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Assessing the soil microbiome population shift after soil disinfestation and beneficial bacteria treatments and their combinations.

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Abstract: This research aimed to investigate the effect of three preplant treatments including anaerobic soil disinfestation (ASD), fumigation, and untreated control with and without bacterial endophyte inoculation on the structure and composition of the soil microbiome. Additionally, the study aimed to assess whether any changes in the soil microbiome populations persisted throughout the entire strawberry growing season. The trial was conducted at the Hampton Roads AREC in Virginia Beach, VA, during the 2022-2023 season. Soil disinfestation treatment beds (ASD, fumigation, and non-disinfested control) were the main plots with four replicates in this study. The sub-plots treatments included: (i) *Bacillus velezensis* only; (ii) TerraGrow (a mixture of five *Bacillus* spp.) at 1.5 lbs/A only; (iii) TerraGrow at 1.5lbs/A plus Oxidate 5.0 (27% hydrogen peroxide and 5% peroxyacetic acid) at 2500gal/A, and (iv) nontreated control. Soil samples were collected for pre-application, post-application, and post-harvest sampling. Three replicates were taken for each treatment and each soil sample consisted of four soil cores, which were collected using a 2.5 cm diameter soil core at a depth of 15 cm. DNA samples were employed as a basis for library construction and Illumina sequencing. The design of fungal internal transcribed spacer region 2 (ITS2) was per the guidelines specified for the Illumina MiSeq system. In general, the preplant ASD treatments had a reduced alpha diversity of fungal populations compared with the fumigated and untreated control soil samples. Additionally, the alpha diversity of fungal communities was found to be significantly reduced in ASD plots treated with *B. velezensis*, which

was also the least diverse among all the ASD treatments and similar to the fumigation plots. These findings suggest that the decrease in alpha diversity observed in communities affected by ASD can be predominantly attributed to endophytic bacteria such as *B. velezensis* treatment and the decrease in soil Eh (redox potential). Reduction of alpha diversity in fungal populations is likely due to the interactions between endophyte bacteria and pathogenic organisms such as competition, parasitism, and predation which are common pathogen control mechanisms by beneficial bacteria.

Introduction: Strawberries are widely consumed and highly favored in both the United States and across the globe. However, the strawberry plant is susceptible to two types of soilborne diseases, namely black root rot complex (BRRC) and crown rot (CR). These diseases are caused by various fungi and can have a significant impact on plant health and productivity, especially if the same land is used for replanting or if the plant is maintained in a perennial production system for a few years. Controlling soilborne pathogens in strawberry production systems is challenging due to the absence of effective, long-lasting control methods. Post-ban of methyl bromide, the development of alternative sustainable chemical methods such as anaerobic soil disinfestation (ASD), beneficial microorganisms, and their combinations should help control soil-borne pests, including the black root rot complex (BRRC), crown rot (CR) diseases of strawberries and weeds, which indirectly improve fruit yield and quality (Rahman et al., 2019).

Strawberry root exudates can attract microbes from the rhizosphere (Lazcano et al., 2021). Engaging beneficial microorganisms such as endophytic bacteria that are considered biostimulants, along with the anaerobic soil disinfestation (ASD) techniques, could generate a powerful tool to control soilborne pathogens and improve the growth and yield of strawberries (Momma et al., 2013). Beneficial microorganisms can improve plant nutrition and support plant development under natural or stressed conditions and can increase the yield and quality of many important crops (Kundan et al., 2015). In the interaction between the beneficial microorganisms and plants, these organisms act as nutrient suppliers, phytohormone producers, plant growth enhancers, biocontrol of phytopathogens, and improvers of soil structure (Jayaprakashvel et al., 2019). Root dipping of seedlings (plug plants), followed by spray treatments of both probiotic bacteria including *Bacillus amyloliquefaciens* (BChi1) and *Paraburkholderia fungorum* (BRRh-4) on strawberry leaves dramatically enhanced the fruit yield by 48%, compared to non-treated controls (Rahman et al., 2018). In the greenhouse, three strains of *Bacillus velezensis*, an endophyte

