in the second year compared with the untreated control. Suppression of pathogenic microbes and plant vigor improvement in treated plots appear to be the factors providing beneficial effects and higher net economic return. Taken together, our results suggest that a combination of beneficial microbes and ASD could be an alternative to synthetic fumigation in a perennial strawberry production system.

Keywords: anaerobic soil disinfestation, biological control, cultural and biological practices, fumigation alternative, fungi, small fruits, soilborne disease, strawberry

The strawberry black root rot (BRR) is an increasing problem in perennial strawberry plantings worldwide. To avoid BRR together with a few other disease problems, strawberry growers in many areas were compelled to adopt an expensive annual hill plasticulture system. This problem has specifically been identified as a limiting factor for sustainable strawberry production in the northeastern United States (Hazelrigg 2013; Hazelrigg and Kingsley-Richards 2015; Howard and Albregts 1984; Pritts and Wilcox 1990). Yield loss from BRR alone can range from 20 to 50% (Hazelrigg and Kingsley-Richards 2015; Louws 2014), which can dramatically increase if crown rot occurs simultaneously. Because several factors are involved with BRR of strawberry, including a range of infectious agents (nematodes, root infecting fungi) and various abiotic factors such as poor soil characteristics (Wing et al. 1994), disease control is complicated, and no general control measure is completely effective. In comparison, crown rot disease of strawberry caused primarily by the fungal species *Colletotrichum gloeosporioides* and *Phytophthora cactorum* (Cannon et al. 2012) can sometimes also incur significant yield loss in strawberry production in the United States and other strawberry-growing countries (Cannon et al. 2012; Legard et al. 2003). Although inoculum sources for crown rot in fruiting fields may be diverse, infected planting stock is the most important source of *C. gloeosporioides* (Debode et al. 2015; Eastburn and Gubler 1990; Freeman et al. 2001; Leandro et al. 2001; McInnes et al. 1992), whereas *P. cactorum* is mostly soilborne and builds up in a strawberry field over time. Colletotrichum crown rot mostly affects strawberries in the Southeast, and Phytophthora crown rot is more damaging in the Northeast together with BRR. Occurrence of crown rot caused by *Fusarium oxysporum* f. sp. *fragariae* is also on the rise in the Northeast (West Virginia University plant diagnostic clinic report). Winter injury to roots reportedly encourages infection by *Fusarium* spp. (Fang et al. 2011; Pscheidt and Ocamb 2018). Control of both BRR and crown rot diseases is very challenging as very little is known about the pest complex, and fungicide application is only partially effective. In many cases where crop rotation was not an option, fumigation of soil was necessary (Mass 1998). Methyl bromide was previously used as a preplant broad-spectrum soil fumigant to control soilborne diseases, nematodes, insects, and weeds in high-value crops such as tomatoes, strawberries, cucurbits, nursery crops, and flowers (Pleog 2008). However, with the phasing out of this highly effective soil fumigant and restrictions on the use of other synthetic fumigants, interest in the development of safe, sustainable, and economically viable fumigation strategies to manage soilborne fungi and nematodes has increased (Pleog 2008; Rodríguez-Kábana 1997). More importantly, the demand for alternative strategies from organic growers and small growers who can't use synthetic fumigants has increased tremendously (Martin 2003). Alternative strategies are also required especially for strawberries as disease-resistant cultivars are not available (Klosterman et al. 2009). As an alternative to soil fumigation with synthetic chemicals,

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glucosinolate-containing *Brassica* spp. is known to release volatile isothiocyanates (ITCs), which are toxic to different pathogens (Kirkegaard et al. 1993; Matthiessen and Kirkegaard 2006). Several lines of evidence suggest that biofumigation with plants producing ITCs has shown promising results against soilborne fungal pathogens (e.g., *Rhizoctonia*, *Verticillium*, *Fusarium*, *Pythium*, and *Phytophthora* spp. [Friberg et al. 2009; Hansen and Keinath 2013; Mattner et al. 2008; Steffek et al. 2006]). However, the concentration of ITCs produced is influenced by mustard variety (Balzano 2017), soil texture, moisture, temperature, soil microbial community, and pH (Bending and Lincoln 1999; Morra and Kirkegaard 2002; Price 1999), resulting in variable soilborne disease control efficacy. Matthiessen et al. (2004) were able to increase soil ITC levels by 20-fold (100 nmol/g soil) using a tractor-drawn tissue pulverizing implement compared with the use of a cutting and chopping implement. In addition, they showed that adding water to soil up to the field capacity was necessary for maximum ITC release from the pulverized tissues.

Another promising nonchemical soilborne disease control alternative is anaerobic soil disinfestation (ASD), which was adapted from the previously described methods of biological soil disinfestation (BSD) and soil reductive sterilization (Goud et al. 2004; Messiha et al. 2007; Momma 2008) to create a treatment suitable for strawberry (Shennan et al. 2014, 2017). A wide range of soilborne plant pathogens and plant parasitic nematodes can be controlled in a variety of crops using ASD (Rosskopf et al. 2015; Shennan et al. 2014). The exact mechanisms that lead to disease suppression with ASD are not clearly understood but may involve production of organic acids and other biologically active volatiles (Hewavitharana et al. 2014) and amplification of specific microbes with biocontrol activity (Momma et al. 2013). Beneficial microorganisms that are used as bio-fertilizers or bio-stimulants possess the ability to colonize the rhizosphere, plant roots, or both when applied to seeds or crops. Some of these microbes have shown potential to promote strawberry plant growth by the release of metabolites into the rhizosphere that may inhibit various pathogens (Esitken et al. 2010; Lingua et al. 2013; Lovaisa et al. 2016; Pešaković et al. 2013). These microbes were reported to improve plant nutrition and support plant development under natural or stressed conditions as well as increase the yield and quality of many important crops including strawberry and thus may play a crucial role in sustainable crop production in the future (de Oliveira-Longati et al. 2015; Kundan et al. 2015; Rahman et al. 2018; Vejan et al. 2016). Some studies indicated that early colonization of the root system might provide better support to plants' growth and development including protection from diseases. Barka et al. (2000) showed that in vitro beneficially bacterized plantlets of grapevine grew faster and sturdier with a better-developed root system and significantly greater capacity for withstanding gray mold fungus than nonbacterized controls. Seed treatment or augmenting beneficial microbial population in soil was also found to reduce seedling mortality from soilborne diseases (Rahman and Punja 2007). In a relevant study, Mukta et al. (2017) found that chitosan and plant probiotic bacteria enhanced growth and yield of strawberry.

