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Insecticidal and Antimicrobial Effects of *Pseudomonas* Species Isolated From Waste Water

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ABSTRACT: In this study, the insecticidal effects of *Pseudomonas aer*uginosa, *Pseudomonas fluorescens*, *Pseudomonas putida* isolated from refinery wastewater on *Leptinotarsa decemlineata* and *Dendroctonus micans* larvae and antimicrobial effects against different microorganisms were investigated. L. *decemlineata* and *D. micans* larvae collected from the Kümbet Plateau of Giresun province were fed with isolate inoculated leaf and bark samples. At the end of 7 days of application, the viability and mortality rates were calculated. Antimicrobial activities of isolates against gram negative, gram positive and fungal species were investigated by disc diffusion method using fermentation broths. As result, at the end of the 7-day application, a mortality rate of 69-85% was obtained for *L. decemlineata* larvae. On the other hand, 49-78% mortality was obtained in *D.micans* larvae. This finding indicates that *D. micans* is more sensitive to isolates. It was determined that the isolates exhibited different levels of antimicrobial activity against gram negative, gram positive and *Candid*a species. These results show that *Pseudomonas* species isolated from refinery wastewater have high potential in biological control against *L. decemlineata* and *D. micans* and exhibit broad-spectrum antimicrobial activity.

Keywords *– Biological control, Dendroctonus micans, Leptinotarsa decemlineata, Pseudomonas sp.*

1. Introduction

Diseases transmitted by insect species have an important place among lethal diseases and such diseases cause millions of deaths every year [1]. For this reason, the control of insects is very important and many chemicals are used in this control. Such excessive use of insecticides causes contamination of water resources and food, and toxic effects on non-target organisms. Biological control based on natural products without side effects is the best alternative to chemical control (Dahmana et al. 2020). As alternatives to synthetic insecticides, semiochemicals such as pheromones that change the behavior of insects or biological control agents are used to reduce the growth rate of the population. Parasites, predators and microorganisms are commonly used in biological control (Bale et al. 2008). Studies on the effectiveness of microorganisms and their metabolites in insect control are increasing day by day, and more than 1500 microorganism species have been identified as potential insecticidal agents. Metabolites produced by microorganisms also show an insecticidal effect, and the insecticidal activity of various metabolites isolated from approximately 942 microorganisms has been determined (Dhanasekaran and Thangaraj, 2014).

Microorganisms with chitinase activity in insecticidal activity are potentially important biological agents. Chitin, which is an important part of the exoskeleton of invertebrates, cell wall structure in protozoa and fungi, consists of unbranched chains of β-1,4-linked N-acetyl-

D-glucosamine units. Hydrolysis of the β-1,4-bonds in the chitin structure results in the deterioration of the cell wall structure and especially the exoskeleton structure in invertebrates. Chitinoltic activity can be catalyzed by chitinase, exochitinase and N-acetyl-glucosaminidase enzymes (Tronsmo and Harman, 1993). Microorganisms synthesizing these enzymes also show high insecticidal activity. Among *Pseudomonas* species, *Pseudomonas aeruginosa, Pseudomonas stutzeri* and *Pseudomonas* fluorescens exhibit chitinolytic activity (Wang and Chang, 1997; Lim et al. 1991).

In this study, the insecticidal effects of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida* isolated from refinery waste waters on *Leptinotarsa decemlineata* and *Dendroctonus micans* larvae were investigated. *L. decemlineata* spends the winter in the soil as an adult. It feeds on the leaves and shoots of plants such as potatoes, eggplants and tomatoes, both in the larva and adult stages, and causes great damage to these plants (Sokolov, 1981). *D. micans* causes great destruction especially in the trunk, thick branches and even thick shoots of spruce trees in forests including Georgia and Turkey (Alkan et al. 2014). In this respect, biological control of these species gains great importance. Within the scope of the study, antimicrobial activity of isolates as well as insecticidal activities were investigated and it was determined that these isolates were promising in terms of insecticidal activity and antimicrobial property.