bacterium, significantly suppressed strawberry pathogen growth (*Colletotrichum gloeosporioides*) and increased marketable fruit yields in the field (Mei et al., 2021). These bacteria increase the concentration of plant available phosphorous in the soil by the release of phosphatase enzymes or organic chelates (Richardson et al., 2011). The gray mold disease in strawberry, caused by *Botrytis cinerea*, was controlled by five different isolates of *Bacillus* spp. via the production of diffusible and volatile antifungal chemicals (de Melo Pereira et al., 2012). However, no research has been done to optimize ASD in combination with beneficial microorganisms. The structure and composition of the soil microbiome appears to be key factor in determining the effectiveness of ASD, beneficial microorganisms, and their combinations to confirm their role in pathogen suppression.

Materials and Methods: We initiated a field study at the Hampton Roads AREC, Virginia Beach, VA, for the 2022-23 growing season in a split-split randomized complete block design with four replicates. Preplant soil disinfestation treatments including ASD, fumigation, and nontreated control comprised the main plots and post-transplant beneficial microbe treatments were the subplots. Strawberry plug plants of ‘Chandler’ were planted on Oct 17, 2022 with the following treatment combinations: (i) non-treated field plots (Control); (ii) plots treated with *Bacillus velezensis* only; (iii) plots treated with TerraGrow 1.5 lbs/A only (TerraGrow is a five-strain formula of *Bacillus* spp., Biosafe systems, CT, USA); (iv) plots treated with TerraGrow 1.5lbs/A and Oxidate® (a.i. hydrogen peroxide and peracetic acid, Biosafe systems, CT, USA): 2500gal/A; (v) plots treated with ASD alone using brewer’s spent grain and paper mulch as a carbon source. ASD treatment duration was three weeks; (vi) plots treated with ASD and inoculated with *B. velezensis*; (vii) plots treated with ASD inoculated with TerraGrow 1.5 lbs/A; (viii) plots treated with ASD and inoculated with TerraGrow 1.5lbs/A and Oxidate®: 2500gal/A; (ix) plots treated with Pic-Clor60 at 175 lbs/A (preplant soil fumigant) (Growers standard); (x) plots treated with Pic-Clor60 at 175 lbs/A and inoculated with *B. velezensis*; (xi) plots treated with Pic-Clor60 175 lbs/A and inoculated with TerraGrow 1.5 lbs/A; (xii) plots treated with Pic-Clor60 175 lbs/A and inoculated with TerraGrow 1.5 lbs./A and Oxidate®: 2500gal/A.

Data collection. Initial soil samples (Sep 2, 2022) were collected for DNA extraction prior to the application of the treatments. Post-application (March 25, 2023), and post-harvest (June 27, 2023) sampling were utilized in the study consisted of three sets of replicates. Each set was composed of

seven soil cores collected from three of the four blocks included in the study. The soil cores were obtained from a depth of 15 cm from the bed top and had a diameter of 2.5 cm. The soil samples were stored at -80°C right after collection from the field until transport to the University of Minnesota Genomics Center for DNA extraction, sequencing, and bioinformatic processing.

Results: The alpha diversity of fungal communities in pre-application and control soils were significantly higher compared to the alpha diversity of fungal communities in all soils that underwent ASD application. In general, this indicates that the application of ASD resulted in a notable decrease in the alpha diversity of fungal communities (Fig. 1). Further, ASD plots that were inoculated with *B. velezensis* generated the lowest alpha diversity of fungal communities which was similar to fumigation plots. The reduction in alpha diversity observed in many studies of ASD is likely because this condition can disrupt the delicate balance of microbial populations (Hewavitharana et al., 2019; Hewavitharana & Mazzola, 2016). As a result, certain species may become overrepresented while others are suppressed, leading to a less diverse overall community. This phenomenon may have important implications for the health of plants with ASD plus *B. velezensis*, as a less diverse microbiome has been linked to a range of negative health conditions. The findings of our study suggest that the decrease in alpha diversity observed in communities affected by ASD was predominantly attributed to treatment with the endophyte bacteria *B. velezensis* and the decrease in soil Eh (redox potential) (data not shown).

