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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Exploring the Potential of *Bacillus amyloliquefaciens* N33 in Biocontrol [*]

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Abstract: The use of biocontrol agents instead of synthetic chemicals in the control of plant diseases and post-harvest preservation of fruits, vegetables and cereals has gained great momentum given their numerous advantages. In the present study, the antifungal activity of *Bacillus amyloliquefaciens* N33 strain isolated from lettuce was tested *in vitro* against *Penicillium citrinum, P. expansum, P. verrucosum, P. digitatum, P. paneum, Fusarium graminearum* and *Aspergillus fumigatus.* The strain showed a high activity against all tested fungi and the inhibition rate ranged between 60.3-94.2%. The highest activity was observed against *P. digitatum* (94.2%) and *P. expansum* (82.5%), which caused significant postharvest losses in fruits. In addition, N33 showed a higher activity against *F. graminearum, P. digitatum, P. expansum* and *P. paneum* than the synthetic fungicide. The findings indicate that *B. amyloliquefaciens* N33 can be a broad spectrum biological control agent against different fungal species.

Keywords: *Aspergillus*, *Bacillus*, biocontrol, *Fusarium*, *Penicillium.*

Bacillus amyloliquefaciens N33'ün Biyokontrolde Kullanım Potansiyelinin Ortaya Çıkarılması [*]

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Öz: Bitki hastalıklarının kontrolünde ve hasat sonrası meyve, sebze ve tahılların muhafazasında sentetik kimyasallar yerine biyokontrol ajanlarının kullanımı, sayısız avantajları göz önüne alındığında büyük bir ivme kazanmıştır. Mevcut çalışmada maruldan izole edilen *Bacillus amyloliquefaciens* N33 suşunun *Penicillium citrinum, P. expansum, P. verrucosum, P. digitatum, P. paneum, Fusarium graminearum* ve *Aspergillus fumigatus*'a karşı antifungal etkinliği *in vitro* olarak test edilmiştir. Suş test edilen tüm funguslara karşı yüksek bir etkinlik göstermiş ve inhibisyon oranı %60,3-94,2 aralığında değişmiştir. En yüksek etkinlik meyvelerde hasat sonrası önemli kayıplara neden olan *P. digitatum* (%94,2) ve *P. expansum*'a (%82,5) karşı görülmüştür. Ayrıca N33 *F. graminearum, P. digitatum, P. expansum* ve *P. paneum*'a karşı sentetik fungusitten daha yüksek bir etkinlik göstermiştir. Elde edilen bulgular *B. amyloliquefaciens* N33'ün farklı fungus türlerine karşı geniş spektrumlu bir biyolojik mücadele etmeni olabileceğini göstermektedir.

Anahtar kelimeler: *Aspergillus*, *Bacillus*, biokontrol, *Fusarium*, *Penicillium.*.

INTRODUCTION

Fungal pathogens, which cause severe yield losses in agricultural production, are the biggest limiting factors in food production and a chronic threat to the global economy and food security (Soliman et al., 2022; Soliman et al., 2023). In particular, *Fusarium* species cause serious diseases such as root rot, wilt and spike blight in many crops, causing significant economic losses (Baard et al., 2023; Krishnan et al., 2024). However, postharvest *Penicillium* and *Aspergillus* species shorten the shelf life of fruits and vegetables and threaten food safety through mycotoxin production (Dukare et al., 2022; Wang et al., 2022).

[*] *This study is derived from Sevda Uçar's PhD thesis titled "Production of chitinase enzyme from Bacillus amyloliquefaciens: Immobilization and potential use in biocontrol of some warehouse diseases and pests"*

Although many strategies have been developed to manage and control yield losses caused by phytopathogens, pesticides are the most widely used (Wei et al., 2023). However, the unconscious and widespread use of pesticides has led to the development of resistance in pathogens, toxicity to non-target organisms and detrimental effects on the environment (Trung et al., 2023; Tang, 2025). Biocontrol, based on the use of beneficial microorganisms or their products (bioactive molecules and hydrolytic enzymes) as an alternative to chemicals, can be defined as an environmentally friendly control that controls plant diseases and pests through non-hazardous mechanisms and is non-toxic to humans and other non-target organisms (Liu et al., 2021).

