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EFFECTS OF *Azotobacter* spp., MYCORRHIZAL FUNGI AND SHADE TREATMENTS ON PLANT GROWTH AND CHLOROPHYLL CONTENT IN BOXWOODS PLANTS

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ABSTRACT

In this study, two important commercial species, *Buxus microphilla* and *B. herlandii*, were evaluated. People do not want polluting and toxic fertilizers used to cultivate the plants they use for nutrition, and they do not want the plants they use as ornamental plants. The study investigated the effects of mycorrhizal and bacterial applications on plant growth and chlorophyll content. As a result of the study, it was concluded that bacterial applications on the development of boxwoods are more effective than mycorrhizal applications. Shading further increased the effect of the applications. In *B. microphilla*, in a 70% shade, plant height increased by 18.5% with mycorrhizal application, and plant width increased by 29.7% with bacterial application. In *B. herlandii*, bacterial application increased plant height by 13.3% and plant width by 20.4%. In shadowless application, the amount of chlorophyll in *B. herlandii* leaves was found to increase by 47.20% with bacteria and in *B. microphilla*, it increased by 65.86. In shadow application in *B. herlandii*, leaves were found to increase by 76.70% with bacteria; in *B. microphilla*, it increased by 94.93%. It was concluded that the bacteria application is more effective than others because *Azotobacters* fix the free nitrogen in the air to the boxwood soil, which needs continuous nitrogen for growth and development. For this reason, N-fixing bacteria applications to the soil can be used as an alternative to chemical fertilizer applications in boxwood cultivation or hedge formation.

Keywords: *Buxus microphilla*, *Buxus herlandii*, biofertilizer, mycorrhiza, bacteria

INTRODUCTION

Boxwoods are evergreen shrubs or tree-shaped plants. The areas where Buxus species are distributed are Europe (Mediterranean Basin) and the Middle East, China, Japan, Korea, Malaysia and the Philippines, Africa, the Caribbean Islands, Mexico and South America, India, the Northwest Himalayas and parts of the former Soviet Union [Köhler 2014]. In addition, boxwoods are very useful and attractive for gardens and landscape plants. In the by of hedges, they are used singly for mass plantings, potted plants, cut foliage, and topiary. In addition, its durable and flamboyant green-leaved branches are used during festivals [Sarı and Çelikel 2019]. More than 13 million boxwood plants are sold in the United States annually, with an annual value of over $141 million [USDA-NASS 2020]. According to a landscape survey by Nursery Management, boxwood is the number one plant purchased by consumers among all woody ornamental plants [Niemiera 2018]. Various studies have shown that nutrient-deficient conditions inhibit plant

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growth and nutrient accumulation [Xu et al. 2017]. Therefore, it is necessary to fertilize the plants to help obtain better quality products [Verma et al. 2021]. In addition, the nutritional requirements of different plants vary depending on their growth period and physiological and biochemical status [Li et al. 2019]. Therefore, scientifically determined fertilizer rates are required to increase fertilizer use efficiency [Yuan 2013]. Chemical fertilizers are used in ornamental plants with a wide usage area to meet the excess demand and increase the yield.

On the other hand, long-term use of chemical fertilizers causes an increase in harmful microorganisms in the soil, leading to an unbalanced distribution of nutrients [Younis et al. 2014, Verma et al. 2018]. New research is necessary for sufficient and sustainable yield and quality in modern ornamental plant cultivation. This need and research have led to the emergence and use of biostimulants or metabolic-enhancing products that do not contain non-polluting chemical fertilizers [Bulgari et al. 2015, Verma et al. 2018]. The role of these products is also to increase the absorption efficiency of nutrients provided by fertilization [Kashyap et al. 2017]. Biofertilizers, or microbes, are applied directly or indirectly to soil [Owen et al. 2015]. Biological methods can be of microbial, plant, or animal origin. Bacteria are one of the most studied microorganisms among these biological methods. Additionally, bacteria are extensively used in the commercial formulations of biological fertilizers, which have become widely used worldwide. In this context, biological control is recommended instead of chemical control, and organic and biological fertilization methods are recommended instead of chemical fertilizer [Gökçe and Kotan 2016]. It has been proven that there is a significant increase in plants' biomass due to using biofertilizers. While biofertilizers suppress harmful soil-borne pathogens, they also increase the number and effectiveness of other beneficial bacteria. In this case, a new microbial dynamic balance is established in the root zone of the plants against the issues that prevent crop growth [Siddiqui 2006]. These fertilizers have an application in agricultural forests and ornamental plants [Reedy 2014].

