In Vitro Biocontrol Potential of Endophytic Methylobacterium sp. Against Plant Pests Pseudomonas savastanoi and Botrytis sp.

Az endofita Methylobacterium sp. in vitro biokontroll-potenciálja a Pseudomonas savastanoi és a Botrytis sp. növényi kórokozók ellen

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Abstract: Fungi and pathogenic bacteria cause numerous plant diseases that significantly affect crop productivity. The use of biological control agents represents an environmentally friendly alternative to chemical pesticides. The present study aimed to evaluate the *in vitro* antagonistic effects of *Methylobacterium sp.* against *Pseudomonas savastanoi* and *Botrytis sp.*, two major phytopathogens affecting olive (*Olea europaea L.*) and faba bean (*Vicia faba L.*), respectively.The bacterial strain *Methylobacterium sp.*, isolated from nodules of *Retama monosperma*, was tested for its antibacterial activity against *P. savastanoi* using the direct method of Fleming et al. (1975) and the indirect method of Barefoot and Klaenhammer (1983). The antifungal potential against *Botrytis sp.* was assessed by the direct confrontation technique on Mueller-Hinton agar medium. The results revealed that no inhibition was observed with the direct method, whereas the indirect method showed a clear inhibitory zone of approximately 5 mm against *P. savastanoi*, indicating that the inhibitory metabolites are mainly intracellular. The confrontation test demonstrated a significant reduction in *Botrytis sp.* mycelial growth; with an inhibition rate exceeding 38%. These findings highlight the potential of *Methylobacterium sp.* as a promising biological control agent against bacterial and fungal phytopathogens. Further studies under *in vivo* conditions are required to confirm its efficacy and identify the active metabolites involved in the antagonistic activity.

***Keywords**: Methylobacterium sp; Pseudomonas savastanoi; Botrytis sp; olive; biological control*

Összefoglalás: A gombák és a patogén baktériumok számos növénybetegséget okoznak, amelyek jelentősen befolyásolják a terméshozamot. A biológiai növényvédő szerek alkalmazása környezetbarát alternatívát jelent a kémiai növényvédő szerekhez képest. A jelen tanulmány célja az volt, hogy értékelje a *Methylobacterium* sp. in vitro antagonisztikus hatásait a *Pseudomonas savastanoi* és a *Botrytis* sp. ellen, amelyek az olíva (*Olea europaea* L.) és a bokorbab (*Vicia faba* L.) két fő növénypatogénjei. A *Methylobacterium* sp. bakteriális törzset, amelyet a *Retama monosperma* gümőiből izoláltak, antibakteriális aktivitás szempontjából teszteltük a *P. savastanoi* ellen a Fleming és munkatársai (1975) által kidolgozott közvetlen módszerrel, valamint a Barefoot és Klaenhammer (1983) által alkalmazott közvetett módszerrel. A *Botrytis* sp. elleni gombaellenes potenciált a közvetlen konfrontációs technikával értékeltük Mueller-Hinton agaron. Az eredmények azt mutatták, hogy a közvetlen módszerrel nem figyeltünk meg

gátlást, míg a közvetett módszer kb. 5 mm-es egyértelmű gátló zónát mutatott a *P. savastanoi* ellen, ami arra utal, hogy a gátló hatású metabolitok főként intracellulárisak. A konfrontációs teszt jelentős csökkenést mutatott a *Botrytis* sp. micélium növekedésében, a gátlási arány meghaladta a 38%-ot. Ezek az eredmények kiemelik a *Methylobacterium* sp. potenciálját, mint ígéretes biológiai növényvédelmi ágens a bakteriális és gombás fitopatogének ellen. További in vivo körülmények között végzett vizsgálatokra van szükség a hatékonyság megerősítésére és az antagonista aktivitásban szerepet játszó aktív metabolitok azonosítására.

***Kulcsszavak**: Methylobacterium sp; Pseudomonas savastanoi; Botrytis sp; olíva; biológiai védelem*

1. Introduction

Pathogenic microorganisms affecting plant health pose a major and ongoing threat to food production and ecosystem stability worldwide. (de Weger et al., 1995; Gai and Wang, 2024). The olive tree (*Olea europaea* L.) and the faba bean (*Vicia faba* L.) are cultivated species susceptible to attacks by phytopathogenic agents such as *Pseudomonas savastanoi* and *Botrytis* sp., respectively. Their rapid and insidious development leads each year to the destruction of hundreds of olive trees and dozens of hectares of faba beans. These pathogens cause significant economic losses, and the chemical methods used to control them can have harmful side effects on the environment and health. Among the alternatives to chemical control, the use of biological protection is an effective solution that helps combat plant pathogens while reducing the use of chemical products. Biological control of plant pathogens is more environmentally friendly than chemical control (Nautiyal, 2000). Among the antagonists present in soils with balanced microflora, the genus *Methylobacterium* sp., PGPR (Plant growth-promoting rhizobacteria) holds an important place due to its beneficial interactions with plants (Han, 2024).In this study, we investigated the in vitro antagonistic effect of *Methylobacterium* sp. against *Pseudomonas savastanoi* and *Botrytis* sp., in order to assess its potential as a biocontrol agent. The study focused on the isolation, pre-identification of the pathogens, and the evaluation of the antibacterial and antifungal activity of *Methylobacterium* sp. using various methods.