Many bacterial genera such as *Pseudomonas*, *Bacillus* and *Streptomyces* have been widely used in biocontrol strategies (Albayrak, 2019). Among these groups, *Bacillus* species remain the most studied and cited genus due to their resistance to adverse environmental conditions such as drought, UV and low nutrient availability and their ability to control a wide range of pathogens (Dobrzyński et al., 2023). *Bacillus* species antagonize phytopathogens by competing for nutrients and niches, producing antifungal compounds (lipopeptides and antibiotics), hydrolytic enzymes and siderophores, or by inducing systemic resistance (Cruz-Martín et al., 2020; Jabnoun-Khiareddine et al., 2023). Therefore, in this study, *B. amiyloliquefaciens* N33 strain isolated from lettuce was tested *in vitro* against 7 different fungi. The aim is to contribute to an environmentally friendly and sustainable plant protection approach with reduced chemical use.

MATERIAL AND METHOD

Bacteria and Fungal Isolates: *B. amyloliquefaciens* N33 strain (Former culture code: M6, Accession number: KX249603) and seven fungal isolates (*P. citrinum, P. expansum, P. verrucosum, P. digitatum, P. paneum, F. graminearum* and *A. fumigatus*) used in the study are stored at -80 °C in the culture collection of Prof. Dr. Neslihan Dikbaş at Atatürk University, Faculty of Agriculture, Department of Agricultural Biotechnology.

Development of Bacteria: *B. amyloliquefaciens* N33 strain was inoculated on Nutrient Agar and incubated at 28 °C for 24 hours. After incubation, the strain was transferred to Nutrient broth and incubated at 28 °C at 120 rpm for 24 hours. The density of the culture medium was adjusted to 10*8* CFU/mL (0.5 McFarland).

In Vitro Antifungal Assay: Agar disk diffusion method was used to test the antifungal effect of *B. amyloliquefaciens* N33 on fungi. The fungi to be used in the experiment were grown in Petri dishes containing Potato Dextrose Agar (PDA) at 24 ºC for 7 days under 15 hours light and 9 hours dark conditions. Mycelial disks with a diameter of 6 mm were taken from the cultures and these disks were placed on two opposite sides of the petri dishes containing PDA medium. The discs were placed in the center of the petri dishes and these discs were impregnated with 50 µL of bacterial suspension adjusted to 10^8 CFU/mL. The petri dishes were then covered with paraffin and incubated in a 15 h light and 9 h dark photoperiod until the mycelial disks in the control treatment coalesced. On the day when the disks in the control treatment coalesced, assessments were made and the colony diameter of the pathogenic fungus was measured in mm (Dikbaş et al., 2023). Purified water and 25% tebuconazole were used as control. Each treatment was repeated three times and the inhibition rate (%) was calculated using the following formula.

$$Inhibition\ rate\ (\%) = \frac{(R1 - R2)}{R1} X100$$

R1: Growth diameter of pathogenic fungus in control (mm)
R2: Growth diameter of pathogenic fungus in treatments (mm)

Statistical Analysis: In this study, all experiments were performed in triplicate. Data were analyzed using analysis of variance (ANOVA) in GraphPad Prism 8.0.2.263. Significant differences between means were determined by Tukey's HSD test. Differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Biological control of fungal diseases is a viable, effective and environmentally friendly alternative to sustainable agricultural methods considering the negative aspects of pesticides (high cost, limited efficacy, residue problems, resistance, non-target species, toxic effects on human health and the environment) (Dadrasnia et al., 2020; Deguine et al., 2021). *Bacillus* species are isolated from different sources and widely investigated for their antifungal activity against fruit, vegetable, grain or plant pathogens (Zeidan et al., 2024). In the present study, *B. amylaqiguefaciens* N33 strain isolated from lettuce was tested against 7 different fungi (*P. citrinum, P. expansum, P. verrucosum, P. digitatum, P. paneum, F. graminearum* and *A. fumigatus*) and its potential to be used in biocontrol was investigated *in vitro*.