2. Material and Methods

In this study, microorganisms isolated from water samples contaminated with Kırıkkale Refinery Industry waste on Kızılırmak by Yalçın and Ergene (2009) were used. Colony morphology, gram staining, oxidase test and pigment status of the isolates were examined and their identification was performed using BD BBL Crystal enteric/Nonfermenter ID System kits, Analytical Profile Index (API) kits and VITEK 2 device (Biomerieux). Stock cultures of all isolates were prepared and stored at +4^{o}C for use in future studies. Within the scope of the study, the insecticidal and antimicrobial activities of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida* isolated from refinery wastewater were investigated.

2.1. Determination of insecticidal activities of isolates

The insecticidal activity of the isolates was tested against the larvae of *Leptinotarsa decemlineata* and *Dendroctonus mic*ans. *L. decemlineata* larvae were collected between May-July 2021, and *D. micans* larvae were collected between July-September 2021from the Kümbet Plateau of Giresun province. The collected larvae were brought to the laboratory in sterile containers, and the healthy ones were used as experimental material as a result of macroscopic examinations. For insecticidal activity, the isolates were incubated for 25 hours in nutrient broth. The cultures prepared from each isolate at a density of 1.8×10^{9} bacteria/mL were centrifuged and the resulting pellet was dissolved in distilled water and used as an insecticidal agent. Potato leaves cut as 5cmx5cm were used for *L. decemlineata* larvae, while fresh spruce peels prepared as 5cmx5cm were used for *D. micans* larvae. Samples of each isolate (5 mL) were spread homogeneously on the surfaces of the leaves and spruce bark. In the experimental stages, a total of 7 groups were formed (Table 1), and leaf and bark samples that did not contain isolates were used in the control groups. For each group, 10 *L. decemlineata* and *D. micans* larvae were placed in sterile containers. Viability and mortality rates were controlled for 7 days. Experiments were carried out in triplicate for each isolate.

Table 1. Experimental groups

Groups	Application
Group I	Control-1 (potato leaf without isolate)
Group II	*P.aeruginosa* inoculated with potato leaf
Group III	*P. fluorescens* inoculated with potato leaf
Group IV	*P. putida* inoculated with potato leaf
Group V	Control-2 (spruce bark without isolate)
Group VI	*P.aeruginosa* inoculated with spruce bark
Group VII	*P. fluorescens* inoculated with spruce bark
Group VIII	*P. putida* inoculated with spruce bark

2.2. Investigation of antimicrobial activities of isolates

Antimicrobial activity of the isolates was studied against gram-negative (*Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC14028, *Klebsiella pneumoniae* ATCC11296) and gram-positive (*Streptococcus pyogenes* ATCC19615, *Bacillus subtilis* ATCC35021, *Staphylococcus aureus* ATCC25923) bacteria and two fungus as *Candida krusei* ATCC6258 and *Candidia albicans* ATCC102316. Antimicrobial activity of isolates was determined by disc diffusion method using fermentation broths. For this purpose, firstly, the fermentation liquid of each isolate was obtained. Samples of each isolate containing 10^7-10^8 units/mL bacteria were incubated for 14 days using Müller Hinton Broth. During the incubation period, the metabolites produced by the bacteria accumulated in the medium and at the end of the 14th day, the supernatant obtained by centrifugation was used as the fermentation liquid. Test bacteria were homogeneously inoculated on Mueller Hinton Agar, 6 mm diameter empty sterile discs were placed in petri dishes, and 50 µL of fermentation liquid was transferred to discs. Petri dishes were kept at 4°C for 1 hour and then incubated at 37°C for 18-24 hours. At the end of the period, the inhibition zones formed on the medium were evaluated in mm. Nystatin (20 µg/mL), Amikacin (20 µg/mL) antibiotics were used as standard.