To demonstrate the highest level of dissimilarity between samples, the beta diversity analysis utilized the Jaccard Emperor method. Component 1 (axis 1) exhibited a maximum component diversity of 10.23% in the Jaccard emperor plots, which was clustered by ASD and control group, while component 2 (axis 2) displayed a slightly lower diversity of 5.566% as observed in fumigation samples. The analysis revealed a noteworthy degree of compositional similarity among the fumigation samples, indicating their proximity in terms of fungal composition. In contrast, the pre-treatment samples formed a distinct cluster, distinguishing them from the other treatment groups (Fig. 2).

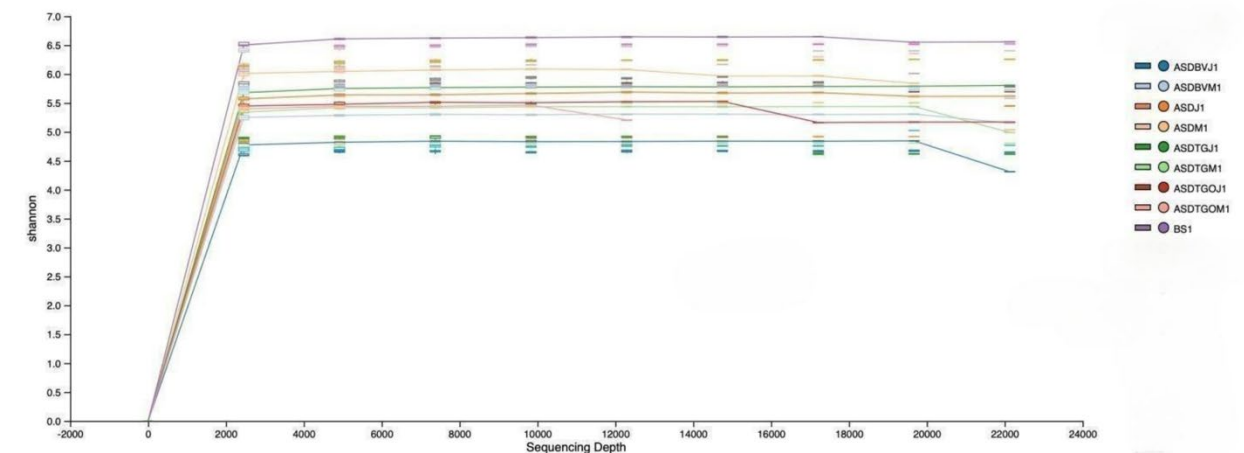


Figure 1: Alpha diversity measures using Shannon's diversity index. The treatments above are as follows. ASDBVJ1(ASD + *B. velezensis*, soil samples collected June 2023): ASDBVM1 (ASD + *B. velezensis*, soil samples collected March,2023): ASDJ1(ASD only, soil samples collected June,2023): ASDM1(ASD only, soil samples collected March,2023): ASDTGJ1 (ASD + TerraGrow, soil samples collected June, 2023): ASDTGM1 (ASD + TerraGrow, soil samples collected March 2023): BS1 (pre-application samples).

