Isolation, Morphological and Cultural Characterization of Azospirillum Isolated from Rhizospheric Soils of Various Non-Leguminous Crops of Ranchi Having Acidic pH

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Abstract

Azospirillum is one of the versatile non-symbiotic, free living diazotrophic bacteria which appears to have a world-wide distribution and occurs in large number in the rhizosphere soil of a variety of grasses and cereals. The present study was carried out during Rabi and Kharif 2016-17 in the Department of Soil Science and Agricultural Chemistry, Birsa Agricultural University, Ranchi, Jharkhand. Efforts were made to screen out the presence of Azospirillum in rhizosphere of various non-leguminous crops and to characterize the isolates on the basis of morphological and cultural behaviours. On the basis of pH range (4.0-5.5), 54 rhizospheric soil samples were tentatively selected out of 100 samples for investigation. From the study conducted, presence of Azospirillum in rhizosphere of acidic pH was confirmed. Morphological characterization revealed that Azospirillum isolated from rhizosphere of various crops were gram negative and vibroid in shape. Cells were encapsulated i.e., were having capsules around them and formed microcyst in aged culture. Cultural characterisation revealed that colonies developed on agar slants were smooth, some of them were having raised while others were having flat elevation. Amount of growth of colonies observed were dense in 43 and thin in 11 colonies while they developed white sub-surface pellicle when grown in semi-solid Okon’s media. Out of 54 colonies, 41 were white, 5 were red and rest colonies were found yellow in colour.

Keywords
Azospirillum, Rhizosphere, Diazotrophic bacteria, Isolation, Characterisation, Microcyst, Capsule

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Introduction

Rhizosphere soil is a “hot-spot” for microbial growth and major microbial activities (Sachdev et al., 2009). It is the narrow zone of soil specifically influenced by the root system (Dobbelaere et al., 2003). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates such as amino acids and sugars providing a rich source of energy and nutrients for bacteria (Gray and Smith 2005). Root exudates are the substrate or fuel for the intense microbial (bacteria, fungi, algae, protozoa, nematodes and arthropods) activity within the rhizosphere. Thus it is the quantity and quality of the exudates and condition of the soil habitat that will determine the colonization potential of the rhizosphere (Lugtenberg et al., 2002). Azospirillum spp. isolated from various geographical regions of the world is one of the best-characterized
genus of plant growth-promoting rhizobacteria (PGPR). They are known to associate with the roots of wheat, tropical grasses, maize, and other cereals (Oh et al., 1999). The soil bacterium *Azospirillum* was first isolated from the Netherlands and originally named as *Spirillum lipoferum* by Beijerinck et al., (1925). Later Schroder (1932) isolated from the soils in Germany and Austria. Till now, they have been isolated from the rhizosphere of many grasses and cereals all over the world, in a wide variety of terrestrial and aquatic habitats of tropical as well as in temperate climates (Yooshinan, 2001). Its occurrence in the rhizosphere varied from 1 to 10 per cent to the total rhizosphere population (Okon, 1985). *Azospirilla* are gram-negative, free-living, nitrogen-fixing rhizospheric bacteria.

They display a versatile C and N metabolism which makes them well adapted to establish in the competitive environment of the rhizosphere (Hartmann and Zimmer 1994). *Azospirillum* flocs comprise a mixture of vegetative and encysted cells surrounded by a polysaccharide-rich network (capsule), conferring advantages such as stress tolerance, extended shelf life and enhanced survivability (Sadasivan and Neyra, 1985). *Azospirillum* cells appear in two distinct forms: the slightly vibroid form (V-form) occurring in young laboratory cultures and on plant roots (Tarrand et al., 1978), and the cyst form (C-form), occurring under stress or in old laboratory cultures (Sadasivan and Neyra, 1985). The C-form may be a survival structure. Occurrence of *Azospirillum* in soil is strongly pH-dependent with a pH around 7, being optimal. However, sporadic occurrence was observed even in soils with pH 4.8 (Magalhaes et al., 1983). Hence the present work was undertaken with a view to screen out the presence, isolate *Azospirillum* spp. from the rhizospheres of acidic soils of Ranchi (Jharkhand) and characterise them on the basis of their morphological and cultural behaviour.