Fusarium graminearum, which causes Fusarium head blight, causes severe yield losses in wheat and produces mycotoxins (deoxynivalenol, etc.) that can have negative effects on human health (Abbas and Yli-Mattila, 2022). In the study, *B. amylaqiguefaciens* N33 was tested against *F. graminearum* and the data obtained from the treatments were statistically analyzed. It was determined that the effect of the treatments on *F. graminearum* was very significant ($p<0.001$) and the inhibition rate varied between 0.0-60.8% according to the treatments (Figure 1, Table 1).

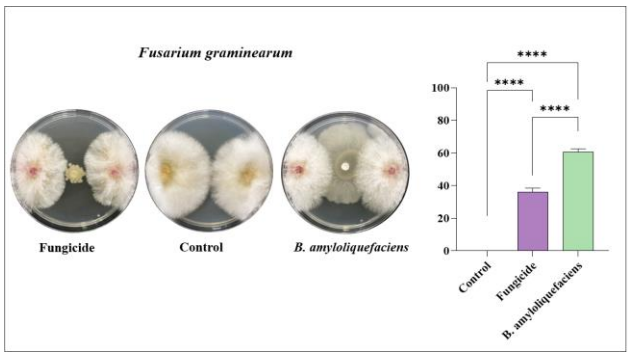

Figure 1. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *F. graminearum* and inhibition rates (****: p<0.001).

The highest inhibition against *F. graminearum* was obtained from *B. amylaqiguefaciens* N33 with a value of 60.8% and the difference with tebuconazole (36.1%) was very significant (p<0.001). In parallel with the results of the study, *B. amyloliquefaciens* MQ01 strain formed a zone diameter of 3.43 mm against *F. graminearum* in PDA medium (Xu et al., 2021). Similarly, *B. amyloliquefaciens* XY-1 strain isolated from wheat roots formed a zone diameter of approximately 30 mm against *F. graminearum* (Xu et al., 2022). In addition, Xie et al., (2022) reported that the inhibition rate of *B. amyloliquefaciens* OR2-30 against *F. graminearum* was 62.8%. Furthermore, Yi et al., (2024) reported that *B. amyloliquefaciens* ZK-9 strain has a broad antifungal activity against *F. graminearum* and filamentous fungi. The results of the present study support the strong antagonistic activity of *B. amyloliquefaciens* N33 against *F. graminearum* in agreement with the literature studies.

Green and blue molds caused by *P. digitatum* and *P. expansum* are considered the most widespread and serious postharvest diseases during storage, handling and marketing of fresh fruits (Settier-Ramírez et al., 2021; Wang et al., 2022). The antifungal activity of *B. amylaqiguefaciens* N33 against *P. digitatum* was tested *in vitro* and the data obtained from the treatments were statistically analyzed. It was determined that the effect of the treatments on *P. digitatum* was very significant (p<0.001) and the inhibition rate varied between 0.0-94.2% according to the treatments (Figure 2, Table 1).

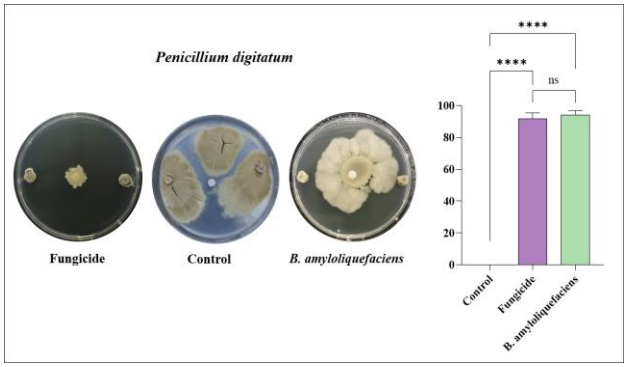

Figure 2. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *P. digitatum* and inhibition rates (****: p<0.001, ns: non-significant).

