



APPLICATION OF INNOVATIVE MICROORGANISMS IN OLIVE TREE (*Olea europaea* L.) CULTIVATION AND PROTECTION UNDER CONTROLLED CONDITIONS

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ABSTRACT

The olive tree (*Olea europaea* L.) is a key crop in Mediterranean and arid regions, valued for its cultural and economic importance and its natural resilience to harsh conditions. However, its cultivation is increasingly threatened by a combination of abiotic stresses – such as drought, salinity, and soil degradation – and biotic factors, particularly soilborne pathogens like *Verticillium dahliae*, which causes vascular wilt and significant yield losses.

To address these challenges, this study evaluated the effects of three microbial consortia, each composed of selected bacterial and yeast strains with plant-beneficial traits, on early olive plant development and disease resistance under sterile, climate-controlled conditions. The bacterial components, derived from the phylum Bacillota (*Oceanibacillus*, *Schouchella*, *Lentibacillus*), are known for stress tolerance, enzymatic activity, and antagonism against pathogens. Each consortium also included strains of the osmophilic yeast *Zygosaccharomyces*, capable of secreting bioactive compounds with antifungal properties.

Over a 90-day period, inoculated olive saplings were assessed for growth (shoot and root biomass, plant height), physiological performance (chlorophyll content, root-to-shoot ratio), and response to *V. dahliae* infection. All consortia significantly enhanced plant growth compared to untreated controls, with one formulation increasing root biomass by up to 40% and improving chlorophyll levels. In pathogen-inoculated plants, microbial treatments reduced disease severity, delayed symptom onset, and significantly lowered pathogen DNA concentrations in root tissues, as confirmed by qPCR.

These results highlight the potential of microbial consortia as effective biostimulants and biocontrol agents in olive cultivation. Their use could reduce dependence on chemical inputs and improve productivity and resilience in both conventional and organic farming systems. Field trials are recommended to validate these findings under diverse environmental conditions.

KEY-WORDS: Microbial consortia; Plant biostimulants; Sustainable agriculture; Olive tree cultivation; *Verticillium* wilt

INTRODUCTION

The olive tree (*Olea europaea* L.) is a foundational species in Mediterranean agriculture, cultivated for both its economic and ecological value. It occupies over 10 million hectares worldwide, particularly in semi-arid and arid regions, and provides high-value products such as olive oil and table olives while supporting rural livelihoods and agrobiodiversity [1]. Despite its adaptation to drought and poor soils, olive cultivation is increasingly threatened by a combination of abiotic and biotic stresses. Climate change has led to rising temperatures, reduced rainfall, and prolonged droughts, while intensive farming practices have contributed to soil degradation and increased disease susceptibility [2, 3].

Pathogens such as *Verticillium dahliae*, *Rosellinia necatrix*, and *Xylella fastidiosa* are responsible for severe yield losses and long-term decline in orchard productivity [4-6]. Traditional disease control measures, including chemical fumigants and synthetic fertilizers, are increasingly viewed as unsustainable due to environmental concerns and regulatory restrictions, particularly in organic and low-input systems. In response, there is growing interest in ecological intensification, which seeks to harness biological processes to enhance productivity

while preserving natural resources. One of the most promising approaches is the use of beneficial microorganisms, including plant growth-promoting rhizobacteria (PGPR), mycorrhizal fungi, endophytes, and biocontrol yeasts [7,8]. These microbes contribute to plant nutrition, phytohormone regulation, stress tolerance, and pathogen suppression through mechanisms such as nitrogen fixation, phosphate solubilization, competition, antibiosis, and induction of systemic resistance [9,10]. More recently, studies have shown that microbial consortia—carefully selected mixtures of functionally complementary microorganisms—can be more effective than single-strain inoculants [11,12]. In particular, bacteria within the phylum Bacillota, such as *Oceanibacillus*, *Schouchella*, and *Lentibacillus*, have attracted attention for their ability to tolerate high salinity, produce biofilms, secrete hydrolytic enzymes, and release antimicrobial compounds [13,14]. In addition to bacteria, non-*Saccharomyces* yeasts like *Zygosaccharomyces* have shown promising plant-associated functions, including competition with pathogens, volatile organic compound (VOC) production, and stress alleviation [15,16]. While historically studied in food spoilage and fermentation, these yeasts are now recognized for their potential in crop protection and root zone conditioning [17].