Lesion nematodes were reported to predispose roots to infection by BRR-causing fungi (LaMondia 2003). The roots develop black cortical lesions that may girdle the whole root resulting in loss of root hair/function and mass giving the root a rat tail appearance. Thus, treatments that control lesion nematodes will be highly welcomed by strawberry growers. Although different control measures such as crop rotation and cultural measures are utilized to reduce the damage caused by the BRR and crown rot pathogens in organic strawberry production (Guerena and Born 2007), very few attempts have been made to test the efficacy of biological control through inoculation of pasteurized growing media to facilitate early root colonization by beneficial microbes and improving plant vigor. Cox et al. (2010) found that approximately 80% of strawberry transplants proved to be colonized by *Pythium*, *Rhizoctonia*, and/or *Colletotrichum* before they were even planted into the test plots, thus indicating a need for treatments of plants with beneficial microbes early in the transplant production cycle. The objectives of this study were to determine if (i) precolonization of strawberry plug plant roots by beneficial microbes reduces plant mortality and improves strawberry yield; (ii) probiotic microbes and ASD improve the availability of soil nutrients to strawberry plants and provide a synergistic effect for enhancing plant vigor and productivity; and (iii) positive net returns can be obtained from treatments by conducting an economic analysis.

Materials and Methods

Experiment site and soil type

We conducted field experiments and collected data for two consecutive years at the certified West Virginia University (WVU) Organic Research Farm. The initial treatment of experimental units and the planting of strawberry plug plants took place in the fall of 2018. First- and second-year harvests of berries were done in 2019 and 2020, respectively. The WVU Organic Research Farm is located at latitude 39.644395 and longitude −79.938739. The soil type of the farm is listed by the Web Soil Survey of the United States Department of Agriculture (USDA) as a Tilsit silt loam (semiactive, fine-silty, mixed mesic typic fragiudults). This farm was converted to an organic production system in 1999 and has been certified since 2004. Since then, no synthetic chemical inputs were used. Due to the increase in demand for organic produce in the state and the whole country, finding organic options for pest management became a pertinent research goal at the WVU Organic Research Farm.

Production of treated plug plants

This study was conducted in two phases. The first phase included strawberry plug plant production from nursery-supplied tips/cuttings at the plug plant production facility of the grower cooperator, Mr. Reuben Martin, located at 8564 Olde Scotland Rd, Shippensburg, PA 17257. The plug production system was slightly modified to meet certified organic production standards and our preplanned treated plant production requirements. Johnny's "512 Organic Mix" (Johnny's Selected Seeds, Winslow, ME) was used for plug plants produced for this experiment. One hundred strawberry plug plants of cultivar Chandler were grown on pasteurized medium that was inoculated with TerraGrow (TG), which is a commercially available microbial inoculant containing five different species of the beneficial bacterial genus *Bacillus*. Two hundred plug plants were grown on nonpasteurized planting mix but treated with TG. Plastic plug trays (50 cells) were used to grow plug plants. The required amount of planting mix (0.016 cubic meters) was moistened and placed in four heat-resistant plastic bags and pasteurized twice at a 12-h interval for 30 min in an oven with a set temperature of 82°C. The planting mix was then allowed to cool down for 24 h after which time mix was spread on greenhouse stainless steel benches in thin layers. Five grams TG was suspended in 5 liters water and sprayed on the mix to evenly distribute the beneficial bacteria on the medium. The medium was then mixed and put in one pile before dispensing evenly in plug trays with 10% extra kept aside. Strawberry cuttings were immersed in TG suspension containing 1 g/liter for 1 min just prior to sticking in plug cells. Each gram of TG contains 1.20×10^9 CFU of *Bacillus licheniformis* and 6.00×10^8 CFU of each of *B. subtilis*, *pumilus*, *amyloliquefaciens*, and *megaterium*. Plug cells were filled with an extra mix as needed and placed under mist following the protocol described by Rowley et al. (2010). Another 250 plug plants without bacterial inoculation were grown on nonpasteurized mix destined for the untreated, biofumigated, and ASD plots in the fruiting field. Thus, combining the plug production and fruiting field, we had six treatments to determine individual treatment effects or synergistic effects of plug and fruiting field treatment with unique treatment acronyms as follows: (i) plug plants grown on untreated/nonpasteurized planting mix and planted in untreated fruiting field plots (Untreated control = non-pasteurized media [NPM] + no treatment [NT] + no field treatment [NF]); (ii) plug plants grown on nonpasteurized untreated planting mix and planted in "Caliente 199" mustard cover crop (MCC) plots (Effect of biofumigation = NPM + NT + MCC); (iii) plug plants grown on nonpasteurized planting mix

treated with TG and planted in untreated fruiting field plots (Effect of TG with normal colonization potential = NPM + TG + NF); (iv) plug plants grown on pasteurized planting mix treated with TG and planted in untreated fruiting field plots (Effect of TG with higher colonization potential = PM + TG + NF); (v) plug plants grown on untreated planting mix and planted in ASD fruiting field plots (Effect of ASD = NPM + NT + ASD); (vi) plug plants grown on nonpasteurized/nonpasteurized planting mix treated with TG and planted in ASD plots (Synergistic effect of TG with normal colonization potential and ASD = NPM + TG + ASD). These acronyms with explicit treatment names can be found in Table 1.

Field trial set up

The second phase of the research was conducted in the fruiting field at WVU Certified Organic Farm in three side-by-side raised plastic mulched beds with a buried drip irrigation line in the middle of each bed. All treatments were replicated three times in a randomized complete block design. Each replicate plot was 6.1 m long, 68.5 cm wide, and 15 cm high, with a 1.5-m center-to-center bed spacing. The popular strawberry cultivar Chandler (BRR susceptible) plug plants that were specifically grown for this study were planted in these plots. Each treatment was randomized on each bed and planted in two staggered rows 60 cm apart within rows and 90 cm apart between rows. Therefore, each treatment unit comprised 20 plants. Plots were maintained at the experiment site with 1.5 m of a nonplanted area between plots and within adjacent beds to minimize interplot interference. Plots that received mustard biofumigation and ASD treatments were prepared accordingly to match the projected planting date of 7 September 2018. Soil incorporation of mustard biomass and mustard meal followed by irrigating and covering with plastic mulch were done 21 days before planting to allow sufficient time to get the ASD process completed and eliminate the probability of phytotoxicity. Plastic mulch was cut at the sites of planting holes 24 h prior to planting to ensure the dissipation of any toxic gas generated in MCC and ASD plots. Immediately after planting with the spacing shown in the field trial setup section, plants were irrigated manually with a watering can as well as through the drip irrigation line to prevent desiccation and aid in establishment. Blood meal (Sta green, 14-0-0 NPK, and C:N = 4:1) was applied manually in the fall of 2018 and the spring of 2019 and 2020 at the rate of 2 g/plant. No other chemical fertilization or pesticides were applied to keep plant management consistent with the regulation of USDA certified organic system.

Table 1. Effect of probiotic bacteria, anaerobic soil disinfestation, and mustard cover crop biofumigation and their combination on soilborne disease (black root rot complex) index[y]

Treatment[z]	Application timing/ sequence	Soilborne disease index	Rank
Untreated check (NPM + NT + NF)	—	5	5
Mustard cover crop (NPM + NT + MCC)	Preplant field	3.5	4
Nonpasteurized media inoculation (NPM + TG + NF)	Plug production stage	2.5	3
Pasteurized media inoculation (PM + TG + NF)	Plug production stage	2.5	3
Anaerobic soil disinfestation (NPM + NT + ASD)	Preplant field	1.5	2
Combination of nonpasteurized media inoculation and ASD (NPM + TG + ASD)	Plug production stage, preplant field	0.5	1

[y] The disease severity index was assigned to each plant and averaged from all plants assessed.