**Materials and Methods**

**Material**

*Azospirillum* species studied in the present investigation were isolated from soil of rhizosphere having pH range of 4.0 to 5.5 of different non-leguminous crops grown in various blocks viz., Kanke, Aangara, Nagri, Bero, Itki of Ranchi district. Details of the location, soil pH and crop grown selected for isolation of *Azospirillum* are mentioned in Table 1.

**Collection of rhizosphere soil**

Rhizosphere soils were collected from the rhizospheric region of the plant at the depth of 5-6 cm near the periphery of roots of different crops from different blocks of Ranchi district in plastic bags. The soil samples were preserved in refrigerator.

**pH of soil samples**

Soil samples were collected from 100 different locations from Ranchi districts for pH analysis. The soil samples were air dried, grounded, sieved for estimation of pH by adopting standard methods. Soil pH was determined in a soil water suspension of 1:2.5 w/v, stirred at regular intervals for 30 minutes using pH meter (Jackson 1973). Details of selected 54 soil samples selected for isolation of *Azospirillum* has been presented in Table 1.

**Isolation of *Azospirillum* spp.**

Isolation of *Azospirillum* species from rhizospheric soils was done following the methods of serial dilution. From the soil samples selected on the basis of pH range (4.0-5.5), 1 g of soil was taken and serially diluted using sterile distilled water upto $10^{-6}$ dilutions. One ml of diluted sample from $10^{-4}$ to $10^{-6}$ dilutions were taken and 1ml of aliquot
was inoculated in tubes containing Okon’s Nfb (Nitrogen free bromothymol) semi-solid media. All the tubes were incubated at 35°C for 48 h and observed the growth by the formation of pellicles. Pellicles formation is considered as positive for *Azospirillum*.

Pellicles were streaked on petriplates containing Nfb Okon’s solid media and incubated at 35°C for 48 hours. Morphologically divergent *Azospirillum* colonies were picked from the plates of dilution 10⁻⁵ and streaked on basal minimal salt agar medium and incubated at 35°C for 24-48 hrs.

After attaining sufficient growth, all the isolates were preserved in a refrigerator for further investigation. The colonies developed on Okon’s agar medium (pH adjusted to 6.8) were transferred to slants of same medium and stored at 4°C.

**Okon’s Media**

Malic acid 5.00 g, KOH 4.00 g, K₂HPO₄ 0.50 g, FeSO₄.7H₂O 0.05 g, MnSO₄.7H₂O 0.01 g, MgSO₄.7H₂O 0.10 g, NaCl 0.02 g, CaCl₂ 0.01 g, Na₂MoO₄ 0.002 g, Bromothymol blue (0.5% in 95% methanol) 2.00 ml, Agar 1.8 g (semi-solid)/18 g(solid), NH₄Cl 1 g, Water 1 litre.

**Purification of the culture**

Purification of the culture was carried out by frequent transfer of colony of *Azospirillum* developed on Okon’s agar media to seal solid nitrogen free malate medium on petriplates (Okon *et al.*, 1977) having the following constituents: K₂HPO₄ 6.0 g, KH₂PO₄ 4.0 g, MgSO₄.7H₂O 0.2 g, NaCl 0.1 g, CaCl₂ 0.2 g, NH₄Cl 0.1 g, NaOH 3.0 g, Yeast extract 0.1 g, FeCl₃ 10.0 mg, Na₂MoO₄ 20.00 mg, MnSO₄ 2.10 mg, H₃BO₃ 2.80 mg, Cu(NO₃)₂ 0.04 mg, Agar 18 g, Water 1 litre

**Morphological characterization**

**Gram reaction**

Smears prepared from 48 hours old cultures were gram stained as per Huker modification (Rangaswami and Bagyaraj, 1996).