Despite their potential, most studies in olive cultivation have focused on single-strain inoculants or field trials under highly variable conditions, limiting reproducibility and mechanistic understanding [18]. There is a clear need for controlled experimental designs that isolate microbial effects on olive growth, physiology, and disease resistance. This study addresses this gap by evaluating three defined microbial consortia—comprising *Bacillota*-associated bacteria and yeasts of the genus *Zygosaccharomyces*—applied to young olive plants under sterile, climate-controlled conditions. The consortia were assessed for their effects on (i) plant growth and morphology, (ii) physiological traits such as chlorophyll content and biomass allocation, and (iii) the suppression of *Verticillium dahliae*. The findings aim to contribute to the development of microbiome-based strategies for resilient and sustainable olive production systems.

MATERIAL AND METHODS

Plant Material and Experimental Design

Young olive saplings (*Olea europaea* L., cv. Arbequina) were selected for uniformity in size and vigor. Seedlings were grown in 2 L pots filled with a sterile substrate mixture of peat moss and perlite (3:1 v/v). The substrate was autoclaved at 121°C for 30 minutes on two consecutive days to eliminate native microbial populations. Plants were maintained in a controlled-environment growth chamber set at 24°C during the day and 18°C at night, with a 12-hour photoperiod (light intensity: 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) and 60% relative humidity.

The trial included five experimental treatments:

1. Control (C): Untreated plants receiving only sterile water.
2. Commercial Biostimulant (CB): Plants treated with a certified microbial-based commercial biostimulant containing *Bacillus subtilis* and *Trichoderma harzianum*, applied at the manufacturer's recommended dosage.
3. Consortium A: Dominated by *Oceanibacillus* and *Lentibacillus* species, aimed at growth promotion.
4. Consortium B: Dominated by *Schouchella* and *Zygosaccharomyces* species, targeting pathogen suppression.
5. Consortium C: A balanced mix of all target genera (*Oceanibacillus*, *Schouchella*, *Lentibacillus*, and *Zygosaccharomyces*) for combined effects.

Each treatment group consisted of 12 replicate plants, divided equally into two subgroups: non-inoculated (healthy) and pathogen-challenged (infected with *Verticillium dahliae*). Plants were arranged in a randomized complete block design within the growth chamber.

Microbial Preparation and Application

Each microbial consortium was cultured individually under sterile conditions: bacteria in nutrient broth and yeasts in YPD medium, both incubated at 28°C for 48–72 h. Suspensions were adjusted to a concentration of 10^8 CFU/mL per strain and mixed prior to application. The commercial biostimulant was diluted and prepared according to the product label.

All microbial treatments were applied via root drenching (50 mL per plant) at the time of transplanting, followed by a second

application at 30 days. The control group received an equal volume of sterile distilled water.

Pathogen Challenge

To test biocontrol activity, six randomly selected plants per treatment group were inoculated with *Verticillium dahliae* (defoliating pathotype). The pathogen was cultured on potato dextrose agar, and conidial suspensions (10^7 spores/mL) were applied (25 mL per plant) to the root zone 10 days after transplanting. Non-inoculated plants served as healthy controls.