[z] NPM = non-pasteurized media; NT = no treatment; NF = no field treatment; PM = pasteurized media; TG = TerraGrow; MCC = mustard cover crop; ASD = anaerobic soil disinfestation.

Weeding was done manually as needed for weeds that grew through planting holes and row middles. Irrigation was provided through the drip tape as needed.

MCC plot establishment and soil incorporation

Field plots were disked twice, and dairy manure compost was applied to all plots at the rate of 22,403 kg/ha in the early spring followed by tilling with a Husqvarna walk behind the rototiller. Plots were marked based on the random distribution of all the treatments in each of the three blocks using a randomized complete block design. "Caliente-199" mustard seeds were seeded in plots that were marked to receive cover crop biofumigation treatments on 1 May 2018. The seeding rate was 11 kg/ha, which is normally used for a cover crop to add biomass in the soil to improve soil health. After plants reached ≥75% flowering, they were mowed on 2 July 2018 with a flail mower to macerate the tissues so that glucosinolate and myrosinase could react and produce ITCs. Flail-mowed mustard residues were then immediately incorporated at a 15-cm depth into the soil with a walk-behind rototiller followed by setting a drip irrigation line in the middle of each bed. Plots were immediately covered with 1.5 mil black plastic mulch (Film Tech, 799 N Broadway St, Stanley, WI 54768; oxygen permeability-44.8 × 10^7 ml m m^{-2} day^{-1} Pa^{-1}) and irrigated through the drip line to bring the soil moisture to field capacity.

Anaerobic soil disinfestation (ASD)

ASD on selected plots was done according to Shennan et al. (2017) in three steps with minor modifications. Briefly, 50:50 mix of *Brassica juncea*/*Sinapis alba* seed meal (C/N ratio of 7.8; %; N-p-K = 8-3-2; S-1.71%, Ca-0.63%, Mg-0.52%) was used at the rate of 4,500 kg/ha as the labile carbon source for ASD. Triumph Italia brand *B. juncea* was procured as Biofence (Agrium Italia, Livorno, Italy) while *S. alba* mustard meal was procured from Farm Fuel, Inc (2897 Freedom Ave, Watsonville, CA 95076) and applied to the plots that were marked to receive the treatment by random distribution. Similar to MCC, mustard meal was rototilled thoroughly to mix with the soil followed by preparing the raised plastic mulched bed with a bed maker where a drip irrigation line was set in the middle of the bed at the same time. The same plastic mulch described above was used for all the treatment units. These plots were irrigated through the drip line up to saturation of soil to make an anaerobic soil environment. The plastic mulch was perforated 3 weeks after the incorporation of mustard meal to ensure the release of residual toxic gas that otherwise has the potential to cause phytotoxicity. Seedlings were transplanted in the holes 48 h after cutting plastic and digging holes.

Soil nutrient analyses before and after treatment

The soil sample was collected from the whole plot before initiating any treatment in the early spring of 2018 by taking multiple samples that were mixed to have a composite sample to represent the baseline nutrient status of the plot. Soil samples were taken again by uprooting plants from each replicate plot of each treatment at the termination of the trial by carefully shaking the rhizosphere for nutrient analysis together with pH and organic matter content. All samples were analyzed at the WVU soil testing laboratory. The Mehlich-3 method (Mehlich 1984) was used for available P, K, and P saturation index and LR Mehlich-1 for pH determination. Organic matter content was determined by following Hoogsteen et al. (2015).

Determination of weed population in treated plots

As plastic mulch was used to cover the beds as well as row middles, the only weed growth observed was through the holes used for planting strawberry plugs. No weeding was done during the fall of 2018. A higher number of weeds were noticed during late spring of 2019 when weeds were uprooted and counted from each planting hole. The number of weeds from all 20 holes was averaged for each replicate plot of each treatment.

Plant mortality and disease progression

Plant stunting due to the BRR and mortality in the fruiting field plots became noticeable in late spring of 2019. First plant mortality was

recorded on 15 June 2019 in a nontreated check plot immediately after the completion of fruit harvest. Mortality data was recorded every 2 months until 15 October 2019 and again in the spring of 2020 from 15 April to 15 July when the trial was terminated. Wilted and dying plants with the root system and rhizosphere soil were dug carefully followed by gentle shaking to remove loose soil particles. The root system and crown were then separated from the foliage and washed under running water. The root system was evaluated for BRR based on the presence or absence of feeder roots and root color. Crowns were then cut longitudinally to check for the presence of crown rot symptoms. To confirm the association of pathogens, symptomatic dissected crowns were incubated in a humid chamber, and some surface sterilized tissues were used for isolation of crown rot-causing pathogens by placing small surface-sterilized pieces on acidified PDA. Area under the disease progress curve (AUDPC) values were determined from plant mortality data taken during the dates specified above and were estimated following the equation suggested by Shaner and Finney (1977) as:

$$\text{AUDPC} = \sum_{i=1}^{n_i-1} [(y_{i+1} + y_i)/2](t_{i+1} - t_i)$$

where y_i is the proportion of the strawberry plants wilted at ith observation, t_i is the time in days after observation of the disease at ith day, and n is the total number of observations. Photos of each representative treatment unit with surviving plants were taken at the end of the 2020 growing season to indicate treatment effects on plant health (Fig. 1).

Economic analysis

To determine the relative economic advantage provided by each treatment compared with the nontreated control, we assessed the cost of the bio-control agent (BCA)-treated transplant production, the cost of biofumigation with a MCC, ASD with the mustard meal, and the combination of ASD and BCA-treated transplant production. Costs related to overall production operations such as planting, intercultural operations, and harvesting that were common to all treatments including the nontreated check were not considered in this analysis. The price of organic strawberry and total revenues were estimated based on the sale price from the local farmers' market. The total strawberry yield from both years was used for economic analysis. Net revenue for a specific treatment compared with the nontreated control was calculated as follows from the modification of the formula from Rysin et al. (2015) and standard costs shown in Djidonou et al. (2013) that were initially developed for grafted tomato:

$$\text{NR} = [(Y_t - Y_{nt}) \times P] - C$$

where NR is net revenue for a specific treatment compared with the nontreated control; Y_t is estimated marketable yield per plant for a specific treatment; Y_{nt} is estimated marketable yield per plant for the nontreated control; P is sale price per kilogram; and C is additional production cost per plant for a specific treatment over nontreated control.