The slides were observed under compound microscope (oil immersion).

**Capsule staining**

Presence of capsules around the cells was observed on acetic crystal violet stained smears under oil immersion.

**Microcyst formation**

Stained smears of two weeks old cultures were observed under oil immersion.

Observations were recorded regarding presence of round thick walled cells as the preparation of microcysts.

**Shape**

Smears prepared from 48 hours old cultures were obtained and examined under oil immersion.

**Cultural characterization**

Different isolates of *Azospirillum* species were grown on respective standard media and their characteristic growth patterns were observed.

Serially diluted isolates of *Azospirillum* species were grown on Okon’s agar medium (Okon *et al.*, 1977) in petriplates and in tubes (for agar strokes) at 35°C for 72 hours then purification of colonies were done.

Observations were made with regard to nature of colonial growth.
Results and Discussion

In the present study, selectivity to grow on specific Nfb (Nitrogen free bromothymol) media and subsequently confirming their morphological, cultural and physiological identity with the type cultures as described in Bergey’s Manual (Buchanan and Gibbons, 1974) and Aquaspirillum taxonomy for Spirillum (Kreig and Hylemon, 1976) were taken as reference for investigation and characterization of Azospirillum isolates. A total of 54 isolates were studied under various morphological and cultural behaviours.

Morphological characteristics

All the isolates were studied for their morphological characteristics and results are presented in Table 2. Isolates were microscopically observed for their gram reaction, cell shape, presence of capsule and microcyst formation. Results revealed that the 54 isolates were gram negative in reaction and cell shape of all the isolates was vibroid when observed under microscope. These findings were confirmed by Rosemary et al., 2013 and Rasool et al., 2015. All the isolates were having capability of forming microcysts. Transition into cyst-like cells were observed in older cultures of Azospirillum was reported by Berlman (2004). Extracellular capsule was present in all 54 isolates which is in confirmity with reports of Madi et al., (1988).

Cultural characteristics

Data related to cultural characterisation has been presented in Table 2.

Colonial morphology

Study revealed that colonies developed on agar slants were smooth, some of them were having raised while others were having flat elevation. Amount of growth ranged from large to slight. 43 colonies were dense and 11 were thin in amount of growth.

Azospirillum displays high degree of pleomorphism with cellular and colony variations among the species as well as within each species depending on the strain, medium composition and culture conditions as reported by Becking, 1985. The same was investigated by Rasool et al., (2015).

Fig.1 White colonies of Azospirillum
Fig. 2 Yellow colonies of *Azospirillum*

Fig. 3 Red colonies of *Azospirillum*
Table 1 Details of 54 rhizospheric soil samples selected for isolation of *Azospirillum*