2.3. Statistical analysis

Analyzes were performed with the "IBM SPSS Statistics 22" package program and the data were given as mean standard deviation (SD). Statistical significance between the means was determined by Duncan's test and One-way ANOVA, and a p value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Insecticidal activities of isolates

The insecticidal effects of isolates on *L. decemlineata* are given in Figure 1. At the end of 7 days of application, 69%-85% mortality of *L. decemlin*eata larvae was obtained. The highest mortality was found in the group treated with *P. aureginosa* isolate (Group II). While no mortality was observed in the control-1 group, *P. aureginosa* administration caused a high mortality in *L.* decemlineata. In Group III and Group IV, in which *P. putida* and *P. fluorescens* isolates were applied, mortality was 76% and 69%, respectively.

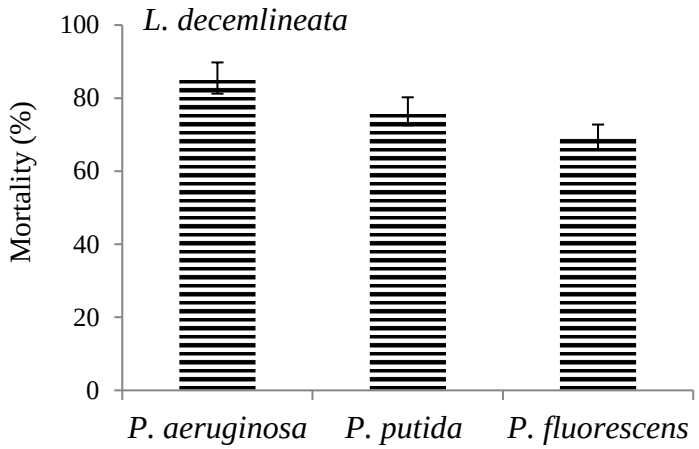

Figure 1. The insecticidal effects of isolates on *L. decemlineata*

The insecticidal effects of isolates on *D. micans* are given in Figure 2. Death was observed in only one larva among all larvae in the control-2 group and this result was statistically insignificant ($p>0.05$). However, 49%-78% mortality was obtained in *D.micans* larvae after 7 days of application in Groups VI, VII and VIII. The highest mortality was found in the group treated with *P. fluorescens* isolate (Group VII). In Groups VI and VIII, in which *P. aeruginosa* and *P. putida* isolates were applied, 65% and 49% mortality were obtained, respectively. These results show that the isolates are more effective against *L. decemlineata* compared to *D.micans*. In addition, while *P. aeruginosa* is more effective in terms of insecticidal effect in *L. decemlineata*, *P. fluorescens* is more effective in *D.micans*. This situation is closely related to the sensitivity of the larval species and the characteristic structures of the isolates.

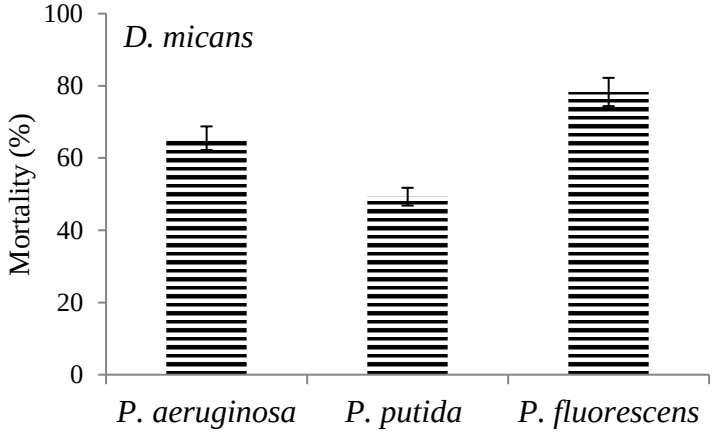

Figure 2. The insecticidal effects of isolates on *D. micans*

In the literature, insecticidal activity of many *Pseudomonas* species against various insect species has been reported. *Pseudomonas* species is a rapidly growing bacterial genus isolated from polluted environments. *Pseudomonas* species are involved in important metabolic