Data collection and statistical analyses

Fruit were harvested twice weekly from 5 May to 10 June 2019 and 25 May to 25 June 2020. Harvest was delayed in 2020 due to early spring frost that killed some blossoms and cooler spring weather that followed. Total yield in a treatment was analyzed based on the cumulative yield from weekly harvests and calculated to represent the average yield per plant. The severity of soilborne disease (BRR/crown rot) was calculated on a 0 to 5 scale where 0 = no disease symptom; 1 = 1 to 5% plants are stunted; 2 = 6 to 10% plants are stunted; 3 = 11 to 25% plants are stunted; 4 = 26 to 50% plants are stunted; 5 = 51 to 100% of plants are stunted and some wilting of the plants. The disease index for each replicate plot was calculated from all symptomatic plants and corresponding disease severity by following the formula shown in Rahman and Punja (2005). As the soilborne disease severity data was collected on an ordinal scale rather than continuous, a nonparametric data analysis was done by PROC RANK in SAS 9.4 (SAS Institute, Cary NC). However, yield and nutrient content data was subjected to analysis of variance to determine differences of means in various treatments. A linear mixed model was used where treatment was considered as a fixed effect and block as a random effect. Percent data obtained from diseased plant count was transformed using angular transformation (arcsine of square-rooted value) prior to the analysis. Means were compared for significant differences by Fisher's protected LSD test ($P = 0.05$).

Results

Weed suppression in treated plots

Significant weed suppression ($P = 0.004$) was observed in ASD field plots alone and a combination of ASD with TG-treated plug plants. The lowest number of weeds were found in untreated non-pasteurized media-grown plug plants planted in ASD plots followed by TG-inoculated nonpasteurized media-grown plug plants planted in ASD plots, thus indicating that TG did not play a role in weed suppression. The weed population in these two treatments did not vary significantly. MCC treated plots also suppressed weed growth to some extent. The difference between MCC and the untreated check was not statistically different. TG treatment of plug plant growing media without any treatment of fruiting field plots did not show any statistical difference in weed count compared with the untreated check although the highest number of weeds were found in the untreated check (Fig. 2).

Fruit yield in the first and second years

Fruit yield in all treatments was within the normal range for organic strawberry production in the mid-Atlantic region. All treatments showed numerical yield enhancement in the first year compared with untreated check. However, only the combination treatment of TG and ASD had significantly higher ($P \leq 0.045$) yield

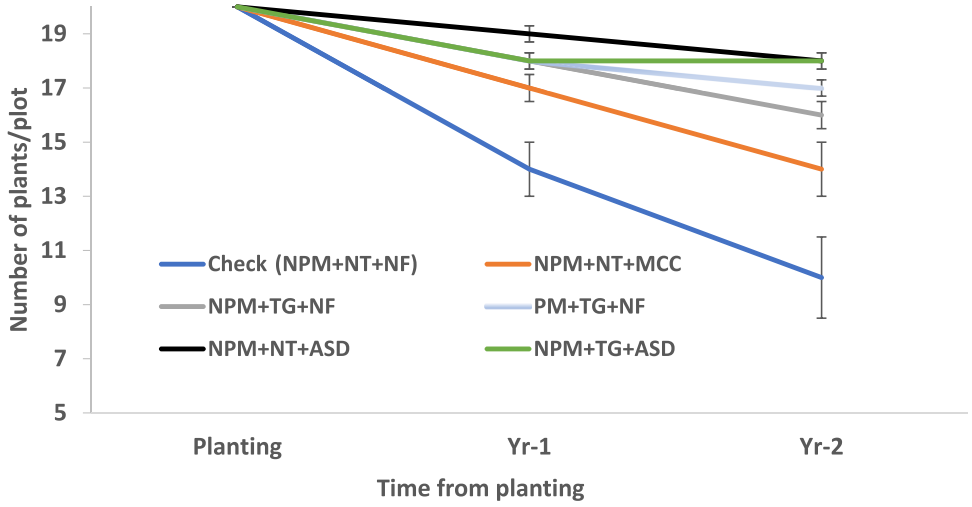

Fig. 1. Plant mortality of strawberry as affected by treatments of probiotic bacteria, anaerobic soil disinfestation, and mustard cover crop biofumigation. Initial planting was done with 20 plants. Healthy plants were counted at the end of the harvest of year 1 and again at the termination of the trial. Treatments are defined in Table 1.

compared with the untreated check, thus indicating a synergistic effect as these treatments separately did not enhance yield (Fig. 3A). As some plants showed diminishing vigor and some died at the end of the first-year fruit harvest, overall yield in the second year was lower in all treatments compared with the first year (Fig. 3B). An unexpected spring frost also killed some blossoms that may have affected yield regardless of treatments. The total yield in a plot was divided by 20 to obtain yield/plant based on the initial number of plants. Fruit yield in the second year showed significantly higher yield in all treatments compared with untreated control except MCC. The highest fruit yield was obtained from the NPM + TG + ASD treatment, which was statistically similar to other treatments except for the MCC and untreated control. ASD alone and TG-inoculated planting mix-grown plug plants had statistically similar fruit yields but were numerically lower than the combination treatment (NPM + TG + ASD), thus indicating a similar synergistic effect as first-year harvest (Fig. 3B).

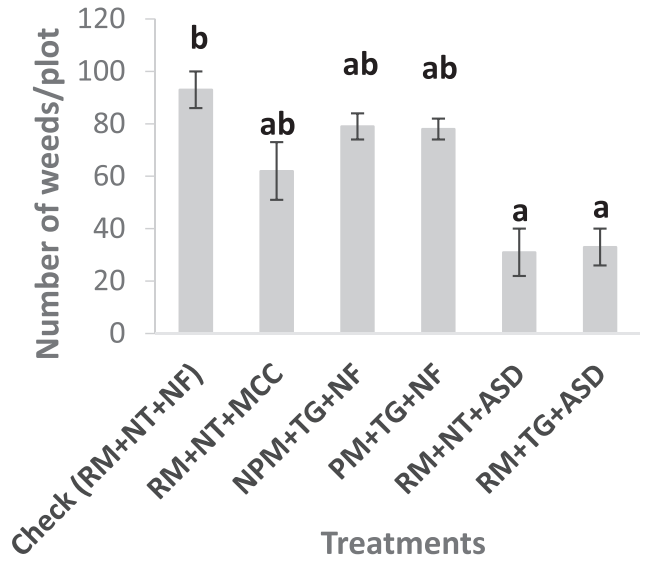

Fig. 2. Effect of probiotic bacteria, anaerobic soil disinfestation, and mustard cover crop biofumigation on weed population grown through planting holes. Vertical bars with different letters are significantly different according to Fisher's protected LSD test (P = 0.05). Treatments are defined in Table 1.