2. Materials and Methods

2.1. Microbial and plant material

The microbial material consists of strains of *Methylobacterium* sp. isolated from *Retama monosperma* nodules. The strain was provided by the LP2VM laboratory. The studied strain was cultured on Mueller-Hinton (MH) (Guiraud, 2003) (Fig.1).

Figure 1 *Macroscopic appearance of Methylobacterium sp. colonies obtained after several subcultures on solid MH medium.*

Pseudomonas savastanoi strains were isolated from infected olive (*Olea europaea* L.) twigs, while *Botrytis* sp. was isolated from faba bean (*Vicia faba* L.) leaves exhibiting characteristic brown spots.

2.2. Isolation and purification

Fragments of infected olive tree branches were disinfected with 95% alcohol, rinsed, and then macerated in distilled water or phosphate-buffered saline (PBS) (EPPO, n.d.) for 30 minutes. The resulting macerate was used for inoculation on Levure Peptone Glucose Agar medium (LPGA) (Guiraud, 2003) and Mueller-Hinton (MH) agar, incubated at 26°C for 24–72 hours. The isolated colonies were purified by successive subculturing on MH medium and stored at 4°C. For *Botrytis* sp., fragments of infected leaves were disinfected in a 2% sodium hypochlorite solution for 3–5 minutes, rinsed three times with sterile distilled water, and then placed on Potato Dextrose Agar (PDA) (EPPO, n.d.) medium. The plates were incubated at 25°C for 5–7 days until mycelium appeared, and then subcultured to obtain pure cultures. All experimental procedures were conducted in the bacteriology laboratory of the Regional Plant Protection Service (SRPV) of Oran, Algeria, in accordance with standard protocol of the European and Mediterranean Plant Protection Organization (EPPO, n.d.).

2.3. Study of antibacterial activity

The antibacterial activity of *Methylobacterium* sp. against *Pseudomonas savastanoi* was evaluated using both direct and indirect approaches. In the direct method (Fleming et al., 1975), colonies of the two strains were placed in direct contact on Mueller-Hinton (MH) agar and incubated at 28°C for 3 days, after which inhibition zones were observed. In the indirect method (Barefoot and Klaenhammer, 1983), the supernatant from a *Methylobacterium* sp. culture or its pure suspension was placed into wells prepared in MH agar seeded with the indicator strain, and the plates were incubated at 28°C for 3 days to assess inhibition. All antibacterial assays were performed in three independent replicates, and representative results are presented.

2.4. Study of antifungal activity

The antifungal activity of *Methylobacterium* sp. against *Botrytis* sp. was evaluated using the direct confrontation method. A fungal disc was placed on a Petri dish containing MH medium, which supports the growth of the bacterial antagonist, at a distance of 3 cm from a bacterial streak. A control containing only the fungus was prepared. After incubation at 26°C for 10 days,

mycelial growth was measured and the inhibition percentage was calculated as described by (Wang et al., 2002).

$$(\%)\ \text{inhibition} = (R_{control} - R_{test}) / R_{control} \times 100$$

- $R_{control}$: maximum radial distance of fungal growth.
- R_{test}: radial distance along a line toward the antagonist.
- Inhibition was considered significant for values ≥20%.

All antifungal assays were conducted in three independent replicates, and representative results are shown.

3. Results

3.1. Antibacterial activity against *P. savastanoi*

Tests conducted using the direct method showed no visible inhibition zones around the *Methylobacterium* sp. colonies (Figure 2). The rapid growth of *P. savastanoi* (24 h) appeared to outpace that of *Methylobacterium sp*. (72 h), preventing effective inhibitory interaction.

In contrast, the indirect method revealed significant antibacterial activity: the pure suspension of *Methylobacterium* sp. produced a clear inhibition zone of about 5 mm in diameter (Figure 3). This result suggests that the metabolites responsible for the inhibition are intracellular rather than extracellular (Mina et al., 2020). Similar results were observed in the other two replicates.