Growth and Physiological Measurements

At 90 days post-transplanting, the following parameters were recorded:

- Plant height (cm)
- Stem diameter (mm, 1 cm above soil surface)
- Leaf chlorophyll content (SPAD index, SPAD-502 meter)
- Shoot and root dry biomass (after drying at 65°C for 72 h)
- Root-to-shoot biomass ratio

Disease Severity and Pathogen Quantification

Plants in the pathogen-inoculated groups were visually assessed weekly for symptoms (chlorosis, wilting, vascular necrosis) using a 0–5 disease severity index (DSI). At harvest, root tissues were sampled from infected plants and subjected to DNA extraction using a CTAB method. Pathogen DNA levels were quantified via qPCR using *Verticillium dahliae*-specific primers (VD-F/VD-R). The relative pathogen load was calculated using the $2^{-\Delta\text{Ct}}$ method with normalization to olive actin gene expression.

Statistical Analysis

Data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Treatment effects were analyzed using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within healthy and pathogen-inoculated subgroups. Statistical analyses were conducted using R software (v4.3.1).

RESULTS

Vegetative Growth and Biomass Accumulation

Application of microbial consortia and the commercial biostimulant led to statistically significant improvements in olive sapling growth compared to the untreated control ($p < 0.001$). Plants treated with Consortium A, which contained growth-promoting *Oceanibacillus* and *Lentibacillus* strains, exhibited the most pronounced vegetative development (Figure 2). Average shoot height in this group reached 46.2 ± 2.1 cm, compared to 36.4 ± 1.7 cm in the control and 39.6 ± 2.0 cm in plants treated with the commercial biostimulant. Stem diameter was also significantly larger in Consortium A-treated plants (5.8 ± 0.2 mm) relative to the control (4.3 ± 0.2 mm) (Figures 1.1, 1.2).

Dry biomass data confirmed these observations. Plants inoculated with Consortium A showed the highest root biomass (6.8 ± 0.3 g), followed by those treated with Consortium C (6.2 ± 0.4 g), while control plants had significantly lower root dry



mass (4.3 ± 0.3 g). A similar trend was observed for shoot dry biomass, with Consortium A yielding the greatest accumulation (10.6 ± 0.6 g)—approximately 40% more than the control (7.6 ± 0.5 g). Although the commercial biostimulant produced a noticeable increase in shoot biomass (9.1 ± 0.5 g), its impact on root growth was less substantial (4.8 ± 0.3 g), suggesting a more limited belowground stimulatory effect (Figures 1.3, 1.4).

Consortium C, which integrated both growth-promoting and protective microorganisms, demonstrated a strong intermediate effect across most parameters. Consortium B, though less impactful on biomass accumulation, still significantly outperformed the untreated control ($p < 0.05$), highlighting its role in general plant vigor.

Leaf Chlorophyll Content and Biomass Allocation

Microbial treatments also had a marked effect on leaf chlorophyll content, measured via SPAD index. All treatments led to a statistically significant increase in chlorophyll levels compared to the control ($p < 0.01$). The highest chlorophyll content was observed in plants treated with Consortium A (SPAD 52.4 ± 1.3), followed closely by Consortium C (50.8 ± 1.6) and Consortium B (48.7 ± 1.4). These values were significantly greater than those in both the control (41.2 ± 1.8) and commercial biostimulant (44.6 ± 1.5) groups. These results suggest enhanced photosynthetic capacity and improved nitrogen assimilation in response to microbial inoculation (Figure 1.5).

The root-to-shoot biomass ratio, a useful indicator of carbon allocation and stress adaptability, was highest in plants treated with Consortium A (0.64) and Consortium C (0.59). These values reflect a more balanced and robust development of both root and shoot systems, compared to the control (0.56) and commercial biostimulant-treated plants (0.50), where allocation favored aboveground growth. A higher root-to-shoot ratio is advantageous under stress-prone conditions and is often associated with improved water and nutrient uptake efficiency (Figure 1.6).

