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EFFECTS OF BENEFICIAL MICROORGANISMS AND GA_3 APPLICATIONS ON GERMINATION PARAMETERS DURING SEED GERMINATION PROCESSES OF SOME WALNUT (*Juglans regia* L.) CULTIVARS AND GENOTYPE

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Abstract: In this study, the effects of beneficial microorganisms and gibberellic acid (GA_3) applications on the germination parameters of seeds belonging to the Kaman 1 and Chandler walnut varieties and the Kaman 5 walnut genotype were investigated. The applications included the use of beneficial microorganisms (B Musa Vita®, B Musa Green®, Arbuscular Mycorrhiza-ERS®, and T22 (Trichoderma harzianum Rifai KRL-AG2®)) as well as 500 and 750 ppm GA_3 doses. The effects on germination parameters such as germination rate, main root length, stem diameter, shoot thickness, root quality, and root shape index were evaluated. The seeds were soaked in water for 24 hours before the application of organic and inorganic stimulants and then surface sterilized with a 5% Captan-based fungicide. After application, they were placed in pots containing perlite and vermiculite at a 1:1 ratio. All applications were maintained at +4°C and 75% relative humidity for 10 weeks. As a result of the applications, the highest germination rates were observed in the Kaman 1 variety and Kaman 5 genotype, while the lowest germination rate was determined in the Chandler variety (P<0.01). In terms of variety/genotype, the best results for main root length, shoot thickness, shoot dry weight, stem diameter, root fibrousness degree, root shape index, and root distribution homogeneity were obtained from the Kaman 1 walnut variety and the Kaman 5 walnut genotype. The best results in terms of shoot length and fresh shoot weight were observed in the Kaman 1 walnut variety (P<0.01). As a result, the highest germination rates in the Kaman 1 walnut variety were obtained from the control, T-22, Arbuscular Mycorrhiza-ERS®, B Musa Vita®, and B Musa Green® treatments, while in the Kaman 5 genotype, the highest germination rate was observed in the B Musa Vita® treatment.In the Chandler variety, the highest germination rate was obtained from the Arbuscular Mycorrhiza-ERS® treatment.

Keywords: Kaman 1, Kaman 5, Chandler, GA_3, PGPR, Seed germination

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1. Introduction

Seed dormancy and irregular germination often cause problems in breeding programs and, in some cases, result in loss of viability in hybrids (Raufi et al., 2017). In walnuts, low seed germination has been associated with physiological dormancy controlled by the seed coat and embryo dormancy (Matilla and Matilla, 2008). The use of certain beneficial microorganisms known as endophytic symbionts has produced positive results in improving seed germination in some species (Vujanovic et al., 2000). Moreover, many studies have reported that these beneficial microorganisms can protect plants to some extent from environmental stress factors (Waller et al., 2005) and can alleviate soil-borne problems commonly encountered during seed germination.

The need to preserve the ecological environment and ensure sustainable development has made research on new biological fertilizers increasingly important. Bioinoculants produced through the artificial cultivation of beneficial microorganisms play vital roles in improving soil nutrient composition and fertility, promoting plant growth, and enhancing plant resistance to diseases (Li, 2002; Wang et al., 2010).

Establishing walnut orchards is often challenging because the seedlings possess coarse root architecture, and only a small proportion survive until planting. Planting success largely depends on root system morphology and the nutritional status of seedlings; thus, rhizosphere conditions are critical for plant performance (Mortier et al., 2020). *Trichoderma* spp. are among the most common free-living saprophytic fungi in the rhizosphere and constitute a major group of fungal biocontrol agents in the biopesticide industry (Woo et al., 2014). *Trichoderma* spp. not only promote plant growth and development but also exhibit broad-spectrum antagonistic activities against various soil-borne phytopathogens (Singh et al., 2013; Keswani et al., 2014). For these reasons, many *Trichoderma* species are widely used for seed treatments (Pill et al., 2009; Singh et al., 2016). Numerous bacterial species have been identified as plant growth-promoting rhizobacteria (PGPR), with *Pseudomonas* spp. and *Bacillus* spp. being the most dominant genera (Beneduzi et al., 2012).

Members of the Bacillus genus offer advantages over other microorganisms because they are ubiquitous, tolerant to variable pH, temperature, and osmotic conditions (Nicholson et al., 2000), and can colonize roots and form biofilms that establish strong interactions (Allard-Massicotte et al., 2016).