The highest inhibition rate was obtained from *B. amylaqiguefaciens* N33 (94.2%), followed by tebuconazole (91.7%). While the difference between both treatments was insignificant (P>0.05), the difference between these treatments and the control was very significant (p<0.001). In the antifungal tests against *P. expansum*, the highest activity was obtained from *B. amylaqiguefaciens* N33 with an inhibition rate of 82.5% and there was no significant difference between it and the fungicide (75%) (p>0.05). The difference between these treatments and the control was very significant (p<0.001) (Figure 3, Table 1).

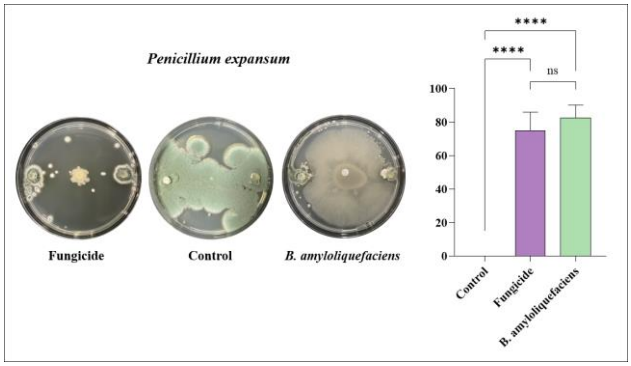

Figure 3. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *P. expansum* and inhibition rates (****: p<0.001, ns: non-significant).

Strain N33 showed a superior biocontrol performance against the respective pathogen compared to the fungicide. There are many reports in the literature on the use of *B. amyloliquefaciens* for the biological control of *P. digitatum* and *P. expansum*. For example, Calvo et al., (2017) determined the inhibition rates of *B. amyloliquefaciens* BUZ-14 strain against *P. digitatum* and *P. expansum* as 54.5% and 56.8%, respectively. Similarly, Chen et al., (2018) reported that *B. amyloliquefaciens* DH-4 strain isolated from citrus rhizosphere soil formed a zone diameter of 6.1 cm against *P. digitatum*. In addition, Huang et al., (2023) reported that *B. amyloliquefaciens* HY2-1 formed a zone diameter of 21.88 mm against *P. digitatum* and exhibited broad-spectrum antifungal activity by producing lipopeptides such as fengycin A and surfactin. In addition, Avan et al., (2024) reported that 250 mL 100 L^{-1} dose of *B. amyloliquefaciens* TV-17C reduced green mold incidence by 90.3% and 89.8% in Hatay and Mersin provinces, respectively, and emphasized the effectiveness of TV-17C strain against green mold in oranges. The results obtained were in line with the literature data and strain N33 was more effective than chemical pesticides against both fungi.

The antifungal activity of *B. amylaqiguefaciens* N33 was also tested against *P. citrinum*, *P. verrucosum*, *P. paneum* and *A. fumigatus* and the results are presented in Figures 4, 5, 6 and 7. The efficacy of N33 and tebuconazole against *P. citrinum* was similar (p>0.05) and the inhibition

rates were 67.5% and 67.1%, respectively (Figure 4, Table 1).

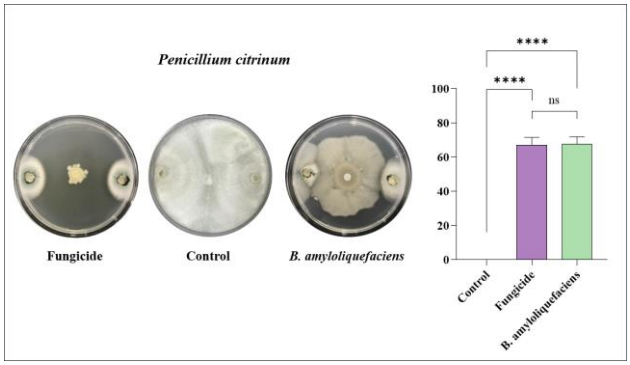

Figure 4. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *P. citrinum* and inhibition rates (****: p<0.001, ns: non-significant).