Mycorrhizal fungi that form mycorrhizas with plants absorb and provide the plant with low- or insufficient solubility nutrients, especially phosphorus, that the plant cannot benefit from [Demir and Onoğur 1999]. Having more mineral sources in the plant encourages growth. Mycorrhizal fungi are active in the root zone, positively affecting the plant [Yeh et al. 2021]. In addition, the use of mycorrhizal fungi instead of chemical fertilizers can potentially reduce the amount of chemical fertilizers used to provide plants with nutrients such as phosphorus, zinc, and copper, which are difficult to obtain from the soil [Yılmaz et al. 2009].

This study aimed to determine the effects of mycorrhizal preparation and bacteria (*Azotobacter* spp.) applications on two commercial boxwood species' plant growth and chlorophyll content. The research aims to contribute to boxwood cultivation by transferring the obtained results into practice.

MATERIAL AND METHODS

The research was conducted at the Black Sea Agricultural Research Institute (Samsun, Türkiye).

Plant materials and experimental design. *Buxus microphilla* and *B. herlandii* were used in the study. Plant materials were obtained from the Forest Nursery Directorate (Gökçebey, Zonguldak).

Four-year-old boxwoods (*B. microphilla* and *B. herlandii*) were used in the study (Fig. 1). Boxwoods were planted in 5 L pots with peat and perlite (3:1, v/v) and placed in a polyethylene greenhouse. The research was established according to a randomized plot design with fifteen replications. A single sapling

Fig. 1. *Buxus microphilla* (A) and *B. herlandii* (B) species used in the study

Table 1. Ingredients declared by commercial companies

Application	Trade name	Ingredient
Mycorrhiza	Endo Roots Soluble (ERS)	total living organism 1×10^4 w/w organism names: *Glomus intradices*, *G. aggregatum*, *G. mosseae*, *G. clarum*, *G. monosporum*, *G. deserticola*, *G. brasilianum*, *G. etunicatum*, *Gigaspora margarita*
Bacteria	Vitomorin Plus	total living organism 10^7 cfu/mL organism names: *Azotobacter chroococcum*, *A. vinelandii*

was used in each replication. Two different microbiological fertilizers, shading, and a control group were used for applications. Plants are divided into two groups. Bacteria (Vitomorin Plus) and mycorrhizal preparation (Endo Roots Soluble) treatments (Table 1) were applied to both groups, and 70% shading was applied to the second group using a shadow net. The application was made at the end of March, before the shoot yield of the plants. No application was made to the control group. In practice, the mycorrhizal preparation was weighed at 5 g and mixed with 1 L of water. A ready-made preparation was used for bacterial application. It is prepared with 5 mL to 1 L of water. Mycorrhizal preparation and bacteria were poured into each pot as 50 mL of liquid on the pot surface (Fig. 2). A subject that was not applied was included in the experiment as a control. The mycorrhizal preparations and bacteria used in the experiment were obtained from commercial companies, and the contents of the products are given in Table 1. While the average temperature inside the greenhouse was 25.6 °C in 2021, the average humidity was 68%. Plant height and plant width measurements were made before and after the application. After the first measurement (March 15) before the application, three measurements were made in 60-day periods.