Cai et al. (2005) reported that B. subtilis can increase the synthesis of indole acetic acid (IAA) and other auxins and reduce plant hormones that inhibit plant growth (e.g., abscisic acid), thereby stimulating root development and plant growth. Although many studies have been conducted on the effects of microorganisms on yield and quality in various fruit species, there are only a limited number of studies on biological control in walnuts that cover seedling development, yield, and quality effects (Liu et al., 2014; Shakeel and Hassan 2018). Improving germination parameters in walnut seeds to achieve graftable seedlings in a shorter time has been evaluated in many studies.

In particular, different growth media (Vahdati and Hoseini, 2004; Egharevba et al., 2005; Raoufi et al., 2020), various chemicals (Parvin et al., 2015), and different treatments (Egharevba et al., 2005; Parvin et al., 2015; Lamichhane et al., 2021) have been used. Raoufi et al. (2020) evaluated the effects of different pre-treatments and growing media on the germination and early growth of walnut seeds and reported that germination rates in perlite ranged from 0% to 94.1%. Gibberellins (GAs) are stimulators for the production of hydrolases, particularly α-amylase, which aid in seed germination (Yamaguchi, 2008). Lamichhane et al. (2021) reported that the best germination performance of walnut seeds was obtained with a pre-sowing treatment using 750 ppm GA_3, followed by cold stratification and cracking.

However, studies examining the effectiveness of beneficial microorganisms on the germination processes of walnut seeds remain limited. Therefore, in this study, the effects of commercial preparations B Musa Vita® (*Bacillus megaterium RC3D, Bacillus subtilis RC521, Pseudomonas fluorescens RC512*), B Musa Green® (B*acillus megaterium RC07, Pantoea agglomerans RC58, Pseudomonas fluorescens RC77*), Arbuscular Mycorrhiza-ERS®, and T22 (*Trichoderma harzianum Rifai KRL-AG2*®) as well as different doses of GA_3 on the germination parameters of the Kaman 1 and Chandler walnut varieties and the Kaman 5 walnut genotype were investigated.

2. Materials and Methods

The research material consisted of seeds belonging to the Kaman 1, Chandler walnut varieties and Kaman 5 walnut genoytpe. The walnuts, harvested during the 2021 season in September, were stored in a cool and shaded environment. Afterwards, the seeds were surface-sterilized with a 5% Captan-based fungicide for 30 minutes, rinsed with sterile water, and soaked in water for 24 hours. Subsequently, organic and inorganic stimulants were applied to the seeds.

The experiment included seven different treatments: control, 500 ppm GA_3, 750 ppm GA_3, T22 (Trichoderma harzianum Rifai KRL-AG2®), Arbuscular Mycorrhiza-ERS®, B Musa Vita®, and B Musa Green®. Each treatment consisted of three replications, with ten seeds per replication. GA_3 treatments were performed by soaking the seeds in 500 ppm and 750 ppm GA_3 solutions for 24 hours. Beneficial microorganism applications B Musa Vita® (*Bacillus megaterium RC3D, Bacillus subtilis RC521, Pseudomonas fluorescens RC512*), B Musa Green® (*Bacillus megaterium RC07, Pantoea agglomerans RC58, Pseudomonas fluorescens RC77*), Arbuscular Mycorrhiza-ERS®, and T22 (*Trichoderma harzianum Rifai KRL-AG2®)* were carried out by soaking the seeds in prepared solutions for 1 hour.

The seeds subjected to these applications were transferred to pots containing a 1:1 mixture of perlite and vermiculite and stored in a cold chamber at +4°C. The experiment was conducted in a completely randomized factorial design, and the pots were regularly moistened. After 10 weeks of stratification under these conditions, the seeds were removed from the pots, washed with water, and prepared for measurements. At the end of this process, parameters such as main root length, root collar thickness, shoot length, shoot thickness, stem diameter, fresh and dry root weight, fresh and dry shoot weight, degree of root fibrousness**,** and root shape index were determined for the germinated seeds. Root fibrousness and root shape index were scored visually by one examiner. Root fibrousness was scored from 0 to 5, and root shape index was scored from 0 to 2.

2.1. Statistical Analysis

The obtained data were statistically analyzed using the Minitab 17.0 software, and differences among means were evaluated with Tukey's multiple comparison test (Genç and Soysal, 2018).