Plant vigor and mortality

Immediately after fruit harvest was completed during the summer of 2019, diminishing plant vigor, decline, and mortality were noticeable in the untreated control plots as well as in a few of the treated plots. Plant mortality in each plot was counted at the end of the summer of 2019 and again at the end of the summer of 2020 at the termination of the experiment. The trial was started with 20 plants in each replicate plot of each treatment. At the end of the first year, an average of six out of 20 plants in the untreated check died, and another four died at the end of the second year leaving only 50% of the plants alive. Plant mortality occurred in other treatments as well but to a lower extent. MCC plots lost three plants/year on average. Other treatments such as the combination of TG treatment with ASD or ASD alone and TG treatments of planting mix with or without pasteurization also had plant mortality in the range of two to four plants/plot on average. The lowest plant mortality was recorded in ASD alone or ASD combined with TG inoculation of planting mix. Overall plant vigor was also lower in treatments that lost more plants compared with the treatments that lost a low number of plants (Fig. 1). Photos taken at the end of the trial showed a remarkable difference in plant health and vigor among treatments (Fig. 4). PROC RANK analysis of soilborne disease severity showed that the nontreated check had the highest rank for disease severity and the lowest was for the combination treatment of biologicals and ASD. There was no difference in the disease severity index of NPM + TG + NF and PM + TG + NF, thus indicating that pasteurization of the medium did not provide extra benefit for root colonization by the biologicals in TG. MCC also did not provide optimum disease suppression as the severity index ranking was the highest for MCC among the treatments. However, the most important and significant finding is the additive effect of biologicals and ASD on lowering the disease severity index (Table 1). However, AUDPC determined from plant mortality data without considering plant stunting and vigor four times from the fruiting field during 2 years of this study indicated a slightly different ranking of the treatments (Fig. 4B).

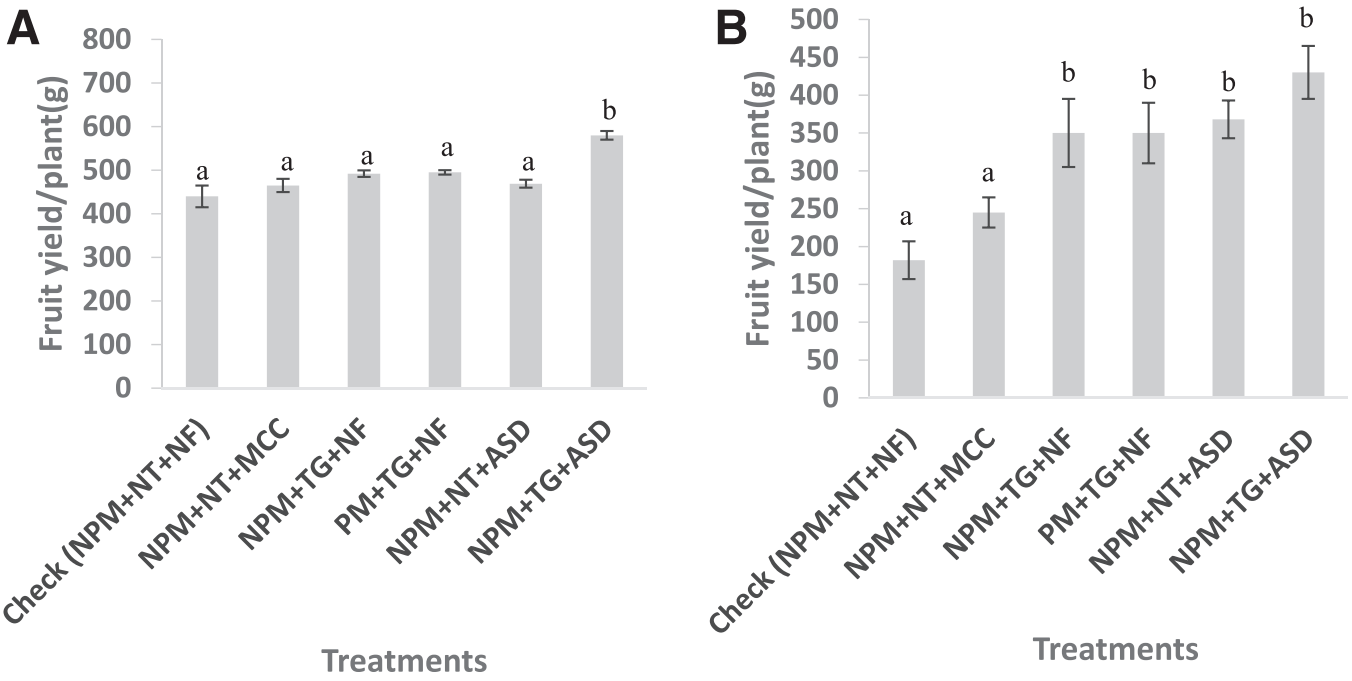

Fig. 3. Effects of probiotic bacteria, anaerobic soil disinfestation, and mustard cover crop biofumigation on strawberry fruit yield (g/plant). **A,** first year (2019) yield; **B,** second year (2020) yield. Vertical bars with different letters are significantly different according to Fisher's protected LSD test (P = 0.05). Treatments are defined in Table 1.

Root disease assessment

Most of the dying and dead plants after 1 year (2019) had Phytophthora crown rot with minor BRR symptoms. However, in year 2 (2020), all the declining plants had BRR, and 10 to 20% had both BRR and Phytophthora crown rot. Isolation from affected roots showed that *Pythium*, *Rhizoctonia*, and *Fusarium* spp. were associated with BRR, whereas *Phytophthora* was consistently isolated from crown rot. Further morphological analysis of the oospore formed by *Phytophthora* indicated the species as *P. cactorum*.

Rhizosphere soil analysis

Rhizosphere soil from each treatment was analyzed for major nutrients, pH, and organic matter content at the end of the trial. A composite sample from the plot prior to treatment application was also collected and analyzed to determine the baseline nutrient status. Phosphorus (P), potassium (K), and organic matter content showed significant differences among treatments but no difference in magnesium (Mg) or pH. P and K contents were higher in all treatments with a significant difference in MCC and ASD compared with the untreated control. Potassium content in all treatments except ASD alone was significantly