activities in such environments and contribute to the degradation of biogenic and xenobiotic pollutants (Timmis, 2002; Li et al., 2005). In literature studies, insecticidal activities of different *Pseudomonas* species against insect species were investigated. Chen et al. (2014) reported that *Pseudomonas taiwanensis* exhibited mortality in the range of 92.7%-94.5% against *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera litura* invading agricultural areas. Schneider and Dorn (2001) reported that although *P. putida* did not have a significant effect on *Oncopeltus fasciatus* nymphs, *P. aeruginosa* killed all experimental animals within 48 hours. Vesga et al. (2021) reported that *P. protegens* and *P. chlororaphis* isolated from agricultural fields, plant roots and soil have lethal effects on various arthropod species. Bensidhoum et al. (2016) determined that *Pseudomonas protegens* isolated from agricultural well waters exhibited significant insecticidal activity on *Galleria mellonella*. The insecticidal activity of many isolates has been investigated in the literature and different cidal effects have been reported. In this study, the insecticidal activity of Pseudomonas species isolated from refinery wastewater is reported for the first time in literature.

3.2. Antimicrobial activity

Antimicrobial activities of microorganisms isolated from refinery wastewater are given in Figure 3. It was determined that the isolates exhibited activity at different rates against each microorganism. While *P. aeruginosa* isolate showed the highest activity against *S.pyogenes* with a zone of 15.6 mm, the lowest activity was obtained against *C. albicans* with 7.6 mm. Similarly, the *P. putida* isolate showed its highest activity against *S.pyogenes* with a zone diameter of 13.5 mm. Amikacin standard antibiotic was formed inhibition zones against *E. coli* as 16.9mm, against *S. typhimurium* as 17.2mm, against *K. pneumoniae* as 15.3mm, against *S. pyogenes* as 15.9mm, against *B. subtilis* as 16.1mm and against *S. aureus* as 14.2mm. The lowest activity was obtained against *C. albicans* in *P. fluorescens* isolate; the highest activity was obtained against *S. aureus* as 11.7 mm. All isolates showed generally lower activity against *Candida* species. This result is closely related to the cell wall of fungi, which is especially rich in chitin and ergosterol. It is known that *Candida* species increase the development of resistance by increasing the chitin levels in the cell wall in the presence of antimicrobial agent (Durhan et al. 2022). The inhibition zones of nystatin standard antibiotic against *C. krusei* and *C. albicans* was 14.3 mm and 13.7 mm, respectively.

The antimicrobial activity of the fermentation broth of the isolates is due to the metabolites produced and passed into the medium. *Pseudomonas* species intensively produce compounds such as primary alcohol, secondary alcohol, alkanes, conjugated alkene and unsaturated esters. These metabolites also have a cidal effect on bacteria. It has been reported in the literature that *P. aureginosa* isolate exhibits antimicrobial activity on different microorganisms, and this effect is related to compounds such as eicosene, nonadecene, hexadecanol produced by the isolate (Amankwah et al. 2022). Antimicrobial effects of *Pseudomonas* species isolated from various sources have been reported in the literature. Bekci et al. (2018) found that *Pseudomonas* strains isolated from milk samples showed a significantly higher inhibition effect on *E. coli* and lactic acid bacteria by agar-well diffusion method. There are similar studies in the literature reporting the antimicrobial activity of different bacteria species isolated from various environments. Motta et al. (2004) determined that isolates obtained from aquatic environments exhibited high cidal activity against Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus cereus*. Singh et al. (2019) determined that the bacterial strain isolated from the lake waters showed antibacterial effect against *E. coli*, *Staphylococcus aureus, Bacillus subtilis*, and antifungal effect against *Candida albicans*.

