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AUTHORS: Hatice ÖGÜTCÜ, Hulya AVSAR

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Characterization and Siderophores Production of *Rhizobium* spp. Isolated from wild Legumes

Hatice OGUTCU*, Hulya AVSAR

Ahi Evran University, Faculty of Agriculture, Department of Field Crops, Kırşehir, Turkey

* Corresponding Author : hogutcu@gmail.com
ORCID: 0000-0001-7100-9318

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Abstract:

Legume plants are very significant not only ecologically but also agriculturally because they are responsible for major change of nitrogen from atmospheric N₂ to ammonia. In this study, total 56 isolates of *Rhizobium* spp. which were previously isolated from wild legumes plant (*Melilotus officinalis*, *Medicago sativa* and *Vicia cracca*) existing in the central and the districts of Kırşehir province (Kaman, Mucur, Akpınar, Akçakent, Çiçekdağı, Boztepe). In order to characterize the isolates; YMA containing Bromothymol blue, Congo red, Gram stain reaction, movement, catalase and oxidase tests were evaluated. In addition, isolates of *Rhizobium* spp. (wild type) were screened for their ability to produce siderophores and it was determined that 50 of 56 isolates in total can produce siderophore.

1. Introduction

Legume crops play major role in sustainable agriculture in drought areas. Root nodule bacteria (*Rhizobium* spp.) are widely used in agriculture practice to increase the ability of legume plants to fix atmospheric nitrogen [1]. Nitrogen (N₂) is necessary nutrient for plant growth. Chemical fertilizers are used to achieve high yield in agricultural applications, but they are both expensive and have a significant detrimental effect on the environment. Therefore, environmentally friendly and sustainable agricultural practices have recently increased the interest in organic farming [2,3]. Expanding and increasing the use of bio-fertilizers (for example *Rhizobium* spp.) decreases need for chemical fertilizers, and also reduces the threatening effects of fertilizers on human, soil and the environment [4]. Nowadays, natural legumes plants and symbionts have attracted attention of ecologists due to their tolerance to extreme environmental conditions such as severe drought, high temperature and salinity [5,6,7]. Iron is necessary for all living organisms [8]. In environments where iron deficiency, bacteria supply

their iron requirement by iron-binding ligands called siderophores [9,10].

Siderophore (production and utilization) is specific attention in *Rhizobium* spp. owing to iron is required for nodule creation, leghemoglobin, nitrogenase, ferredoxin other electron transport, and symbiosis [9,11,12]. The objective of this study is to determine the phenotypic properties of natural *Rhizobium* spp. isolated from different ecological areas and their ability to produce siderophore.

2. Material and methods

2.1. Isolation of *Rhizobium* spp. and morphological, physiological, biochemical characterization

The isolates (from nodules of root) were sum up from natural vetch (*Vicia cracca*), yellow melilot (*Melilotus officinalis*) and alfalfa (*Medicago sativa*) plants existing which were previously isolated in the central and the districts of Kırşehir province (Kaman, Mucur, Akpınar, Akçakent, Çiçekdağı, Boztepe), Turkey. The isolates of *Rhizobium* spp. is done on YEMA (Yeast Extract Mannitol Agar) media through streak plate method then plates were incubated at 28°C for 24-72 hours and colony growth

is followed. Single colonies were defined and controlled for purity on YEMA medium [13,14] and colony morphology, Congo red reaction, Gram-stain reaction. Colony property (color, mucosity, borders, transparency and elevation), movement, catalase, oxidase tests and acid / alkaline reaction were evaluated on YMA containing bromthymol blue (0.00125 mg kg⁻¹) as indicator [15]. All of isolates stored at -20°C in 25 % glycerol-YM broth.

2.2. Siderophore production

The experiment reported by Schwyn and Neilands [16] were applied for the qualitative detection of siderophore production (Chrome Azurol S Dye (CAS) Agar) in different strains. A loop was obtained from 7 day old YMA plaques were infected into the middle of the CAS medium and were incubated for 5-7 days at 30 °C. The colony dia was observed and the halo region formed around the colony was measured as defined by Van-Rossum et al. [8,17].