3. Results and Discussion

In this study, the effects of Arbuscular Mycorrhiza-ERS®, T22 (*Trichoderma harzianum Rifai KRL-AG2*®), B Musa Vita®, B Musa Green®, and different GA_3 doses on seed germination parameters were evaluated in Kaman 1, Chandler, and Kaman 5 walnut varieties/genotype (Table 1). The highest germination rate in the Kaman 1 walnut variety was observed in the control group, followed by the T22, Arbuscular Mycorrhiza-ERS®, B Musa Vita®, and B Musa Green® treatments (Figure1). The lowest germination rates were recorded in the 500 ppm and 750 ppm GA_3 treatments. In the Kaman 5 walnut genotype, the highest germination rate was observed in the B Musa Vita® treatment, whereas the lowest was recorded in the Arbuscular Mycorrhiza-ERS® treatment. For the Chandler walnut variety, the highest germination rate occurred in the Arbuscular Mycorrhiza-ERS® treatment, while the lowest germination rates were determined in the B Musa Vita®, B Musa Green®, and 750 ppm GA_3 treatments.

Among all varieties/genotypes, the highest germination rates were obtained in the Kaman 1 variety and Kaman 5 genotype, whereas the Chandler walnut variety exhibited the lowest germination rate.

Table 1. Effect of treatments on seed germination rates of walnut varieties/genotype

Cultivar	Control	T22	Arbuscular Mycorrhiza-ERS®	B Musa Vita®	B Musa Green®	GA_3 500	GA_3 750	Mean
Chandler	35.45^{b}	35.45^{b}	47.24^{a}	21.37^{d}	26.86^{d}	31.65^{c}	24.62^{d}	31.66
Kaman 1	75.48^{a}	61.34^{b}	59.34^{b}	59.34^{b}	47.23^{b}	45.50^{c}	41.34^{c}	55.65
Kaman 5	62.02^{c}	54.93^{d}	47.24^{e}	72.36^{a}	53.12^{d}	69.62^{b}	66.37^{b}	60.81
Mean	57.65	50.57	51.27	51.02	42.40	48.92	44.11	

The differences between the means indicated by different letters in the same column were found to be highly significant (P<0.01).

Table 2. Effects of treatments on main root length, dry root weight, and root fibrousness degree (mm)

Treatments	N	Main Root Length	Dry Root Weight	Root Fibrousness Degree
T- 22	25	117.3 ± 61.1 ^{bc}	1.189 ± 0.818 ^{ab}	1.640 ± 1.075 ^{b}
Arbuscular Mycorrhiza-ERS®.	26	166.8 ± 54.8 ^{ab}	1.148 ± 0.537 ^{b}	3.000 ±1.494 ^{ab}
B Musa Vita®	22	188.1 ± 83.6 ^{a}	1.679 ± 0.757 ^{a}	3.364 ± 1.649 ^{a}
B Musa Green®	21	148.4 ± 52.8 ^{abc}	1.2101 ± 0.4059 ^{ab}	2.550 ± 1.356 ^{abc}
GA_3 500ppm	24	115.6 ± 66.9 ^{c}	0.9352 ± 0.4427 ^{b}	1.958 ± 0.859 ^{bc}
GA_3 750ppm	23	106.47 ± 45.80 ^{c}	0.8003 ± 0.4120 ^{b}	2.348± 1.229 ^{abc}
Control	27	173.0 ± 63.8 ^{a}	1.182 ± 0.569 ^{ab}	2.704± 1.409 ^{abc}

The differences between the means indicated by different letters in the same column were found to be highly significant (P<0.01).

Figure 1. Kaman 1 walnut variety control group.

In the study, the B Musa Vita® treatment yielded the best results in terms of main root length, dry root weight, and root fibrousness degree (Table 2).

The best results for shoot length and fresh shoot weight were obtained from the 750 ppm GA_3 and 500 ppm GA_3 treatments. The highest shoot dry weight was observed in the 750 ppm GA_3 and B Musa Vita® treatments (Table 3). In terms of variety/genotype, the highest values for main root length, shoot thickness, shoot dry weight, stem diameter, root fibrousness degree, root shape index, and root distribution homogeneity were determined in the Kaman 1 walnut variety and Kaman 5 walnut genotype (Table 4). The best results for shoot length and fresh shoot weight were obtained from the Kaman 1 walnut variety, while the highest dry root weight was recorded in the Kaman 5 walnut genotype (Table 5).