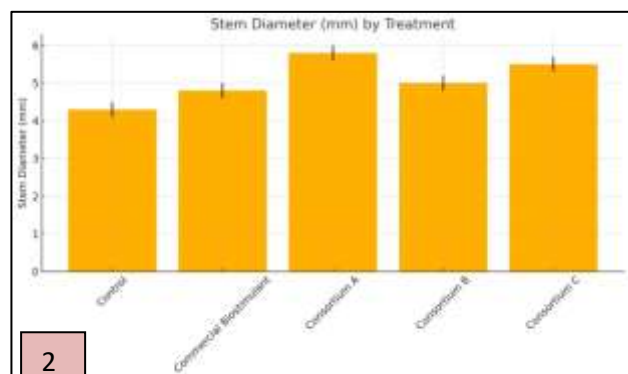
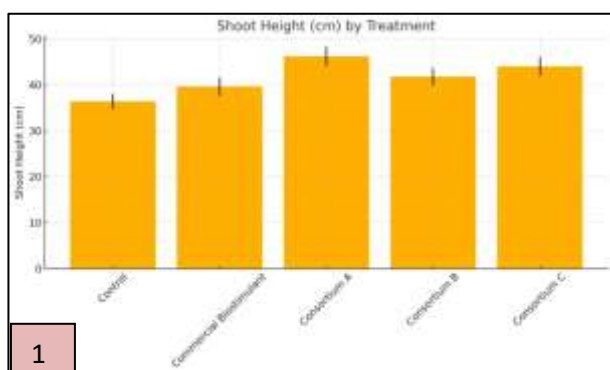
Disease Severity Following *Verticillium dahliae* Inoculation

Following artificial inoculation with *Verticillium dahliae*, typical wilt symptoms began to appear in control plants within 21 days. Symptoms included progressive leaf chlorosis, wilting, and internal vascular discoloration. By the end of the 90-day experimental period, the untreated control group exhibited a high disease severity index (DSI) of 4.3 ± 0.4 , indicative of advanced disease progression and considerable physiological stress.

All microbial treatments significantly reduced disease severity ($p < 0.001$), though to varying degrees. Consortium B, which included antagonistic *Schoucheilla* and *Zygosaccharomyces* strains, demonstrated the highest protective efficacy with a mean DSI of 1.8 ± 0.3 , representing only mild leaf curling and localized chlorosis. Consortium C also conferred strong protection (DSI 2.0 ± 0.5), while the commercial biostimulant and Consortium A resulted in more moderate disease reduction (DSI 2.7 ± 0.4 and 3.1 ± 0.6 , respectively) (Figure 1.7). These results indicate that microbial treatments not only limited symptom progression but also delayed the onset of visible disease.

Quantification of *Verticillium dahliae* in Root Tissue

Quantitative PCR (qPCR) analysis of root tissue corroborated visual assessments of disease severity. Relative *V. dahliae* DNA levels were significantly lower in all microbial treatment groups compared to the control ($p < 0.01$). The greatest suppression of pathogen colonization was achieved with Consortium B, which reduced fungal DNA concentration by approximately 72% (Figure 1.8). Consortium C followed closely with a 65% reduction, while the commercial biostimulant and Consortium A achieved 51% and 36% reductions, respectively. These molecular data confirm the biocontrol potential of the consortia, especially Consortium B, and highlight the capacity of specific microbial formulations to limit pathogen establishment in root tissues.