3. Results and discussion

3.1. Morpho-physiological, biochemical characterization

In this study, it was determined that all of the 56 isolates tested had normal colonies, producing creamy, medium to high mucus. When all 56 samples showing the presence of Rhizobia were subjected to Gram staining all isolates were found to be Gram negative, because the cells appeared pink. After 3 to 5 days of growth at 28°C in YMA, all isolates acidified medium (as shown by bromothymol blue). With respect to biochemical characterization, all the isolates showed positive for catalase and oxidase as reported in the Bergey's Manual Systematic Bacteriology (Table1) [16,17,6,18]. Similarly, Prajapati et al., (2018) reported that *Rhizobium* spp. isolates were positive for oxidase and catalase test [19].

3.2. Production of siderophores

The detection of siderophores utilizing the Chrome Azurol S (CAS) agar plaques was based on capacity of the siderophores to act as chelating agents with changing affinity for iron. Presence of iron chelator is shown by coloring the blue colored ferric CAS complex and this is determined by the formation of an orange halo around the colonies in the CAS plates. In this work, siderophore produce ability of 56 isolates were tested and 50 of them were positive and 6 were negative on CAS agar.

Table 1. Morpho-physiological, biochemical characteristics of *Rhizobium* spp.

| Isolate No. | Brom th. blue | Cong o red | Cell mor | Gram react | Move | Catal | Oxid |
|-------------|---------------|------------|----------|------------|------|-------|------|
| HR 1 | yellow | white | rod | - | + | + | + |
| HR 2-1 | yellow | white | rod | - | + | + | + |
| HR 2-2 | yellow | white | rod | - | + | + | + |
| HR4 | yellow | white | rod | - | + | + | + |
| HR 7a | yellow | white | rod | - | + | + | + |
| HR 7b | yellow | white | rod | - | + | + | + |
| HR 14 | yellow | white | rod | - | + | + | + |
| HR 16-1 | yellow | white | rod | - | + | + | + |
| HR 16-2 | yellow | white | rod | - | + | + | + |
| HR 19 | yellow | white | rod | - | + | + | + |
| HR 28 | yellow | white | rod | - | + | + | + |
| HR 29 | yellow | white | rod | - | + | + | + |
| HR 31 | yellow | white | rod | - | + | + | + |
| HR 33-2a | yellow | white | rod | - | + | + | + |
| HR 33-2b | yellow | white | rod | - | + | + | + |
| HR 33-3aA | yellow | white | rod | - | + | + | + |
| HR 36-1 | yellow | white | rod | - | + | + | + |
| HR 36-2 | yellow | white | rod | - | + | + | + |
| HR 37 | yellow | white | rod | - | + | + | + |
| HR 38-1a | yellow | white | rod | - | + | + | + |
| HR 38-2 | yellow | white | rod | - | + | + | + |
| HR 40-1a | yellow | white | rod | - | + | + | + |
| HR 40-1b | yellow | white | rod | - | + | + | + |
| HR 40-2 | yellow | white | rod | - | + | + | + |
| HR 41-1 | yellow | white | rod | - | + | + | + |
| HR 41-2 | yellow | white | rod | - | + | + | + |
| HR 42 | yellow | white | rod | - | + | + | + |
| HR 43-2 | yellow | white | rod | - | + | + | + |
| HR 45-1 | yellow | white | rod | - | + | + | + |
| HR 48-1 | yellow | white | rod | - | + | + | + |
| HR 48-2 | yellow | white | rod | - | + | + | + |
| HR 49 | yellow | white | rod | - | + | + | + |
| HR 50-1 | yellow | white | rod | - | + | + | + |
| HR 50-2 | yellow | white | rod | - | + | + | + |
| HR 51 | yellow | white | rod | - | + | + | + |
| HR 52-2 | yellow | white | rod | - | + | + | + |
| HR 59-1a | yellow | white | rod | - | + | + | + |
| HR 59-1b | yellow | white | rod | - | + | + | + |
| HR 59-2 | yellow | white | rod | - | + | + | + |
| HR 102-1 | yellow | white | rod | - | + | + | + |
| HR 102-2 | yellow | white | rod | - | + | + | + |
| HR 103 | yellow | white | rod | - | + | + | + |
| HR 104-2b | yellow | white | rod | - | + | + | + |
| HR 105-2 | yellow | white | rod | - | + | + | + |
| HR 106-1 | yellow | white | rod | - | + | + | + |
| HR 106-2 | yellow | white | rod | - | + | + | + |
| HR 107 | yellow | white | rod | - | + | + | + |
| HR 109 | yellow | white | rod | - | + | + | + |

