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Research article

Rhizospheric microbiota and its diversity associated with *Zephyranthes rosea* Lindl.: A medicinally important bulbaceous plant

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Abstract: *Zephyranthes rosea*, belonging to family Amaryllidaceae commonly known as 'Rain lily', is a bulbaceous species native to Peru and Columbia. In India, the plant species is used in folk medicine along with *Z. flava* for treatment of diabetes, ear and chest ailments and viral infections. A study was conducted to see the diversity of microbes associated with rhizosphere of this plant. It was observed that fungal species such as *Alternaria zinniae*, *Aspergillus niger*, *Penicillium* sp., *Paecilomyces* sp. were present in the rhizosphere. Among the bacterial diversity, 3 different species of *Bacillus* and only 1 *Streptobacillus* sp. was isolated. Some endomycorrhizal species *i.e. Gigaspora gigantea*, *Acaulospora bireticiulata*, *Glomus macrocarpa*, *Glomus* sp.-1, *Sclerocystis coccogenum*, *Sclerocystis sinuosa*, *Glomus mosseae*, *Glomus* sp.-2, were isolated from the rhizospheric soil samples. The roots were detected for extreme arbuscular mycorhizal endomycorrhizal fungal infection with Arum type colonization having rectangular and profuse vesicles with hyphae entering the cortical cells of root. About 3 different species of Actinomycetes *i.e. Streptomyces* spp. were too observed in the rhizosphere. The study indicates that the target plant species is heavily dependent on endomycorrhizal fungi and other microbes (fungal, bacterial and actinomycetes) and the microbial interaction can be attributed to the presence of some toxic alkaloids in the bulbs of the plant as per the literature review and thus, this area needs to be further explored.

Keywords: Actinomycetes - Alkaloids - ArbuscularMycorrhizae - Endomycorrhizae - Microbiota.

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INTRODUCTION

The rhizosphere is generally defined as the narrow zone of soil directly adjacent to roots which is affected by plant roots. Rhizospheric soil is known to contain higher population densities of microbes (Curl & Truelove 1986). Microbes play an essential role in the functioning of plants by influencing their working and growth. Many phytopathogenic fungi and bacteria are the source of metabolites which are phytotoxicas well as possessing some other interesting biological activities (Schrader *et al.* 2010). Rhizospheric microbes have been known to play a significant role in altering the mobility and bioavailabity of heavy metals to enable better phytoremediation (Rajkumar *et al.* 2012). Several members of the rhizosphere microbiome are advantageous to plant growth while others may be pathogenic. To improve plant growth and health, it is necessary to know which microorganism is present in the rhizospheric microbiome and what they are doing (Mendes *et al.* 2013). According to Dojima & Craker (2016), inoculated medicinal and aromatic plants with nurturing rhizospheric microorganisms enhance growth, development and secondary metabolite production through increased nutrient and moisture availability, repressing pathogens, improving stress tolerance and increasing phytochemical synthesis. There have been many examples of plant growth promotion in bulbous plants with the help of rhizospheric microbes. Arbuscular mycorrhizal (AM) inoculation in *Allium cepa* L. (Shuab *et al.* 2014) leading

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to significant improvement in growth and development. Scagel (2003) studied the Soil pasteurization and inoculation with *Glomus intraradices* N.C. Schenck & G.S. Sm. to alter flower production and bulb composition of *Zephyranthes* species and concluded that all three studied *Zephyranthes* species *i.e. Z. candida* (Lindl.) Herb. (White Rain Lily), *Z. robusta* (Herb. ex Sweet) Baker (Pink Fairy Lily), *Z. sulphurea* Noter (Yellow Zephyr Lily) showed significant effect on AM inoculation in terms of flower production and bulb composition with *Z. Sulphurea* showing the most consistent responses to AM inoculation.