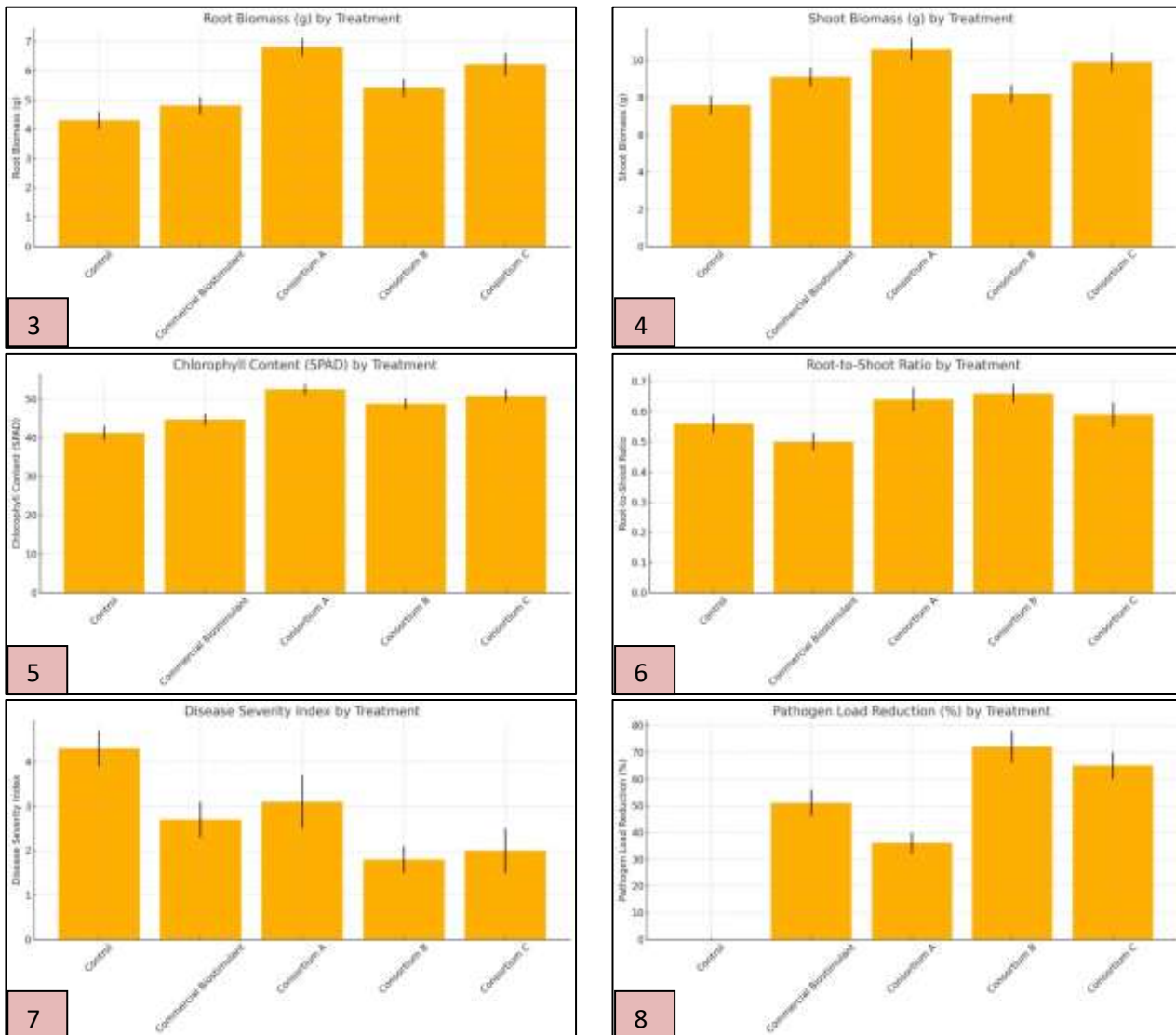


Figure 1 – These visualizations provide a clear comparative summary of treatment effects. 1. Shoot Height (cm); 2. Stem Diameter (mm); 3. Root Biomass (g); 4. Shoot Biomass (g); 5. Chlorophyll Content (SPAD); 6. Root-to-Shoot Ratio; 7. Disease Severity Index; 8. Pathogen Load Reduction (%)



Figure 2 – Comparison between the height and vegetative development obtained with the Consortium A treatment and the commercial biostimulant treatment.