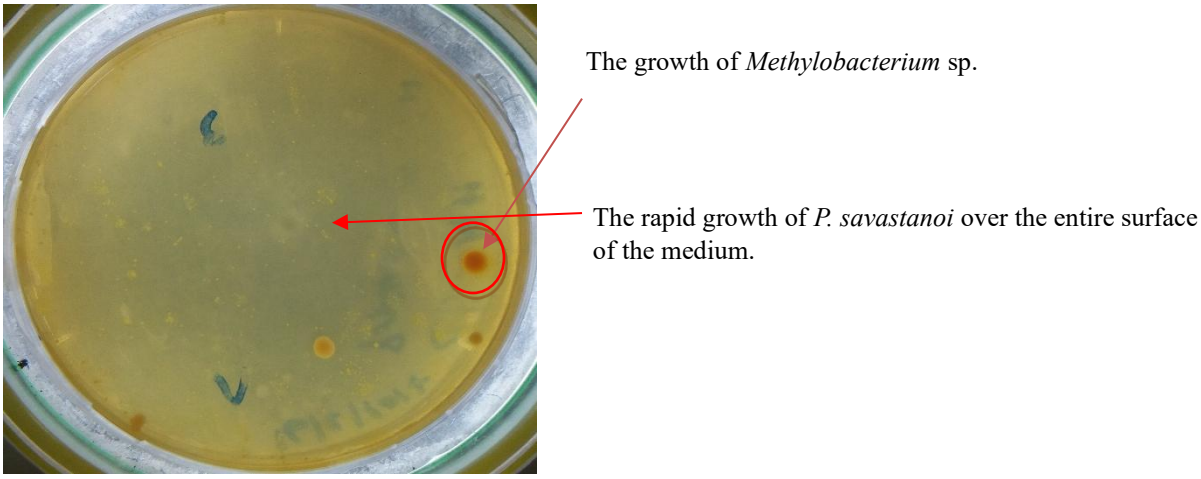

Figure 2 *Results obtained by the spot method according to Fleming et al. (1975).*

Figure 3 *Antibacterial activity of Methylobacterium sp. against P. savastanoi using the Barefoot and Kaenhammer (1983) method, adapted.*

3.2. Antifungal activity against *Botrytis* sp.

The assessment of the antagonistic effect of *Methylobacterium* sp. against *Botrytis* sp. through direct confrontation showed a clear inhibition of mycelial growth (Figure 4). The calculated average inhibition percentage exceeded 38%, indicating a significant antagonistic interaction. Although the inhibition percentage reported here corresponds to one representative replicate, similar trends were observed in other repetitions (data not shown). The comparison with the control (Figure 5) confirms that the growth of the fungus is significantly reduced in the presence of *Methylobacterium* sp., demonstrating its potential to limit the development of *Botrytis* sp. Comparable inhibition was observed in the other replicates.

A *B*

A. ***Figure 4*** *Representation of the results of the direct confrontation on the Botrytis sp. Strain.*

B. ***Figure 5*** *The control only contains the phytopathogenic fungus after 6 days.*

4. Discussion

Our results are in line with those of Poorniammal et al. (2009) and Egamberdieva et al. (2015), and align with more recent findings (Ehinmitan et al., 2025; Photolo et al., 2020),who also observed an antifungal effect of *Methylobacterium* strains against various pathogens, suggesting that this activity could be related to the production of secondary metabolites or competition for nutrients. Based on our results, we can suggest that *Methylobacterium* sp. could exhibit an eco-friendly approach by modulating certain experimental parameters such as pH, incubation time, temperature, and the concentration of the inhibitory strain. These factors strongly influence the production and diffusion of antibacterial and antifungal metabolites, as reported by Ehinmitan et al. (2025) and Egamberdieva et al. (2015). Furthermore, co-inoculation with *Trichoderma* sp. could enhance the effectiveness of biocontrol. According to (Mahmoudi, 2012; Risoli et al., 2022), Trichoderma species exhibit strong antagonistic effects against *Botrytis* sp. Similarly, Schierling et al. (2024) demonstrated that Trichoderma spp. and beneficial bacteria (*Kosakonia* sp.) can act complementarily within integrated management strategies against B. cinerea, even when combined with reduced fungicide use. These findings suggest that combining *Methylobacterium* sp. with *Trichoderma* sp. could represent a promising, eco-friendly biocontrol approach.

5. Conclusion

The study highlighted the in vitro antagonistic potential of *Methylobacterium* sp. against two major phytopathogenic agents, *Pseudomonas savastanoi* and *Botrytis* sp. The results show significant antibacterial activity against *P. savastanoi* using the indirect method and an inhibition of mycelial growth of *Botrytis* sp. greater than 38%.These observations confirm that *Methylobacterium* sp. could serve as a promising biological control agent, potentially complementing or partially replacing chemical treatments. Further studies, particularly in vivo, are needed to confirm the efficacy of this strain, characterize the metabolites responsible for the inhibitory activity, and optimize application conditions in a sustainable agricultural context.