Among the different *Zephyranthes* species, *Zephyranthes rosea* Lindl. is one of the least explored plant species. *Z. rosea*, commonly known as rain lily is a species native to Peru and Columbia. The plant species is widely cultivated as ornamental plants and have become naturalized in tropical regions worldwide (Gary 2005). These plant species are tending to bloom only after heavy rainfall. They are common in those areas that receive periodic rainfall which includes recently disturbed land and grassy areas (like lawns and meadows). *Z. rosea* can reach a height of about 15–20 cm and has a very short life span. The flower develops from spherical tunicate bulbs and is bright pink in colour with six petals and a distinctive fragrance. The flowers evolve into capsules. The capsules are divided deeply into three lobes. The seeds of *Z. rosea* are flattened and shiny black. Propagation of the plant species occurs by division in clumps of bulbs, but can also be grown from seeds. When compared with other species of *Zephyranthes*, *Z. rosea* is found out to be less tolerant of colder temperatures. The plant species is having lethal toxins as the bulb contains various toxic alkaloids including Lycorine and Haemanthamine. The plant species are also used in folk medicine in India. Katoch & Singh (2015) reported that the bulbs of *Z. rosea* contain chemical compounds *e.g.* Lycorine, Galanthamine, Epimaritidine, Crinamine, Haemanthamine, Maritidine. Mujib *et al*. (2014) had previously worked on the plant *Z. rosea* on its organogenesis and plant regeneration aspects whereas no basic microbiological work has been done till date. The knowledge concerning the dynamics of soil microbial community structure has potential to yield great benefit in the development of methodologies for the manipulation of such populations as a means to enhance plant health (Mazzola 2004). Thus, this study is aimed to explore the diversity of microbes (fungal, bacterial, mycorrhizal and actinomycetes) associated with rhizosphere of this plant species.

MATERIALS AND METHODS

Sample collection

The rhizospheric soil samples of *Zephyranthes rosea* were collected from naturally growing plants alongside the Tons River, Doon valley, Dehradun, Uttrakhand.

Isolation and identification of fungal isolates

The isolation of the fungi associated with rhizospheric soil was done by taking 1 g of soil sample poured in 10 ml of distilled water and making serial dilutions from 10^{-1} to 10^{-5} and spread uniformly (Waksman 1922, Warcup 1950). These were kept in incubator for 2–4 days at 25°C.The fungi observed on PDA were then sub-cultured for identification and transferred to the culture slants for preservation and storage. Identification of the fungal species was done on the basis of morphological characteristics of the colony and microscopic examinations (Diba *et al*. 2007). The fungi were identified with the help of various taxonomic keys available in laboratory (Gilman 1957, Subramanian 1971, Singh *et al*. 1991, Watanabe 1993, Domsch *et al*. 2007).

Isolation and identification of bacterial isolates

Similarly, for bacterial isolation, nutrient agar (NA) was used as the growth medium and the dilutions were uniformly spread onto the medium. The isolation was done by using a dilution plate technique as given by Johnson & Curl (1972) at 10^6 dilutions on nutrient agar. The NA plates were incubated at 30±1 ºC for 48 hours. The pure cultures of bacteria were preserved at 40°C in NA slants after observing the abundance of bacterial growth and colony morphology. The isolated bacteria were preserved in 15% (v/v) glycerol in nutrient broth (NB) at -20°C. The identification of bacteria is based on examination of external morphology as well as biochemical tests. The physiological and biochemical characteristics of isolates were examined according to Cappuccino & Sherman (2004) and Bergey's Manual of Systematic Bacteriology, 1934. A modified method of Gram staining (Moyes *et al.* 2009) was also performed in order to differentiate Gram-positive and Gram-negative strains of bacteria.

Isolation and identification of actinomycetes isolates

The agar medium was used for isolation of actinomycetes (Porter *et al*. 1960). About 0.5 g of soil samples was suspended in 9.5 ml of sterile distilled water and was 1000-fold diluted, 0.1 ml of the dilutions was spread on the culture medium. The plates were incubated at 28ºC for 2 weeks. Generic level identification of

actinomycetal isolates was done through referring Bergeys Manual of Determinative Bacteriology (Brown 1939).

Isolation of Arbuscular Mycorrhizal spores

The Arbuscular Mycorrhizal (AM) spores were isolated by 'Wet sieving and decanting technique' of Gerdemann & Nicolson (1963). About 50 g of soil was mixed with 500 ml of water in the 1000 ml Beaker. The soil mixture was agitated vigorously to free the AMF spores from soil and allowed to settle for approx a day and the supernatant was decanted through standard sieves. By using a dissecting microscope and stereozoom microscope, AM spores were picked up by means of hypodermic needle.

Root colonization by Arbuscular Mycorrhizal fungi assessment

The root colonization by Arbuscular Mycorrhizal fungi was studied through 'Rapid clearing and staining technique' (Philips & Hayman 1970). The roots attached to the bulbs of *Z. rosea* were dug out and collected in the sample bag. The roots were then cut from the bulbs and washed thoroughly under the tap water. It was then submerged in 10% KOH solution in a petri dish and kept for 6–8 hours or overnight for field grown roots. KOH solution was used for clearing the tannins present in the roots. The roots were soaked in 1% HCl for 5–10 minutes. After 5–10 minutes, HCl was removed from the plate. The roots were then allowed to simmer in Trypan blue or Cotton blue stain for 6–8 hours.