Related to the study, Li et al., (2016) reported that *B. amyloliquefaciens* SYBC H47 strain isolated from raw honey inhibited *P. citrinum* by ~25%. The rate of inhibition obtained by the researchers was much lower than the results obtained in the present study. This shows that strain N33 has a good antagonistic property against the related fungus.

Penicillium verrucosum is an important fungal pathogen that widely affects cereal crops during harvest and storage under favorable environmental conditions and produces citrinin, ochratoxin A, patulin and penicillic acid (Mosa et al., 2023). In the antifungal activity test against *P. verrucosum*, the highest activity was obtained from tebuconazole (71.1%) and the difference with N33 (61.4%) was significant (p<0.05). The difference between these treatments and the control was very significant (p<0.001) (Figure 5, Table 1).

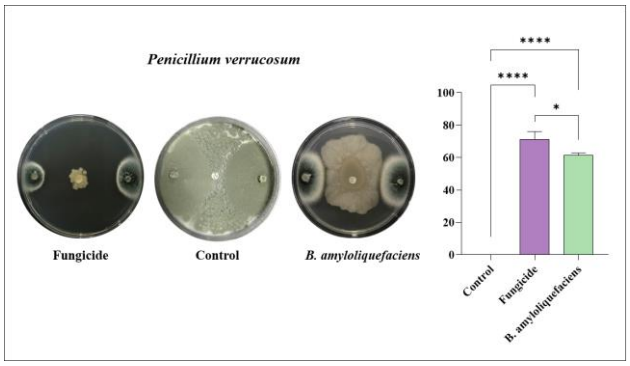

Figure 5. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *P. verrucosum* and inhibition rates (*: p<0.05, ****: p<0.001).

In the literature, different Bacillus species were tested against *P. verrucosum* and positive results were reported. For example, Ul Hassan et al., (2019) reported that *B. licheniformis* BL350-2 strain inhibited *P. verrucosum* by 53% in their study. Similarly, Saleh et al., (2021) reported that volatile organic compounds of *B. megaterium* BM344-1 inhibited the growth of *P. verrucosum*.

Penicillium paneum is a major contaminant of cereal grains, thriving at low temperature and pH and high carbohydrate levels, and some species are known to produce mycotoxins that can be harmful to humans and animals (Zhao et al., 2024). In the study, *B. amylaqiguefaciens* N33 strain exhibited a high inhibition rate (73.9%) against *P. paneum* and the difference with fungicide (66.7%) was insignificant (p>0.05). However, the difference between both treatments and the control was very significant (p<0.001) (Figure 6, Table 1).

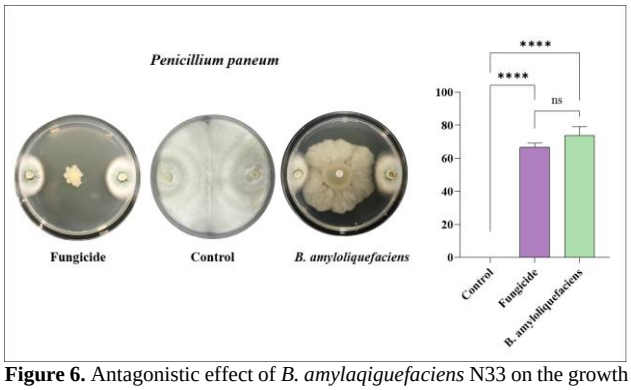

Figure 6. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *P. paneum* and inhibition rates (****: p<0.001, ns: non-significant).

The highest efficacy against *A. fumigatus* was obtained from the fungicide treatment (73.0%) and the difference with the bacterial treatment (60.3%) was very significant (p<0.001). However, the bacterial treatment achieved a very good inhibition rate compared to the control and the difference with the control was very significant (p<0.001) (Figure 7, Table 1). Similarly, Singh et al., (2015) reported that the lysate of *B. amyloliquefaciens* DSM-1067 strain completely inhibited the growth of *A. fumigatus* and *A. flavus*.