Fig. 2. General view of the applied boxwood

Chlorophyll content. Before applying microbiological fertilizer to the boxwoods, chlorophyll content was measured separately in control plants and in the plants to be included in each application. Measurements were made on ten leaves from each plant. The relative chlorophyll contents in the leaf were measured using the SPAD-502 Chlorophyll Meter (Minolta Camera Co., Ltd., Japan). The top four rows of leaves were used to measure the SPAD index.

The first analysis determined Chlorophyl Concentration Index (CCI) for *B. herlandii* and *B. microphilla* as 25.93 and 41.10 CCI, respectively.

Data analysis. Analyses were performed using SPSS 20.0 statistical software. Differences between treatments were compared with the Duncan multiple comparison test (within 1% and 5% error limits).

RESULTS

Height and width of plants. In *B. microphilla*, the effects of applications (mycorrhizal preparation and bacteria) ($p < 0.01$), shade ($p < 0.05$) and application × shade interaction ($p < 0.01$) on all examined characteristics were found to be statistically significant. Although mycorrhizal application increased plant height in both 70% shade and shadowless, the highest result was obtained in 70% shade application. While mycorrhizal preparation showed an 18.5% improvement in plant height compared to the control in plants with 70% shade, this rate was 17.9% in the shadowless. Regarding plant width, bacteria application improved by 29.7% compared to the control in plants with 70% shade, while it was found to be 25.9% in the shadowless. According to the shade application averages, 8.3% higher plant height and 8.9% higher plant width were obtained in plants with 70% shade applica-

Table 2. Effect of bacteria and mycorrhizal preparation treatments on plant height and plant width in *Buxus microphilla*

Application	Plant height			Plant width		
	70% shade	shadowless	average	70% shade	shadowless	average
Control	33.6 c	31.9 b	32.8 c	45.5 c	44.8 c	45.2 b
Bacteria	36.7 b	32.4 b	34.6 b	59.0 a	56.4 a	57.7 a
Mycorrhiza	39.8 a	37.6 a	38.7 a	56.2 b	46.4 b	51.3 b
Average	36.7 a	33.9 b	–	53.6 a	49.2 b	–
Application		**	–	–	*	–
Shade		*		–	*	–
Application * shade interaction		**		–	*	–