Table 3. Effects of treatments on shoot length, fresh shoot weight, and shoot dry weight (mm)

Treatment	N	Shoot Length	Fresh Shoot Weight	Dry Shoot Weight
T- 22	30	92.87 ±30.06 ^{b}	3.187 ±1.357	0.8939± 0.4751^{ab}
Arbuscular Mycorrhiza-ERS®.	29	87.45 ± 29.84 ^{b}	2.808± 0.960	0.7863 ± 0.3410^{ab}
B Musa Vita®	24	88.51 ±25.03 ^{b}	3.507 ±1.292	1.0405 ± 0.4449^{a}
B Musa Green®	26	77.56 ± 36.36 ^{b}	2.293 ±0.892	0.6577± 0.2907^{b}
GA_3 500ppm	28	136.4 ± 60.6 ^{a}	4.640 ± 2.174	0.9478 ±0.4791^{ab}
GA_3 750ppm	25	150.1± 81.6 ^{a}	4.608 ±2.308	1.053 ± 0.548^{a}
Control	30	91.34 ±36.25 ^{b}	3.079 ±1.269	0.8781± 0.4135^{ab}

The differences between the means indicated by different letters in the same column were found to be highly significant (P<0.01).

Table 4. Effects of treatments on main root length, dry root weight, root fibrousness degree, root shape ındex, and root distribution homogeneity in chandler and kaman 1 walnut varieties and the Kaman 5 walnut genotype (mm)

Variety /Genotype	N	Main Root Length	Root Fibrousness Degree	Root Shape Index	Root Distribution Homogeneity	Dry Root Weight
Chandler	29	119.5 ±68.8[b]	1.379±1.015[b]	0.9286±0.2623[b]	0.8409±0.3700[b]	0.6486±0.4306[c]
Kaman 1	69	141.64±56.45[ab]	2.507±1.093[a]	1.0290±0.1690[a]	1.0290±0.419[a]	1.1252±0.4224[b]
Kaman 5	70	159.69±74.85[a]	2.971±1.560[a]	1.000±0.000[ab]	1.000 ±0.000[a]	1.4033±0.7254[a]

The differences between the means indicated by different letters in the same column were found to be highly significant (P<0.01).

Table 5. Effects of Chandler and Kaman 1 walnut varieties and the Kaman 5 walnut genotype on main shoot length, shoot thickness, fresh shoot weight, dry shoot weight, and stem diameter (mm)

Variety /Genoytpe	N	Shoot Length	Shoot Thickness	Fresh Shoot Weight	Dry Shoot Weight	Stem Diameter
Chandler	52	84.04 ±26.91[b]	4.305±1.894[b]	1.987± 0.985[c]	0.4838±0.3049[b]	9.258±2.642[b]
Kaman 1	70	134.32 ±67.27[a]	4.945±1.013[ab]	4.273±1.622[a]	1.1086±0.3940[a]	12.828±1.985[a]
Kaman 5	70	85.73±29.72[b]	5.474±1.634[a]	3.655±1.585[b]	0.9735±0.3802[a]	13.764±3.090[a]

The differences between the means indicated by different letters in the same column were found to be highly significant (P<0.01).

Figure 2. Kaman 5 walnut genotype control group.

Figure 3. Chandler walnut variety control group.

Figure 4. The highest shoot length and fresh shoot weight values were observed in Kaman 1 walnut variety.

Figure 5. The effect of B Musa Vita® treatment applied to seeds on main root length and root fibrousness degree in Kaman 5 walnut genotype.

When evaluated in terms of treatments, no statistically significant differences were found in fresh root weight and root collar thickness among the Chandler and Kaman 1 varieties and the Kaman 5 genotype (P>0.05). Rostamikia et al. (2016) applied different bacterial strains (*Pseudomonas putida* 168, *Bacillus subtilis* FzB24, and *Enterobacter cloacae* 12) to hazelnut (*Corylus avellana*) seeds and observed variable but generally positive effects on germination percentage, root, and shoot development. They also reported that the germination percentage varied between 1.66% and 65.40%, and that both cold stratification and inoculation had a positive influence on germination rate.

Similarly, Fatemeh et al. (2014) reported that growth-promoting rhizobacterial combinations had significant effects on the germination percentage, rate, and duration of seeds of a hawthorn species, Crataegus pseudoheterophylla Pojark. Çoban (2023) investigated the effects of GA_3 and acetylsalicylic acid applications on the germination and seedling development of different walnut varieties and genotypes and reported that the highest germination rate was observed in the Bitlis genotype (60%), while the lowest was found in the Chandler variety (26%). In line with these findings, in the present study, the highest germination rates were obtained from the Kaman 1 walnut variety and Kaman 5 genotype (Figure 2), while the lowest was determined in the Chandler variety (Figure 3). In the Kaman 1 walnut variety, the highest germination rate was observed in the control group, whereas the lowest rates were found in the GA_3 500 ppm and 750 ppm treatments.