| Sl. No. | Sample No. | Place of collection | pH of the soil | Crop (previous/ present) |
|---------|------------|---------------------|----------------|--------------------------|
| 1       | AZM5       | B.A.U Campus, SSAC, Kanke block | 5.4             | Maize                    |
| 2       | AZM6       | B.A.U Campus, SSAC, Kanke block | 5.3             | Maize                    |
| 3       | AZM10      | B.A.U Campus, SSAC, Kanke block | 5.5             | Rice                     |
| 4       | AZM15      | B.A.U Campus, Tech park, Kanke block | 5.1             | Rice                     |
| 5       | AZM16      | B.A.U Campus, Tech park, Kanke block | 5.4             | Ragi                     |
| 6       | AZM17      | R.A.C Farm, W-section, Kanke block | 5.3             | Rice                     |
| 7       | AZM18      | R.A.C Farm, W-section, Kanke block | 5.1             | Ragi                     |
| 8       | AZM19      | R.A.C Farm, W-section, Kanke block | 5.2             | Rice                     |
| 9       | AZM22      | R.A.C Farm, W-section, Kanke block | 5.5             | Wheat                    |
| 10      | AZM23      | R.A.C Farm, W-section, Kanke block | 5.4             | Wheat                    |
| 11      | AZM25      | Chamghati, Aangara block | 5.5             | Rice                     |
| 12      | AZM26      | Chamghati, Aangara block | 5.3             | Rice                     |
| 13      | AZM27      | Chamghati, Aangara block | 5.1             | Rice                     |
| 14      | AZM29      | Chamghati, Aangara block | 5.2             | Rice                     |
| 15      | AZM30      | Chamghati, Aangara block | 5.4             | Rice                     |
| 16      | AZM32      | Chamghati, Aangara block | 5.4             | Rice                     |
| 17      | AZM33      | Chamghati, Aangara block | 5.3             | Rice                     |
| 18      | AZM34      | Chamghati, Aangara block | 5.2             | Rice                     |
| 19      | AZM35      | Chauli patra, Nagri block | 5.9             | Pea                      |
| 20      | AZM36      | Chauli patra, Nagri block | 4.6             | Ragi                     |
| 21      | AZM39      | Itki mor, Itki block | 4.7             | Potato                   |
| 22      | AZM40      | Itki mor, Itki block | 4.6             | Ragi                     |
| 23      | AZM42      | Itki mor, Itki block | 4.7             | Mustard + Pea            |
| 24      | AZM45      | Garhgao, Itki block | 4.6             | Pea + Sugarcane          |
| 25      | AZM46      | Garhgao, Itki block | 5.1             | Wheat                    |
| 26      | AZM53      | Devali, Itki block | 5.4             | Ragi                     |
| 27      | AZM55      | Devali, Itki block | 4.7             | Potato                   |
| 28      | AZM56      | Devali, Itki block | 4.8             | Maize                    |
| 29      | AZM60      | Bhandra, Itki block | 4.2             | Maize                    |
| 30      | AZM61      | Bhandra, Itki block | 4.7             | Onion                    |
| 31      | AZM62      | Karmatoli, Bero block | 4.1             | Pea + Potato             |
| 32      | AZM63      | Karmatoli, Bero block | 4.0             | Potato                   |
| 33      | AZM64      | Karmatoli, Bero block | 4.0             | Potato                   |
| 34      | AZM65      | Kalanji, Bero block | 4.0             | Ginger                   |
| 35      | AZM66      | Didhiya, Bero block | 4.1             | Mustard + Pea            |
| 36      | AZM70      | Tuko, Bero block | 5.1             | Pea                      |
| 37      | AZM71      | Tuko, Bero block | 4.4             | Potato                   |
| 38      | AZM75      | Parepara, Bero block | 4.6             | Pea                      |
| 39      | AZM76      | Parepara, Bero block | 4.9             | Potato                   |
| 40      | AZM77      | Parepara, Bero block | 4.7             | Lentil                   |
| 41      | AZM80      | Jainathpur, Bero block | 4.8             | Pea                      |
| 42      | AZM81      | Jainathpur, Bero block | 4.9             | Mustard                  |
| 43      | AZM83      | Bhaishmuro, Bero block | 4.4             | Ginger                   |
| 44      | AZM84      | Bhaishmuro, Bero block | 4.8             | Mustard                  |
| 45      | AZM85      | Bhaishmuro, Bero block | 4.4             | Pea                      |
| 46      | AZM87      | Bhaishmuro, Bero block | 4.1             | Ragi                     |
| 47      | AZM88      | Bhaishmuro, Bero block | 4.3             | Potato                   |
| 48      | AZM89      | Bhaishmuro, Bero block | 4.4             | Potato                   |
| 49      | AZM90      | Bhaishmuro, Bero block | 4.1             | Potato                   |
| 50      | AZM93      | Kundo, Bero block | 4.2             | Potato                   |
| 51      | AZM94      | Kundo, Bero block | 4.8             | Ragi                     |
| 52      | AZM95      | Kundo, Bero block | 4.6             | Maize                    |
| 53      | AZM99      | Bero, Bero block | 4.3             | Potato                   |
| 54      | AZM100     | Bero, Bero block | 4.7             | Ragi                     |
**Table.2** Morphological and cultural characterization of the new isolates of *Azospirillum*