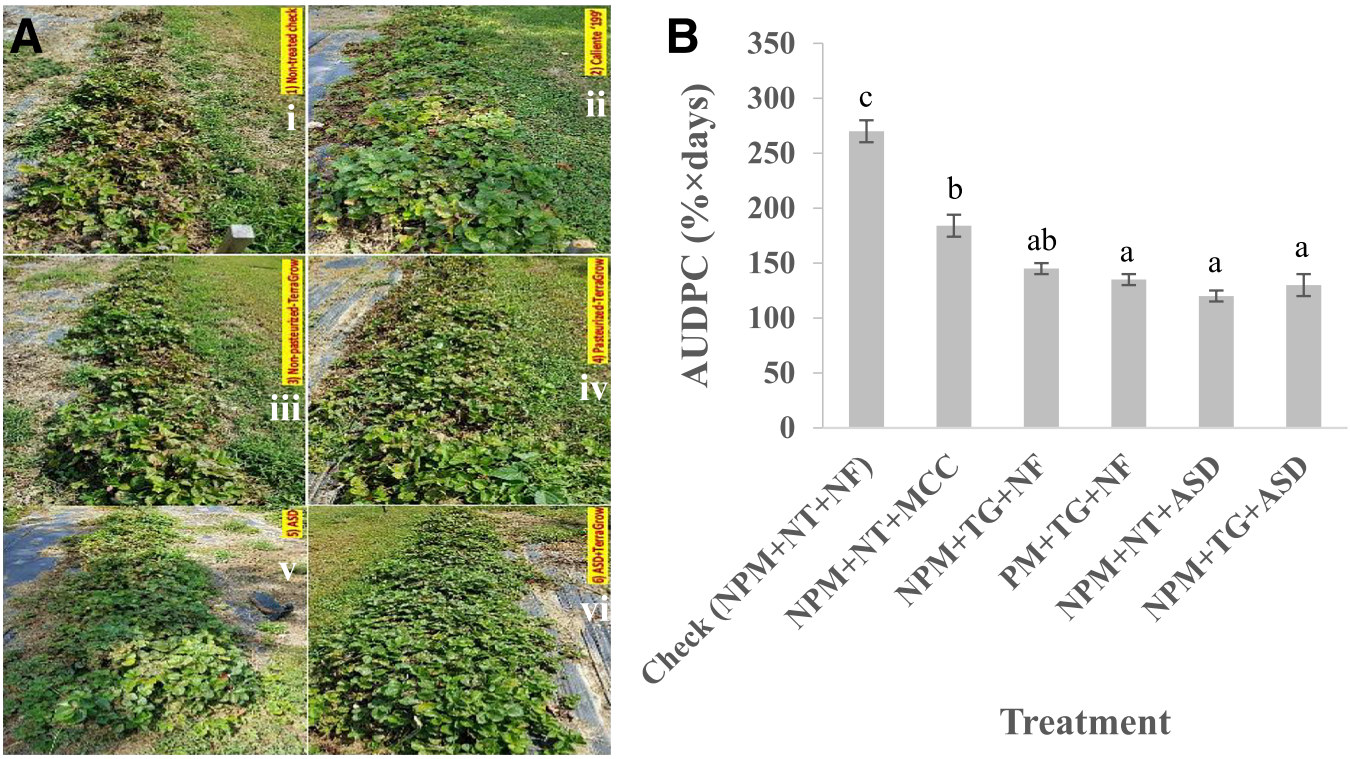

Fig. 4. A, Appearance of strawberry plots before termination of the trial during the summer of 2020. Nontreated plots showed very high plant mortality and low vigor; Roman numerals on the photos follow the same sequence as mentioned in materials and methods section, the production of treated plug plants, as well as on all the tables and figures; **B,** area under the disease progress curve (AUDPC) for the plant mortality data in different treatments in the fruiting field in 2019 to 2020. Vertical bars with the same letter above them are not significantly different from one another according to Fisher's protected LSD test ($P = 0.05$). Treatments are defined in Table 1.

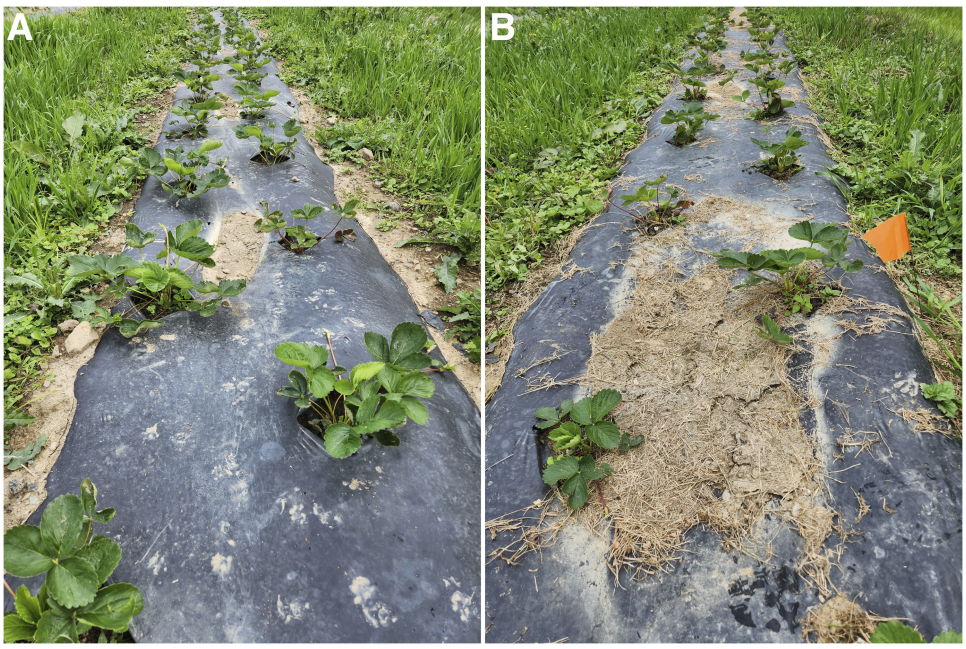

Fig. 5. Vigor improvement in strawberry plants after 35 days of field set. **A,** Nonpasteurized planting mix treated with TerraGrow to grow plug plants; **B,** nontreated plug plants. Treatments are defined in Table 1.

higher ($P = 0.003$) compared with the untreated check. Organic matter content was increased numerically in all treatments compared with the untreated check. However, only the difference between the untreated check and the combination treatment of probiotic bacteria and ASD was statistically significant (Table 2).

Economic analysis

Strawberry BRR and crown rot could be greatly eliminated to improve plant health and fruit yield for better economic return by some of the treatments included in this study. The highest net revenue/plant was provided by the combination treatment due to the additive effect of probiotic media-treated plug plants and field soil ASD (Table 3), followed by pasteurized and nonpasteurized media inoculation by TG. ASD alone and MCC treatment provided low net revenue due to the higher cost involved with the application of these treatments compared with BCA only but relatively low yield enhancement. For the BCA treatment, the production cost of plug plants was slightly higher with higher net revenue on pasteurized media. However, this treatment option may not be suitable for growers due to the lack of media pasteurization capacity. The lowest yield advantage and net revenue in our study was obtained from the MCC treatment. As MCC requires significant effort in seeding, time to grow up to flowering, and incorporation in the field with very low net advantage, this treatment may not be an acceptable alternative option to growers either.