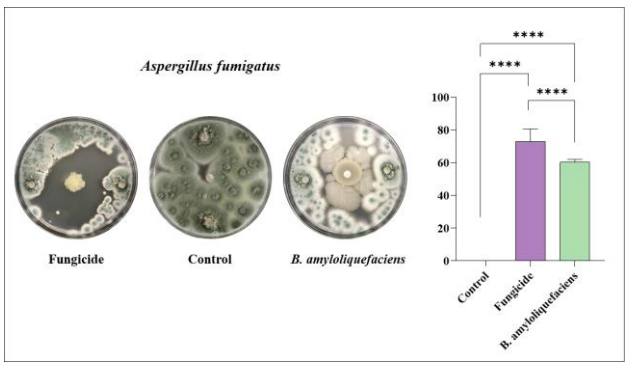

Figure 7. Antagonistic effect of *B. amylaqiguefaciens* N33 on the growth of *A. fumigatus* and inhibition rates (****: p<0.001).

Considering all the results obtained in the study, *B. amyloliquefaciens* N33 showed the highest inhibition rate against *P. digitatum*, followed by *P. expansum*, *P. paneum*, *P. citrinum*, *P. verrucosum*, *F. graminearum* and *A. fumigatus* in descending order (Table 1). The difference between the inhibition rates obtained against *P. expansum* and other fungi was statistically significant (p<0.05). In the

fungicide treatment, the highest efficacy was again obtained against *P. digitatum* and the difference between the inhibition rates obtained against other fungi was significant ($p<0.05$). (Table 1). The efficacy of the fungicide against *P. citrinum*, *P. expansum*, *P. verrucosum*, *P. paneum* and *A. fumigatus* was statistically similar ($p>0.05$).

Table 1. Efficacy of fungicide and bacterial treatments on different fungi.

Fungi	Inhibition Rates (%)		
	Control ± SD	*B. amyloliquefaciens* ± SD	Fungicide ± SD
F. graminearum	0 ± 0^{c}	$60.8\pm1.8^{D,a}$	$36.1\pm2.5^{C,b}$
P. digitatum	0 ± 0^{b}	$94.2\pm2.6^{A,a}$	$91.7\pm3.8^{A,a}$
P. expansum	0 ± 0^{b}	$82.5\pm10.8^{B,a}$	$75\pm7.6^{B,a}$
P. citrinum	0 ± 0^{b}	$67.5\pm4.3^{CD,a}$	$67.1\pm4.4^{B,a}$
P. verrucosum	0 ± 0^{c}	$61.4\pm1.4^{D,b}$	$71.1\pm4.9^{B,a}$
P. paneum	0 ± 0^{b}	$73.9\pm5.2^{BC,a}$	$66.7\pm2.6^{B,a}$
A. fumigatus	0 ± 0^{c}	$60.3\pm1.8^{D,b}$	$73.0\pm7.7^{B,a}$

According to the HSD Tukey multiple comparison test, capital letters in the table represent differences within each column, and lowercase letters represent differences within each row, **SD:** Standard deviation.

Considering all the results obtained within the scope of the study, it was observed that *B. amyloliquefaciens* N33 showed good activity against all tested fungi and supported the results in the literature.

CONCLUSION

In this study, *B. amyloliquefaciens* N33 strain isolated from lettuce was tested against 7 different fungi (*P. citrinum*, *P. expansum*, *P. verrucosum*, *P. digitatum*, *P. paneum*, *F. graminearum* and *A. fumigatus*) and high inhibition rates were obtained. The strain showed higher activity than tebuconazole against *F. graminearum*, *P. digitatum*, *P. expansum* and *P. paneum*, while its activity against other fungi (*P. citrinum*, *P. verrucosum* and *A. fumigatus*) was lower or similar to tebuconazole. The broad-spectrum antifungal activity of the strain strengthens its potential for use in the biological control of many disease agents. Further research is needed to evaluate the effects of N33 on relevant disease agents *in vivo* and to fully characterize its antifungal activity.

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