Significance: ** $p < 0.01$, * $p < 0.05$, ns – no significance

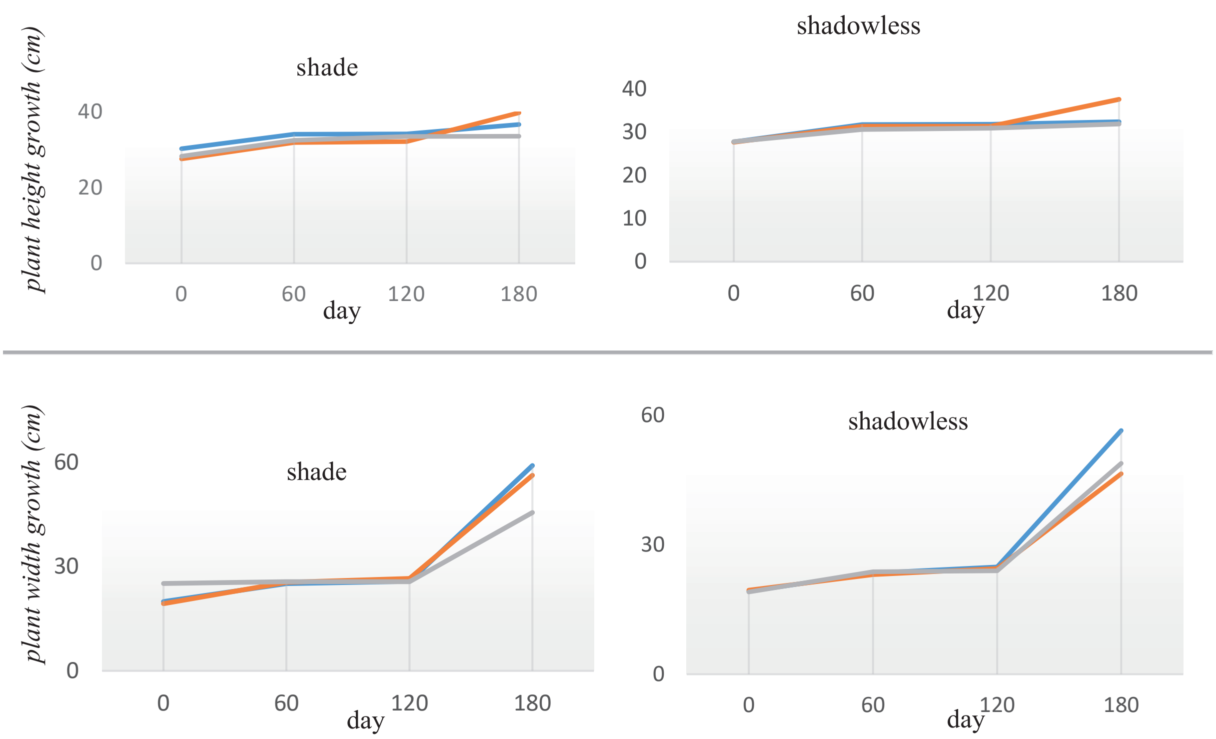

Fig. 3. The effect of applications on the developmental process in *Buxus microphilla*

tion compared to plants shadowless. Regarding application averages, it provided the highest improvement in plant height by 18.2%, while bacterial application provided the highest improvement in plant width by 27.8% compared to the control (Table 2, Fig. 3).

In *Buxus herlandii*, the effects of applications (mycorrhizal preparation and bacteria) ($p < 0.05$), shade ($p < 0.05$) and application × shade interaction ($p < 0.05$) on all examined characteristics were found to be statistically significant. While the best results were obtained from applying bacteria in 70% shade application on plant height, the effects of bacteria and mycorrhizal preparation application on plant height were similar in the environment where no shade was applied. Bacteria application on plant height provided a 13.3% improvement compared to the control in

Table 3. Effect of bacteria and mycorrhizal preparation treatments on plant height and plant width in *Buxus herlandii*

Application	Plant height			Plant width		
	70% shade	shadowless	average	70% shade	shadowless	average
Control	27.0 c	25.6 b	26.3 c	31.4 c	29.8 b	30.1 b
Bacteria	30.6 a	28.6 a	29.6 a	37.8 a	34.0 a	35.9 a
Mycorrhiza	28.2 b	28.2 a	28.2 b	35.2 b	28.2 c	51.7 b
Average	28.6 a	27.5 b	–	34.8 a	30.7 b	–
Application	*			–	**	–
Shade	*		–	–	*	–
Application * shade interaction	*			–	**	–