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References

Barefoot, S. F., Klaenhammer, T. R. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus. Applied and Environmental microbiology* **45**, 1808–1815. https://doi.org/10.1128/aem.45.6.1808-1815.1983

de Weger, L.A., van der Bij, A. J., Dekkers, L. C., Simons, M., Wijffelman, C. A., Lugtenberg, B. J. 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiology Ecology* **17**, 221–227.

Egamberdieva, D., Wirth, S., Alqarawi, A. A., Abd_Allah, E. 2015. Salt tolerant *Methylobacterium mesophilicum* showed viable colonization abilities in the plant rhizosphere. *Saudi Journal of Biological sciences* **22**, 585–590. https://doi.org/10.1016/j.sjbs.2015.06.029

Ehinmitan, E., Siamalube, B., Losenge, T., Mamati, E., Juma, P., Ngumi, V. 2025. *Methylobacterium spp.* in sustainable agriculture: strategies for plant stress management and growth promotion. *The Microbe*, 100476. 100476. https://doi.org/10.1016/j.microb.2025.100476

European and Mediterranean Plant Protection Organization (EPPO). n.d. EPPO Standards – Diagnostic protocols for regulated pests (PM 7). https://www.eppo.int/RESOURCES/eppo_standards/pm7_diagnostics

Fleming, H., Etchells, J., Costilow, R. 1975. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Applied microbiology* **30**, 1040–1042. https://doi.org/10.1128/am.30.6.1040-1042.1975

Gai, Y., Wang, H. 2024. Plant disease: A growing threat to global food security. *Agronomy*. **14**. 1615.

Guiraud, J.-P. 2003. Microbiologie alimentaire: milieux et techniques générales de culture. Dunod, Paris, pp. 178–180.

Han, L. 2024. Harnessing the power of PGPR: unraveling the molecular interactions between beneficial bacteria and crop roots. *Molecular Soil Biology*. **15**(1). 8–16. https://doi.org/10.5376/msb.2024.15.0002

Mahmoudi, M. E. 2012. Contribution à l'étude de Botrytis cinerea Pers agent de la pourriture grise. PhD diss., École Nationale Supérieure Agronomique, Algiers. Available at: https://theses-algerie.com/1847331316348892/memoire-de-magister/ecole-nationale-superieure-agronomique---alger/contribution-%C3%A0-l-%C3%A9tude-de-botrytis-cinerea-pers-agent-de-la-pourriture-grise

Mina, D., Pereira, J. A., Lino-Neto, T., Baptista, P. 2020. Screening the olive tree phyllosphere: search and find potential antagonists against *Pseudomonas savastanoi* pv. savastanoi. *Frontiers in Microbiology* **11**, 2051. https://doi.org/10.3389/fmicb.2020.02051

Nautiyal, C. S. 2000. Biocontrol of plant diseases for agricultural sustainability. In: *Biocontrol Potential and its Exploitation in Sustainable Agriculture: Crop Diseases, Weeds, and Nematodes.* 9–24. Springer US, Boston, MA. https://doi.org/10.1007/978-1-4615-4209-4_2

Photolo, M. M., Mavumengwana, V., Sitole, L., Tlou, M. G. 2020. Antimicrobial and antioxidant properties of a bacterial endophyte, Methylobacterium radiotolerans MAMP 4754, isolated from *Combretum erythrophyllum* seeds. *International Journal of Microbiology* 2020, 9483670. https://doi.org/10.1155/2020/9483670

Poorniammal, R., Sundaram, S., Kumutha, K. 2009. In vitro biocontrol activity of *Methylobacterium extorquens* against fungal pathogens. *International Journal of Plant Protection* **2**, 59–62.

Risoli, S., Cotrozzi, L., Sarrocco, S., Nuzzaci, M., Pellegrini, E., Vitti, A. 2022. Trichoderma-Induced Resistance to Botrytis cinerea in Solanum Species: A Meta-Analysis. Plants 11, 180. https://doi.org/10.3390/plants11020180

Schierling, T. E., Vogt, W., Voegele, R. T., El-Hasan, A. 2024. Efficacy of *Trichoderma* spp. and *Kosakonia* sp. both independently and combined with fungicides against *Botrytis cinerea* on strawberries. *Antibiotics* 13, 912. https://doi.org/10.3390/antibiotics13090912

Wang, S.-L., Hsiao, W.-J., Chang, W.-T. 2002. Purification and characterization of an antimicrobial chitinase extracellularly produced by *Monascus purpureus* CCRC31499 in a shrimp and crab shell powder medium. *Journal of agricultural and food chemistry* **50**, 2249–2255. https://doi.org/10.1021/jf011076x