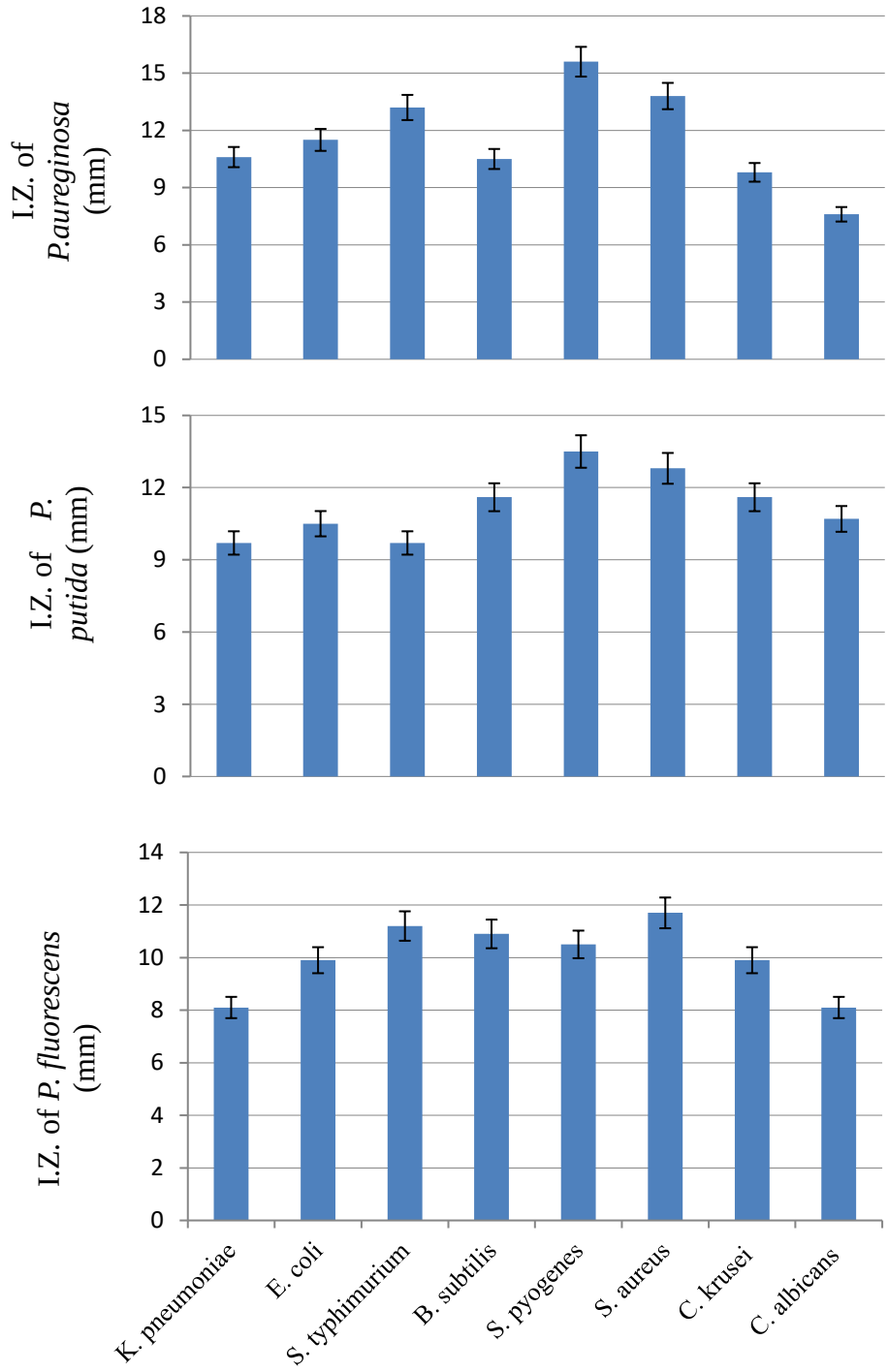

Figure 3. Inhibiton zones of isolates against microorganisms

4. Conclusion

In our study, the insecticidal activities and antimicrobial activities of *Pseudomonas* species isolated from refinery wastewater were evaluated. It was determined that the isolates exhibiting a broad spectrum antimicrobial activity showed a higher cidal effect on L. *decemlineata* larvae compared to *D. micans* larvae. The use of natural agents instead of chemical insecticides and more research for biological control will make very positive contributions to the environment and human health.

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