Significance: ** $p < 0.01$, * $p < 0.05$, ns – no significance

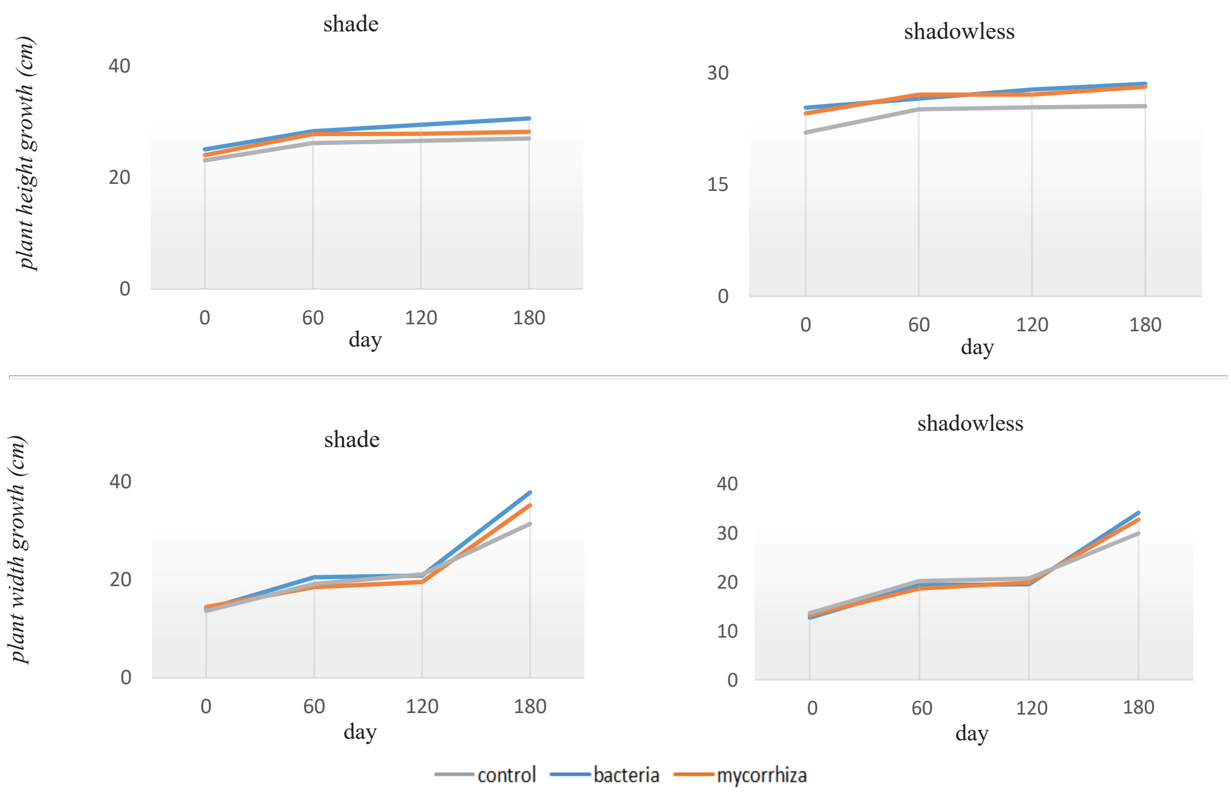

Fig. 4. The effect of applications on the developmental process in *Buxus microphilla*

plants with 70% shade, while this rate was found to be 11.7% in the shadowless environment. Regarding plant width, bacteria application improved by 20.4% compared to the control in plants with 70% shade, while it was found to be 14.1% in the shadowless. On average, plants with 70% shade application had a 4% higher plant height and a 13.4% higher plant width than plants shadowless. Regarding application averages, bacterial application provided the highest improvement in plant height by 12.5% compared to the control and the highest improvement in plant width by 19.3% (Table 3, Fig. 4).

Table 4. The effect of bacteria and mycorrhizal applications on the amount of chlorophyll

Application	*Buxus herlandii*		*Buxus microphilla*		Average
	shadowless	shadow	shadowless	shadow	
	Chlorophyll Concentration Index				
Control	27.29 c	32.15 c	45.29 c	55.19 c	39.98 c
Bacteria	38.17 a	45.82 a	68.17 a	80.12 a	58.07 a
Mycorrhiza	33.04 b	40.14 b	50.04 c	62.40 b	46.41 b
Average	32.83 d	39.37 c	54.50 b	65.90 a	–
Application	**		**		
Shade	**		**		
Application * shade int.	**		**		

Significance ***p* < 0.01, **p* < 0.05, ns – no significance

Chlorophyll contents. In both species, the effects of applications (mycorrhizal preparation and bacteria), shade and application × shade interaction ($p < 0.01$) on the amount of chlorophyll were found to be statistically significant. The amount of chlorophyll in *Buxus herlandii* leaves increased by 5.24% with the control, whereas it increased by 47.20% with bacteria and by 27.41% in mycorrhized plants (Table 4). In *B. microphilla* leaves, it increased by 10.19% with control application, whereas it increased by 65.86% with bacteria and by 21.75% in mycorrhized plants (Table 4).