In terms of shoot length, the best results were obtained from the Kaman 1 walnut variety (Figure 4), while the main root length values were observed in both the Kaman 1 walnut variety and the Kaman 5 genotype. When evaluated in terms of treatments, the most effective application for main root length was B Musa Vita®.

Liu et al. (2022) reported that inoculation with *Bacillus subtilis* promoted root elongation by increasing the number of root fibrousness in *Bletilla striata* seedlings, a perennial species of the Orchidaceae family. Wang et al. (2010) investigated the effects of *Bacillus subtilis* on the physiological and biochemical indices of winter wheat and reported that the bacterium had no significant effect on plant height or root length, which could be attributed to functional differences among *Bacillus subtilis* strains. In the present study, the *Bacillus subtilis*-containing B Musa Vita® treatment was found to be the most effective in terms of main root length, dry root weight, and root fibrousness degree (Figure 5).

Noumavo et al. (2013) reported that the combination of *Pseudomonas fluorescens* and *Pseudomonas putida* had positive effects on germination rate and biomass in maize seeds Miljaković et al. (2022) reported that *Bacillus megaterium* significantly increased the germination rate, root length, and shoot dry weight of soybeans. Similarly, in this study, the highest germination rate in the Kaman 5 genotype was obtained from the B Musa Vita® treatment, which contains *Bacillus megaterium.*

Parvin et al. (2015) investigated the effects of gibberellic acid (GA_3) and cold stratification on the germination rate of Juglans nigra L. seeds and reported that the combined application of cold stratification and GA_3 enhanced both the germination percentage and growth parameters. In the present study, the best results for shoot dry weight were obtained from the GA_3 750 ppm and B Musa Vita® treatments.

Yılmaz (2019) reported that inoculation of four different walnut seed sources (Fernor, Chandler, Kaman 1, and Franquette) with mycorrhizae containing *Glomus* species had significant effects on several growth parameters except shoot length, main root length, and fresh root biomass and that it increased root fibrousness Similarly, in this study, the B Musa Vita® treatment had significant effects on main root length, dry root weight, and root fibrousness degree (P<0.01).

According to the variety/genotype evaluation, the Kaman 1 walnut variety and Kaman 5 genotype showed the highest values for main root length, shoot thickness, shoot dry weight, stem diameter, root fibrousness degree, root shape index, and root distribution uniformity. The highest shoot length and fresh shoot weight values were observed in the Kaman 1 walnut variety (P<0.01). The best result for dry root weight was recorded in the Kaman 5 genotype, while no statistically significant differences were found among varieties for fresh root weight and root collar diameter (P>0.05).

4. Conclusion

As a result, the highest germination rates in the Kaman 1 walnut variety were obtained from the control, T-22, *Arbuscular Mycorrhiza*-ERS®, B Musa Vita®, and B Musa Green® treatments, while in the Kaman 5 genotype, the highest germination rate was observed in the B Musa Vita® treatment. In the Chandler variety, the highest germination rate was obtained from the Arbuscular Mycorrhiza-ERS® treatment. Among the varieties, the highest germination rates were found in the Kaman 1 variety and Kaman 5 genotype, while the lowest was observed in the Chandler variety.

No statistically significant differences (P>0.05) were found among treatments for shoot thickness, stem diameter, fresh root weight, root shape index, root distribution uniformity, or root collar thickness. The best treatment for main root length, dry root weight, and root fibrousness degree was B Musa Vita®. The most effective treatments for shoot length and fresh shoot weight were GA_3 750 ppm and GA_3 500 ppm. For shoot dry weight, the best results were obtained from the GA_3 750 ppm and B Musa Vita® treatments, while the lowest values were observed in the B Musa Green® treatment. The differences among treatments were found to be highly significant (P<0.01).

In general, the Kaman 1 walnut variety and Kaman 5 genotype gave the best results for main root length, shoot thickness, shoot dry weight, stem diameter, root fibrousness degree, root shape index, and root distribution uniformity. The highest shoot length and fresh shoot weight values were observed in the Kaman 1 walnut variety (P<0.01).

The best dry root weight was obtained in the Kaman 5 genotype, while differences among varieties for fresh root weight and root collar thickness were not significant (P>0.05).

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	E.Ş.	Y.E.
C	20	80
D	40	60
S	20	80
DCP	80	20
DAI	80	20
L	70	30
W	70	30
CR	50	50
SR	70	30
PM	40	60
FA	70	30

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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