Discussion

Strawberry production in the mid-Atlantic and northeastern states of the United States takes place in limited land on a recurrent basis due to the lack of sufficient suitable sites, the need for irrigation, and for U-pick marketing. Recurrent use of land for the same crop without an effective rotation plan diminishes fruit yield over time due to the buildup of soilborne pathogen populations that cause BRR or crown rot. Use of the highly effective soil fumigant methyl bromide in most cases could suppress these pathogens. However, due to its ozone-depleting properties, this product was banned by the Montreal protocol (Minuto et al. 2006). Furthermore, use of chemicals for soil fumigation is not feasible for small and organic growers due to the added cost and federal regulations, which justify the search for alternate options such as biological and biorational methods. This study demonstrated that BCAs, ASD, and their combination can provide remarkable protection of strawberry plants from soilborne pathogens in a perennial organic production system. Results from our study showed initial vigor improvement of plug plants grown on probiotic bacteria-treated planting mix after field set and complemented with superior protection from soilborne pathogens to achieve higher yield. One of the most interesting and important findings of this study is the synergistic effect of early colonization of strawberry plug root system by probiotic bacteria and ASD due to the field set of these plug plants in ASD field soil. Although utilization of BCA (Mukta et al. 2017; Rahman et al. 2018), biofumigation (Giovannini et al. 2021), and/or ASD (Shennan et al. 2014) have been reported for soilborne pathogen control in strawberry, this study for the first time assessed the comparative performances of these management options for disease suppression and yield improvement of strawberry in an organic production system. In addition, suppression of weeds in ASD treatment provided support for this treatment as an alternative to fumigants. As the mustard meal was used in ASD treatment as a carbon source, quick decomposition and production of toxic gas in ASD may have effectively suppressed weed seed germination. However, due to the use of a certified organic production system to carry out this study, it was not possible to compare the efficacy of synthetic chemical-based fumigants with the biorational treatments. Nevertheless, our results provided strong evidence that biological and biorational treatments and their combination can benefit growers compared with a nontreated check. Thus, the replacement of synthetic chemical-based soil disinfestation with suitable organically acceptable protocols shown in this study creates an opportunity for further refinement to enhance efficacy. Further study is now underway to enumerate and characterize the ratio of harmful and beneficial microbial components in soil samples of these treatments, which will be reported elsewhere in a separate article. Additional studies will be needed to prove if this approach can provide sustainable control of soilborne pathogens in fruiting fields for the recurrent production of strawberry where crop rotation is not an option for growers.

Research on MCCs for biofumigation has been going on for a long time with mixed results for both weed and disease control (Balzano 2017; Hansen and Keinath 2013; Hartz et al. 2005). Our results in a comparative setting with other biorational options demonstrated that a MCC, although adding biomass and nutrients to soil, may not provide any disease management or weed control benefit to growers

Table 2. Nutrient contents in the rhizosphere soil from different treatments[y]

Treatment[z]	P (ppm)	K (ppm)	Mg (ppm)	Average soil pH	OM (%)
Untreated check (NPM + NT + NF)	130 c	230 c	190 a	6.3 a	7.7 b
Mustard cover crop (NPM + NT + MCC)	210 a	340 a	195 a	6.6 a	8.6 ab
Nonpasteurized media inoculation (NPM + TG + NF)	160 bc	315 ab	188 a	6.3 a	8.5 ab
Pasteurized media inoculation (PM + TG + NF)	154 bc	301 ab	187 a	6.5 a	8.3 ab
Anaerobic soil disinfestation (NPM + NT + ASD)	170 b	277 bc	213 a	6.3 a	9.5 ab
Combination of NPM inoculation and ASD (NPM + TG + ASD)	185 ab	310 ab	225 a	6.4 a	9.8 a

[y] Soil samples were collected by shaking the root system after carefully digging the plants at the termination of the trial.
[z] NPM = non-pasteurized media; NT = no treatment; NF = no field treatment; PM = pasteurized media; TG = TerraGrow; MCC = mustard cover crop; ASD = anaerobic soil disinfestation.

Table 3. Summary of the economic advantage provided by specific treatment relative to nontreated control in a perennial organic strawberry production system[w]

Treatment[x]	Yield/plant (kg)	Yield gain/plant (over nontreated)	Strawberry price/kg[y]	Additional cost over nontreated[z]	Net revenue
Untreated check (NPM + NT + NF)	0.59	0	$7.00	0	0
Mustard cover crop (NPM + NT + MCC)	0.72	0.13	$7.00	$0.30	$0.61
Nonpasteurized media inoculation (NPM + TG + NF)	0.86	0.27	$7.00	$0.10	$1.79
Pasteurized media inoculation (PM + TG + NF)	0.91	0.32	$7.00	$0.30	$1.94
Anaerobic soil disinfestation (NPM + NT + ASD)	0.84	0.25	$7.00	$0.30	$1.40
Combination of NPM inoculation and ASD (NPM + TG + ASD)	1.04	0.45	$7.00	$0.35	$2.80

[w] Cumulative yield from 2019 to 2020 were used in calculating the net revenue.
[x] NPM = non-pasteurized media; NT = no treatment; NF = no field treatment; PM = pasteurized media; TG = TerraGrow; MCC = mustard cover crop; ASD = anaerobic soil disinfestation.
[y] Small growers sell their organic strawberries in local farmers' markets for a premium price.
[z] Additional cost included the cost of inputs, supplies, as well as labor for application that were not needed in nontreated plants/plots.

as we did not obtain any significant weed suppression or yield enhancement. Many growers have a similar experience with mustard biofumigation (M. Rahman, personal communication). A large body of literature suggests that the biofumigation effect of *Brassica* is derived from the release of ITCs due to the hydrolysis of glucosinolates present in the tissue that can suppress a range of soil pathogens (Matthiessen and Kirkegaard 2006; Morra and Kirkegaard 2002), and efficacy is partly dependent on good tissue maceration and immediate incorporation into soil that has optimum moisture (Matthiessen et al. 2004). Despite being very careful about meeting these requirements, we did not get optimum disease suppression. Hartz et al. (2005) also reported that they did not find any positive effects of using MCCs for suppression of soilborne diseases (Verticillium and Fusarium wilt) or yield when used for tomato soilborne disease management.