In shade application, the amount of chlorophyll in *B. herlandii* leaves increased by 23.98% in control plants, whereas it increased by 76.70% with bacteria and by 54.80% in mycorrhizal application (Table 4). In *B. microphilla* leaves, it increased by 34.28% with control, whereas it increased by 94.93% with bacteria and by 51.82% in mycorrhized plants (Table 4).

DISCUSSION

The effect of applications on growth. Mycorrhizal, bacterial, and shade treatments in boxwoods significantly affect plant height and width. Boxwoods show continuous growth under suitable conditions. Therefore, there is a need for balanced nutrition, especially nitrogen. Previous studies have shown that soil microbes can help plants adapt to nutrient stress and improve the absorption and utilization of soil nutrients [Sun 2017, Hijri and Bâ 2018]. According to this study, the best plant height and width results were obtained from the bacterial application.

In contrast, mycorrhizal application at plant height gave a better result only in *B. microphilla*. This effect of bacterial application can be explained as follows: *Azotobacters* are non-symbiotic nitrogen-fixing bacteria that live freely in the soil and fix nitrogen from the air. Mycorrhizal fungi are normal root symbiotic inhabitants that assist plants primarily in the uptake of water and mineral nutrients. This information and the results of our application reveal a difference between these two applications in terms of the mechanism of the action. As a result, bacterial application is thought to provide nitrogen to the plant by using the free nitrogen from the air, apart from the nutrients in the plant soil.

Contrary to this, mycorrhizal application only improves root activities and provides nutrients to the plant in the soil. It has been determined that bacteria provide better and more sustainable growth and development as they can provide extra nutrients other than those in the environment. In the application of mycorrhizal, lower growth and development occurred because they only helped provide the nutrients in the environment to the plant, and the nutrients in the environment are more limited. Bacteria contribute to the nitrogen cycle due to the nitrogen they attach to the soil. In addition to fixing nitrogen, *Azobacter chroococcum* produces phytohormones, siderophores, antifungal agents, and other beneficial functions [Lakshminarayana 1993]. It has been determined that various *Azotobacter* species improve the nitrogen, phosphorus, and potassium content of the soil, provide biomass increase in various agricultural, industrial, and forest plants and different parts of the plants, and are especially effective in the increase of antioxidant enzymes, carotenoids, chlorophyll pigments, soluble protein, and dry matter in plants [Mrkovački et al. 1997]. Mycorrhizal fungi, on the other hand, can provide the plant with the nutrients found in the environment and needed by the plant. Previous studies have shown that mycorrhizal fungi can help plants adapt to nutrient stress and improve the absorption and utilization of soil nutrients [Sun 2017, Hijri and Bâ 2018]. *Acinetobacter calcoaceticus* bacteria increased the seedling height by 32.9% in *Dahlia variabilis* 'Violet' cv. [Alkaç et al. 2022]. It was determined that *Azotobacter* increased the seedling height by 37.17% compared to the control [Asif et al. 2013]. Again, different biofertilizers containing bacteria and their doses applied to *Cupressus arizonica* and *Acer saccharum* seedlings increased the seedling height by 11–13%, respectively, compared to the control [Özgün 2020]. The study's results support previous researchers' findings that biofertilizers consisting of beneficial microorganisms increase plant properties. However, it has been determined that the result varies according to the desired result, dose, and plant type, both in this study and in the researchers' studies. On the other hand, it was concluded that applying bacteria to boxwood is more beneficial than applying mycorrhizal.

Shade application had a significant effect on plant height and width. 70% shade application significantly increased plant height and width compared to control. In Abkenar and Abdinejad's [2011] research, where the effects of different light intensities were tested on *Buxus sempervirens*, low, medium, high, and very high light intensities were applied. The effect of light intensity on stem quality, branching, canopy symmetry, plant height, and sapling stem diameter values was investigated. The best results in all measured properties were obtained from the low-light-intensity application [Abkenar and Abdinejad 2011]. It has been reported that while planting depth is not effective on stem elongation in tulips, and shading is effective, stem length varies between 28.4 and 32.3 cm, stem lengthens as the shading rate increases, and the difference between applications is significant [Cavins and Dole 2002]. Again, a study on the *Begonia rex* flower found that decreased light intensity increased the effect of apical dominance [Srivastava 2002]. The results obtained by the researchers and the findings of this study overlap.