DISCUSSION

This study demonstrates that microbial consortia composed of stress-tolerant bacteria (*Oceanibacillus*, *Schouchella*, *Lentibacillus*) and osmotolerant yeasts (*Zygosaccharomyces*) can significantly enhance the growth, physiological status, and disease resistance of young olive trees under controlled conditions. These results align with growing evidence supporting the use of microbial inoculants as key tools for improving crop performance in sustainable agricultural systems [20,21]. The observed improvements in biomass accumulation and chlorophyll content—particularly in plants treated with Consortium A—are consistent with the known plant growth-promoting mechanisms of *Bacillota* species, such as phytohormone production, phosphate solubilization, and enhanced nitrogen assimilation [22,23]. *Oceanibacillus* and *Lentibacillus* are halophilic and thermotolerant genera, commonly found in saline or degraded soils, and have been associated with improved root system architecture and plant resilience under osmotic stress [24,25]. The enhanced root-to-shoot ratio in Consortium A and C-treated plants supports this, suggesting better belowground investment and potential water-use efficiency. Interestingly, Consortium B, despite exerting only moderate effects on growth, showed the most pronounced biocontrol activity against *Verticillium dahliae*. This result is likely due to the presence of antagonistic *Schouchella* species and *Zygosaccharomyces* yeasts, which are known for their capacity to produce hydrolytic enzymes, volatile organic compounds (VOCs), and antifungal peptides [26,27]. Several studies have reported that yeast-based inoculants can suppress soilborne pathogens via competition and induction of systemic resistance, often without significantly altering plant morphology [28,29]. The combined effect observed in Consortium C, which performed well in both growth promotion and disease suppression, suggests that multi-strain formulations can leverage synergistic interactions between bacteria and yeasts. This supports previous findings that consortia with complementary functional traits often outperform single-strain inoculants, especially under biotic or abiotic stress conditions [30,31]. Quantitative PCR data confirmed that microbial treatments significantly reduced root colonization by *V. dahliae*, particularly in Consortium B and C groups. These findings reflect earlier work showing that biocontrol organisms can limit pathogen establishment through rhizosphere colonization and root immune priming [32,33]. The reduction in disease severity and pathogen DNA correlated well, validating both the visual assessments and molecular approach used in this study. While the commercial biostimulant also conferred some degree of growth stimulation and disease protection, its performance was consistently lower than that of the microbial consortia developed for this trial. This outcome may reflect limitations in the breadth or adaptability of commercial formulations, which often rely on a narrow range of microbial taxa [12]. In contrast, consortia composed of native, stress-adapted strains may offer broader functional compatibility with host plants, especially in challenging environments. Overall, these results underscore the potential of using custom-designed microbial consortia as biofertilizers and bioprotectants in olive cultivation. Their ability to simultaneously promote plant development and reduce pathogen impact makes them particularly valuable for

integrated and organic farming systems. Future work should focus on validating these effects under field conditions and over multiple growing seasons to assess persistence, scalability, and interactions with native soil microbiota.

CONCLUSIONS

This study clearly demonstrates that microbial consortia composed of stress-adapted bacterial and yeast strains can significantly enhance both vegetative growth and disease resistance in olive trees under controlled conditions. Among the treatments tested, Consortium A was the most effective in stimulating plant development, resulting in the highest shoot height, root biomass, and chlorophyll content. In contrast, Consortium B provided the strongest biocontrol activity against *Verticillium dahliae*, as evidenced by a substantial reduction in disease severity and pathogen load. Consortium C delivered a balanced effect, promoting both plant growth and pathogen suppression. While the commercial biostimulant yielded modest improvements in growth and partial disease mitigation, it was consistently outperformed by the tailored microbial consortia across most parameters. These results highlight the advantages of using carefully designed, multi-strain microbial formulations that combine complementary functional traits to enhance plant health and resilience. In conclusion, the use of microbial consortia represents a promising and sustainable strategy for improving olive tree performance and protecting against soilborne pathogens. Future research should focus on field-scale validation, assessment of long-term microbial persistence, and integration into diverse agricultural systems, including organic and regenerative farming practices.

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