Biocontrol of soilborne phytopathogens with beneficial microorganisms is considered an alternative to environmentally hazardous synthetic fungicides (Weller 1988). Many microorganisms have been tested in recent decades for their capacity to suppress soilborne pathogens in diverse crop production systems (Lee et al. 2008). However, microorganisms that show biocontrol potential during in vitro tests or in bioassays often fail to produce consistent results under field conditions. Large-scale use of BCA and adoption of the technology in agriculture has been hindered by this (Weller 1988), which warrants microclimate-based determination of efficacy for a specific host-pathogen combination. In the current study, we used the bio-fungicide TG proactively to facilitate early root colonization of plug plants complemented with planting hole application in an organically managed production system. This product has five different beneficial bacteria belonging to the genus *Bacillus*. Root system colonization by these microbes usually wards off harmful microbes when planted in contaminated soil, makes nutrients available to plants, and in some cases provides stress tolerances by producing growth hormones and other plant-beneficial enzymes. Consistent with the recommendations for biological control to occupy the root surface of a plant prior to pathogen infection, we applied BCAs to the plug production planting mix, as well as in the field twice by watering manually with a watering can. Investigators on similar diseases obtained significant disease reduction on other crops as well. For example, Rose et al. (2004) found that stem and root rot diseases of cucumber caused by *Pythium aphanidermatum* and *F. oxysporum* f. sp. *radicis-cucumerinum* were reduced by the application of Prestop (*Gliocladium catenulatum* Strain J1446) at the seeding time of greenhouse-grown cucumber. Rahman and Punja (2007) found that augmenting beneficial microbial populations on the seed surface and in soil could reduce seedling mortality, which corroborates findings from the current study. To maximize the planting mix colonization potential, we eliminated resident microbial competition by autoclaving media. Our results, however, did not show any advantage of media pasteurization on colonization and thereby reduction of soilborne disease and yield enhancement. Inoculation of our planting mix with probiotic bacteria with or without pasteurization provided a similar benefit, thus indicating that plug producers can directly mix BCA product with the planting mix before dispensing in the plug tray for sticking tips/cuttings. Due to the ease of application method, there is a high potential for plug producers to adopt this technology in their nonpasteurized plug production protocol. To obtain the additive effect, fruit growers, especially those who can't use synthetic chemical-based fumigation, may place these strawberry plug plants in ASD field plots. Outreach efforts targeted toward plug producers should make a huge impact, as plug plant buyers in most cases pay attention to the recommendation made by plug suppliers. We did not explore the specific mode of action of disease suppression by beneficial microbes in this study. However, Lahlali et al. (2013) pointed out that control of clubroot disease on canola by the biofungicide Serenade (*Bacillus subtilis* QST713) occurred via antibiosis and induced systemic resistance. Barka et al. (2000) reported that grapevine plantlets that were inoculated in vitro with beneficial microbes grew faster than noninoculated controls, were sturdier, and developed a better root system. Similar findings were obtained with banana plantlets treated with endophytic *Pseudomonas* and *Bacillus* species. These plants not only showed improved physiological attributes and vegetative growth but also had strong resistance against bunchy top diseases in field conditions (Kavino et al. 2007). In our study, we did not get the expected vigor enhancement during the plug production phase. However, within a month of field set, probiotic-treated plants showed enhanced vigor compared with nontreated (Fig. 5). Most importantly, these plants remained more vigorous throughout the duration of the study and produced more fruit with a concurrent reduction of the disease severity index. In a similar study for managing tomato bacterial wilt, we documented clear vigor enhancement during the seedling stage in the greenhouse and found that pasteurized media inoculation with beneficial microbes provided higher vigor compared with the nonpasteurized medium. It appears from this work that seedlings started from seeds versus cuttings are different considering beneficial microbe colonization. Although not much information is available on substrate inoculation after autoclaving to facilitate quick rhizosphere colonization in a less competitive environment, the difference between seed-grown seedlings in tomatoes and vegetative propagation for strawberry plug plants may be responsible for the difference. More research is needed to unravel the differences in mechanisms involved with the inoculation of pasteurized medium for seedling and vegetatively propagated plants such as strawberry.

In this study, we used a commercially available formulation containing multiple *Bacillus* species that was easy to incorporate into the planting mix to facilitate early root colonization. Baysal et al. (2008) tested *B. subtilis* strain EU07 and QST 713 for controlling tomato root and crown disease caused by *F. oxysporum* f. sp. *radicis-lycopersici* at 10^6 CFU/ml. Strain EU07 reduced disease incidence by 75%, whereas QST 713 reduced incidence by only 52% when applied as an inoculant compared with nontreated. This example shows the potential and prospect of biocontrol if the right strain can be selected and utilized. Although the commercial product used for this study contained multiple strains that individually were proven to provide beneficial effects to plants, their addition in a single product did not seem to provide any additive effect.

An added advantage of managing soilborne fungal disease with a bacterial biocontrol agent is that conventional growers can combine these agents with fungicides for additive effects as confirmed in other studies (Omar et al. 2006). Although *Bacillus* species have been found effective against soilborne pathogens by many authors worldwide, the application method of many of those biocontrol agents including *B. amyloliquefaciens* FZB42 played a key role in the efficacy (Fira et al. 2018). Berendsen et al. (2012) suggested that microflora in the rhizosphere of young plants needs to be stabilized for colonization of the rhizosphere by the inoculated strain in a sufficiently high number. These studies also identified multiple modes of action or mechanisms of pathogen suppression that included direct competition of beneficial microbes for space or nutrients on the root surface, production of volatile organic compounds, and antibiotic metabolites.

Our comparison of some options feasible to any grower for managing soilborne diseases of strawberry revealed that plug plants grown on probiotic-inoculated media followed by planting in ASD fruiting plots provided superior disease resistance and yielded the highest in both years. Many investigators reported that ASD could provide some protection to strawberry from Verticillium wilt and other possible soilborne pathogens (Shennan et al. 2017). In the current study, we compared multiple nonchemical strategies to manage soilborne diseases in strawberries under a certified organic production system and demonstrated results to potential users of these technologies. Yield increase (1.5 times in the first year and up to 2.4 times in the second year) in the combination treatment over the nontreated check is a clear indication that the combination treatment can be an important component together with a few others such as probiotic media inoculation for growing plug plants by plug producers that can be acceptable to fruit producers interested in nonchemical strategies to manage strawberry soilborne diseases. However, more studies comparing the effect of probiotic bacteria and combination treatments through on-farm trials may support the adoption of the technology. Significant increases in

nutrient contents in MCC and ASD can be explained by the addition of a large amount of biomass in the soil. As we added lots of biomass in MCC and ASD plots, higher nutrient and organic matter contents in these treatments are expected. However, it is very interesting that probiotic bacteria-treated planting mix grown plug plants when planted in the field plots likely helped in the solubilization of P and K from parent materials and increased their concentration in rhizosphere soil. Probiotic bacteria, especially *Bacillius* spp., are known to solubilize P and K from soil parent materials. It appears from the results that nutrient content did not play a pivotal role in yield enhancement, and plant health as MCC treatment did not provide any significant yield advantage. Pathogenic microbes may rather have played a bigger role in influencing plant productivity.

Economic analysis to determine the net advantage obtained from each treatment clearly showed that the combination of probiotic and ASD treatment can provide the highest financial return to strawberry growers who are not able to fumigate fields with synthetic fumigants. The margin of net revenue from such treatment can be even wider if growers use the same piece of land year after year and inoculum builds up to create higher disease pressure. As such, growers may not hesitate to spend additional money for buying treated plug plants to plant in a perennial organic production system. In comparison, the use of MCC keeps the land occupied for a few months that may prevent growers growing other short-term crop such as leafy greens, and our results do not support the use of MCC for managing soilborne disease in strawberry, as variation within the trial was too wide to negate any positive effect. This corroborates the findings of Sun et al. (2018) that suppression of soilborne diseases by organically acceptable methods may result from their multiple complex interactions with soil components that are not well understood. Therefore, further detailed work is needed to gain additional insights into the mechanism of strawberry BRR and crown rot suppression and yield increase by the organically acceptable biorational approaches (probiotic bacteria, ASD, and combination treatment of both probiotic bacteria and ASD) described in this report. However, the use of the combination treatment was shown to be the best method for preventing soilborne disease and boosting yield for net economic benefit in our study. The net revenue obtained in this study may only be applicable for organic strawberry production in soil infested with soilborne pathogens such as those causing BRR or crown rot.

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