| Sl. No. | Azospirillum isolates | Gram reaction | Capsule | Microcyst formation | Shape of cell | Solid agar media | Semi-solid media | Color of colony |
|---------|----------------------|---------------|---------|---------------------|---------------|------------------|------------------|----------------|
| 1.      | AZM 5                | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 2.      | AZM 6                | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 3.      | AZM 10               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 4.      | AZM 15               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 5.      | AZM 16               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 6.      | AZM 17               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 7.      | AZM 18               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 8.      | AZM 19               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 9.      | AZM 22               | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 10.     | AZM 23               | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 11.     | AZM 25               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 12.     | AZM 26               | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 13.     | AZM 27               | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 14.     | AZM 29               | Negative      | Present | +                   | Vibroid       | Smooth, Raised, Dense | White sub-surface pellicle | White           |
| 15.     | AZM 30               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 16.     | AZM 32               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 17.     | AZM 33               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 18.     | AZM 34               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 19.     | AZM 35               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 20.     | AZM 36               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 21.     | AZM 39               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 22.     | AZM 40               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 23.     | AZM 42               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 24.     | AZM 45               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
| 25.     | AZM 46               | Negative      | Present | +                   | Vibroid       | Smooth, Flat, Dense  | White sub-surface pellicle | White           |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 26. | AZM 53 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 27. | AZM 55 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 28. | AZM 56 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 29. | AZM 60 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 30. | AZM 61 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 31. | AZM 62 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 32. | AZM 63 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 33. | AZM 64 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 34. | AZM 65 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 35. | AZM 66 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 36. | AZM 67 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 37. | AZM 68 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 38. | AZM 69 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 39. | AZM 70 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 40. | AZM 71 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 41. | AZM 72 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 42. | AZM 73 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 43. | AZM 74 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 44. | AZM 75 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 45. | AZM 76 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 46. | AZM 77 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 47. | AZM 78 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 48. | AZM 79 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 49. | AZM 80 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 50. | AZM 81 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 51. | AZM 82 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 52. | AZM 83 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 53. | AZM 84 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 54. | AZM 85 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 55. | AZM 86 | Negative | Present | + | Vibroid | Smooth, Flat, Dense | White sub-surface pellicle | White |
Colour production by colonies

Out of 54 colonies, colour of 41 was white, 5 were red and rest were yellow in colour (Fig. 1, 2 and 3). Tarrand et al., (1978) have reported that colonies of different N₂ fixing Azospirillum strain showed pink, deep pink, red or yellow colour. This was due to presence of different carotenoid pigment in that isolates as reported by Baldani et al., (1986) and Rasool et al., (2015).

Growth in semi-solid media

Investigation revealed that all the 54 isolates of Azospirillum were developed as white sub-surface pellicle in semi-solid agar media. In this zone the concentration of dissolved oxygen permits optimal respiration rates without inhibiting nitrogen fixation (Day and Dobereiner, 1976).

As growth continues and more oxygen is consumed, the pellicle moves towards the surface where a dense pellicle forms. This growth pattern of Azospirillum in semi-solid media was reported by Hossain et al., (2015). Free living diazotroph, Azospirillum are able to survive even at pH 4.0 i.e., under highly acidic conditions and they have wider availability in rhizospheric soils of different blocks of Ranchi district. They are negative to Gram’s reaction.

They are vibroid shaped cells having capsule and are able to form thick walled microcysts during unfavourable conditions which is their adaptive mechanism to survive in adverse conditions. Azospirillum spp. show high degree of polymorphism in respect to their colonial patterns, elevation etc which may be attributed to their isolation from different rhizospheric and soil conditions where they were surviving. Colour development in few colonies is due to presence of carotenoid pigments.

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