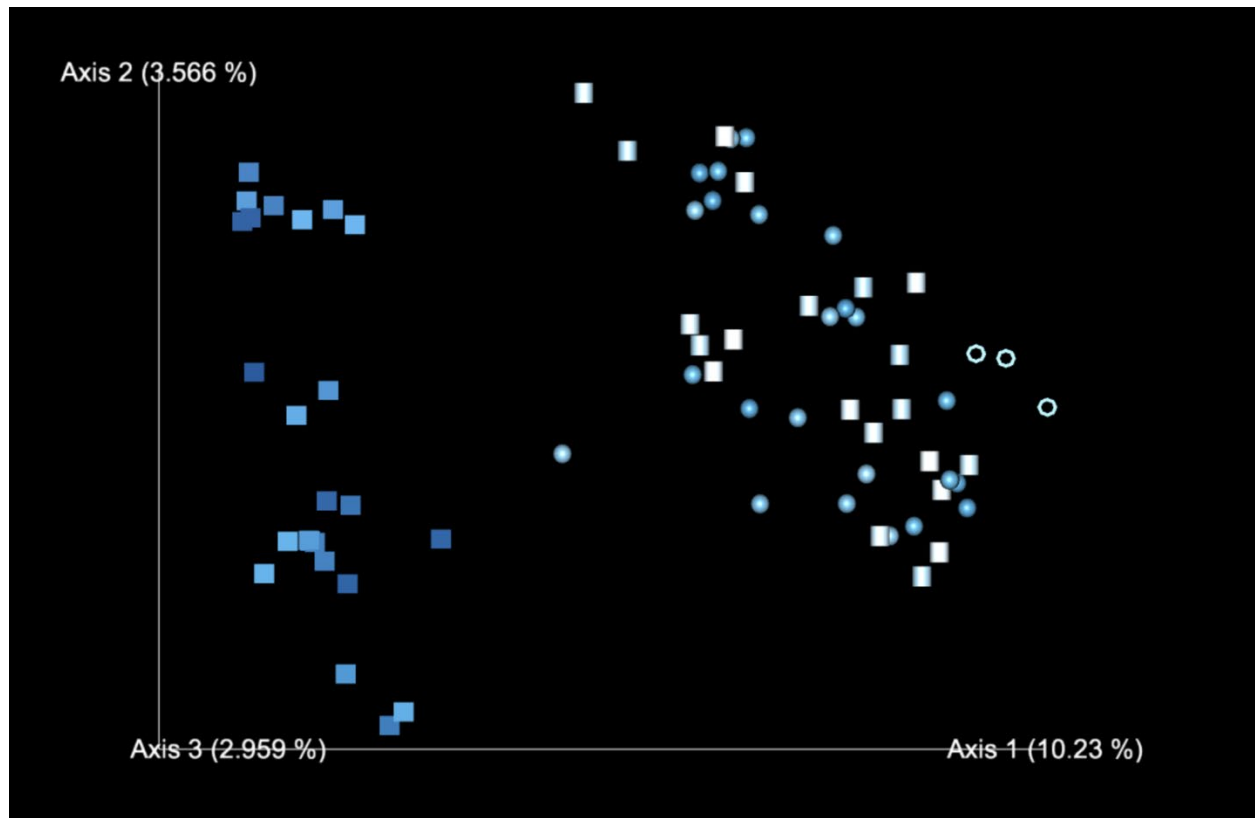


Figure. 2: The Jaccard Emperor PCoA plots exhibit the highest level of dissimilarity between samples. The first axis represents the maximum dissimilarity between samples, followed by the second and third axes. The Fumigation samples (■), ASD (■), Untreated control (●), and pre-application (●).

Conclusion: The implementation of ASD resulted in a decrease in the alpha diversity of fungal populations when compared to both the untreated control samples and the pre-application samples. Among all the ASD treatments, the plots treated with *B. velezensis* exhibited the lowest diversity, similar to the fumigation plots. Our study indicates that the reduction in alpha diversity observed in communities affected by ASD was mainly due to the introduction of the endophyte bacteria *B. velezensis* and the decrease in soil Eh (redox potential). The potential for interactions between endophytic bacteria and pathogenic organisms, including competition, parasitism, and predation, may play a significant role in controlling pathogens.

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