| | | | | | | | |
|-----------|--------|-------|-----|---|---|---|---|
| HR 111 | yellow | white | rod | - | + | + | + |
| HR 123 | yellow | white | rod | - | + | + | + |
| HR 124 | yellow | white | rod | - | + | + | + |
| HR 125 | yellow | white | rod | - | + | + | + |
| HR 132-2a | yellow | white | rod | - | + | + | + |
| HR 138-1 | yellow | white | rod | - | + | + | + |
| HR 143-1 | yellow | white | rod | - | + | + | + |
| HR 143-2 | yellow | white | rod | - | + | + | + |

Table 2. Siderophore production (halozone- mm) by different *Rhizobium* spp. on CAS agar plate

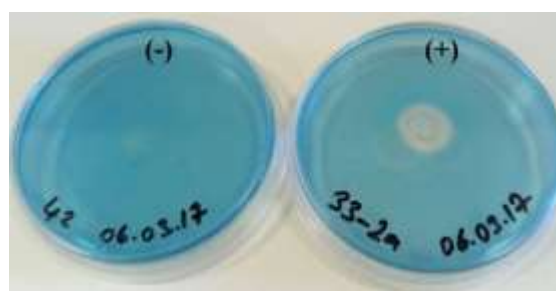
| Isolate No | Halozone (mm) | Isolate No | Halozone (mm) |
|------------|---------------|------------|---------------|
| HR 1 | 12 | HR 45-1 | 9.5 |
| HR 2-1 | - | HR 48-1 | 10.5 |
| HR 2-2 | 11.5 | HR 48-2 | - |
| HR 4 | 11.5 | HR 49 | 11 |
| HR 7a | 10 | HR 50-1 | 12 |
| HR 7b | 10 | HR 50-2 | 12.5 |
| HR 14 | 11 | HR 51 | 10.5 |
| HR 16-1 | 12 | HR 52-2 | 8.5 |
| HR 16-2 | 12 | HR 59-1a | 10.5 |
| HR 19 | 10 | HR 59-1b | 12 |
| HR 28 | 9.5 | HR 59-2 | 11.5 |
| HR 29 | 9.5 | HR 102-1 | 12 |
| HR 31 | 12 | HR 102-2 | 11 |
| HR 33-2a | 12 | HR 103 | 9 |
| HR 33-2b | 10.5 | HR 104-2b | - |
| HR 33-3a A | 9 | HR 105-2 | 9 |
| HR 36-1 | 11.5 | HR 106-1 | 10 |
| HR 36-2 | 10.5 | HR 106-2 | 10.5 |
| HR 37 | 9.5 | HR 107 | 11.5 |
| HR 38-1a | 11 | HR 109 | 10 |
| HR 38-2 | 10.5 | HR 111 | 9.5 |
| HR 40-1a | 11.5 | HR 123 | 9.5 |
| HR 40-1b | 12.5 | HR 124 | 9.5 |
| HR 40-2 | 10 | HR 125 | - |
| HR 41-1 | 9.5 | HR 132-2a | - |
| HR 41-2 | 9.5 | HR 138-1 | 10 |
| HR 42 | - | HR 143-1 | 12 |
| HR 43-2 | 9 | HR 143-2 | 9.5 |

Table 2 and Figure 1 show the production of siderophore of different *Rhizobium* spp. isolates on CAS agar plates. All isolates were to produce siderophore except for 6 isolates. Isolates HR 2-1, HR 42, HR 48-2, HR 104-2b, HR 125, HR 132-2a have not grown on the CAS agar plates. All of isolates showed ratio between 8.5mm to 12.5mm halo zone area. Similar results have been declared by Dhul et al. [8], Jossi et al. [12] and Harshitha et al. [20] in various *Rhizobium* spp.

4. Conclusions

When the studies carried out in recent years are examined, siderophores attract attention because of the emergence of different application areas (plant growth improvement, biocontrols, biosensors, bioremediation, chelation agents).

The results of this work showed that *Rhizobium* spp. isolated from wild legume plants secrete wide siderophores. The capacity to use siderophores enables natural *Rhizobium* spp. to grow better in iron limited conditions in the presence of those siderophores



(+): Positive controle (halo zone); (-): Negative control (halo zone)

Figure 1. Halo zone production by the isolates in CAS Agar

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