RESULTS AND DISCUSSION

Several fungal, bacterial and actinomycetes colonies were isolated from the rhizospheric soil of this plant species which were later sub-cultured and identified (Fig. 1; Table 1). Among the fungal isolates, colonies of *Alternaria zinniae*, *Aspergillus niger*, *Penicillium* species and *Paecilomyces* species were observed. All the isolated fungal colonies are filamentous fungi belonging to sub-Phylum Pezizomycotina and are commonly recognized soil fungi (Egbuta *et al*. 2016). Such type of fungal association have already been reported in the rhizospheric microbiome of various plants species like *Oroxylum indicum* (Debi & Parkash 2017), Soyabeen (Al-Sheikh & Abdelzaher 2010), *Jatropha curcas* (Venkatesan 2013).

Table 1. Isolated rhizospheric microflora.

Fungal isolates	Bacterial isolates	Actinomycetes isolated	Endomycorrhizal (AM) spores	AM fungal colonization
Alternaria zinniae Ellis	*Bacillus* sp. -1	*Streptomyces* sp. -1	*Gigaspora gigantia* Gerd. & Trappe	Extreme infection observed. Arum type colonization having rectangular and profuse vesicles with hyphae entering the cortical cells of root
Aspergillus niger van Tieghem	*Bacillus* sp.- 2	*Streptomyces* sp. -2	*Acaulospora bireticiulata* Rothwell & Trappe	
Penicillium sp.	*Bacillus* sp. -3	*Streptomyces* sp. -3	*Glomus macrocarpum* Tul. & C. Tul.	
Paecilomyces sp.	*Streptobacillus* sp.	----------	*Sclerocystis coccogenum* Pat.	
----------	----------	----------	*Glomus* sp.	
----------	----------	----------	*Glomus mosseae* Gerd. & Trappe	
----------	----------	----------	*Sclerocystis sinuosus* Gerdemann and Bakshi	

The isolated bacterial colonies have been shown in table 2. It is evident from the table that three different *Bacillus* species and one *Streptobacillus* species were present in rhizospheric soil. *Bacillus* species-1 was having an elevated glistening yellow colour colony. The *Bacillus* species-1 was gram-negative in nature with long rods and clustered arrangement. A mucoid creamy coloured translucent colony was observed in *Bacillus* species-2 which was gram-positive species with long rods and clustered arrangement. Another gram-positive bacterial colony with the clustered arrangement was observed in case of *Bacillus*-3 but it had short rods and a shiny yellow colony. This was also a *Bacillus* species but different from the first two isolated *Bacillus* species in terms of colony morphology, shape of rods and gram staining, thus the fourth isolated bacterial species was *Streptobacillus* species which was gram-negative in nature and had opaque creamy coloured colony. The rods were short and were found in chains. Similar bacterial association in soil rhizosphere has also been observed in *Abroma augusta* (Parkash & Saikia 2018).

The Actinomycetes isolated from the rhizosphere of *Zephyranthes rosea* are listed in table 3. Three different *Streptomyces* species were isolated and all were gram-positive in nature. The main difference between the three

species was in terms of colony morphology, shape of rods and their arrangements. *Streptomyces* species-1 had spider web-like colonies which were convex, serrated and had white aerial mycelium. The rods were long in clustered arrangement. Whereas, round convex serrated colonies were observed in case of *Streptomyces* species-2, which also had long rods but in chains. The *Streptomyces* species-3 was short rods in chains and the colony was convex and serrated but slimy. Jeffrey (2008) worked on isolation, characterization and identification of