After the bacteria and mycorrhizal applications, it was determined that there was an improvement until the first 60th day. At the same time, the growth remained stable between the 60th and 120th days, and there was a re-development between 120 and 180 days. When the applications and plant development periods were evaluated, it was determined that control, bacteria, and mycorrhizal treatments had similar but different effects throughout the development process. For this reason, it has been found that the application does not cause the growth periods occurring during these periods but is related to the plant's development physiology.

Change in the amount of chlorophyll. According to the results, fertilizer-containing bacteria was the most effective application, and the improvement in plant leaves could also be detected visually. Thus, a significant improvement in plant appearance occurred. Although there was an increase in chlorophyll in all other applications, it was also revealed that the bacteria application gave a better result in the shaded. Clark and Zheng [2015] studied 'Gro-Low' scented sumac (*Rhus aromatika* Aiton), (*Spiraea* × *bumalda* Burv.) and 'Bloomerang' purple lilac (*Syringa* × 'Penda') for spirea and 'Goldmound' spirea. It has been reported to correspond to visual nutrient deficiency in plants with leaf CCI values ≤ 7.6 and ≤ 17.8, respectively. In addition, 'Bloomerang' purple lilac was found to show visual nutrient deficiencies with CCI values ≤ 16.0, suggesting N deficiency. The efficiency of mineral nutrients is vital in synthesizing chlorophyll and carotenoids. It has been reported that N is the most essential element in chlorophyll biosynthesis [Waraich et al. 2015] and that N fertilization increases the formation of active photosynthetic pigments in leaves [Cooke et al. 2005, Razaq et al. 2017]. In addition, findings show that P applications increase the total chlorophyll content and plant growth in many woody plants and decrease the protein and chlorophyll content in plants with P deficiency [Dutt et al. 2013, Razaq et al. 2017]. These results are exceptionally high in boxwood, which is evergreen and shows continuous growth under suitable conditions, compared to other nutrients. If there is not enough N in the soil, this can only cause a certain amount of chlorophyll to increase.

As a result, mycorrhizal application provided the plant with available nitrogen and other nutrients from the soil. However, the increase in chlorophyll remained low compared to the bacterial application since there were no more nutrients. In the bacterial application, since Azotobacter can fix the nitrogen in the atmosphere to the soil, even if the amount of N available in the soil decreases, they can supplement it and thus increase the amount of chlorophyll more than in other applications. It has been reported that bacteria contribute significantly to the nitrogen cycle in nature due to the nitrogen they attach to the soil [Sun 2017, Hijri and Bâ 2018]. In addition, *Azotobacter* species were found to increase the biomass in different parts of the plants by improving the phosphorus and potassium values of the soil, as well as the nitrogen values of the soil. They were especially effective in increasing antioxidant enzymes, carotenoids, chlorophyll pigments, soluble proteins, and dry matter in plants [Lakshminarayana 1993, Mrkovački et al. 1997].

CONCLUSIONS

This study evaluated two commercial products used as microbiological fertilizers in cultivating two essential species of boxwood (*B. microphilla* and *B. herlandii*) worldwide. As a result of the study, it was concluded that bacterial applications were more effective than mycorrhizal products in the development of boxwoods. Shadowing further increased the effectiveness of the application of microbiological fertilizers. It was concluded that bacterial application was more effective than other mycorrhizal products because *Azotobacter* fixed free nitrogen in the air into the soil of boxwoods, which need constant nitrogen for growth and development. For this reason, soil N-fixing bacterial applications have the potential to be used as an alternative to chemical fertilizer applications in boxwood cultivation.

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