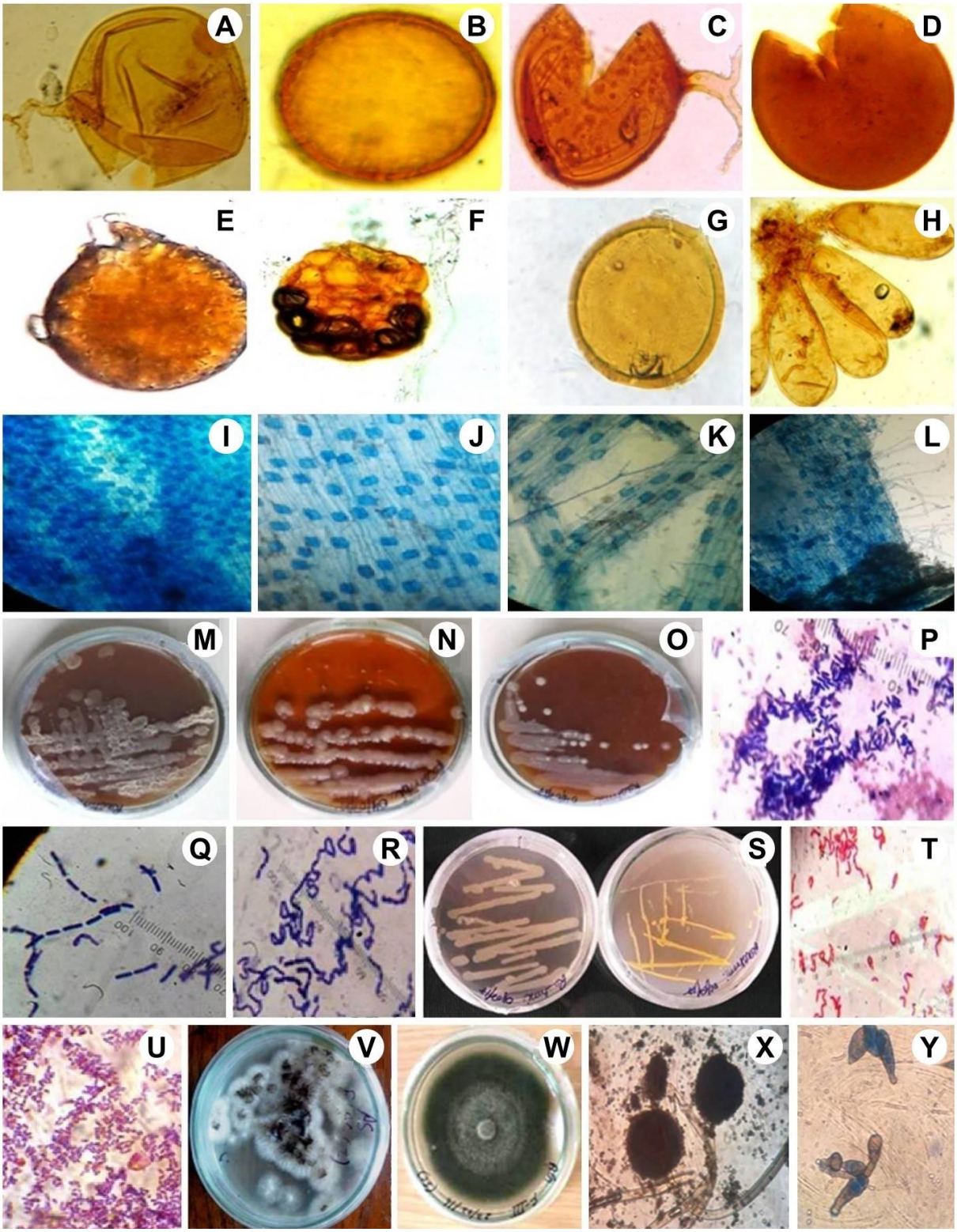

Figure 1. Microbial diversity of Rhizospheric soil samples of *Zephyranthes rosea* Lindl.: **A**, *Gigaspora gigantia*; **B**, *Acaulospora bireticiulata*; **C**, *Glomus macrocarpum*; **D–E**, *Glomus* sp.; **F**, *Sclerocystis coccogena*; **G**, *Glomus mosseae*; **H**, *Sclerocystis sinuosus*; **I–L**, AM Fungal Infection; **M–O**, *Streptomyces* spp. onto culture medium **P–R**, Microscopic images of *Streptomyces* spp.; **S**, *Bacillus* spp. on Nutrient Agar; **T**, Microscopic view of *Streptobacillus* sp.; **U**, Microscopic image of *Bacillus* sp. **V–W**, *Aspergillus niger* and *Alternaria zinniae* on culture medium; **X–Y**, Microscopic images of *Aspergillus niger* and *Alternaria zinniae*.

Actinomycetes from agriculture soils at Semongok, Sarawak and found that all the isolates belonged to genus *Streptomyces*. In this study too, all Actinomycetes isolates belonged to one single genus *Streptomyces* indicating wide host range of this genus of Actinomycetes.

Table 2. Isolated colonies of Bacteria.

Morphology of colony	Shape	Arrangement	Gram stain	Suggested identity
Yellow, glistening, elevated	Long rods	Clusters	Gram negative	*Bacillus* sp.-1
Creamy, translucent, mucoid	Long rods	Clusters	Gram positive	*Bacillus* sp. -2
Yellow, shiny	Short rods	Clusters	Gram positive	*Bacillus* sp. -3
Creamy, opaque	Short rods	Chains	Gram negative	*Streptobacillus* sp.

Table 3. Isolated colonies of *Actinomycetes*.

Morphology of colony	Shape	Arrangement	Gram stain	Suggested identity
Serrated, convex, white aerial mycelium, spider web like colonies	Long rods	clusters	Gram positive	*Streptomyces* sp.-1
Serrated, convex, round	Long rods	Chains	Gram positive	*Streptomyces* sp.-2
Serrated, convex, slimy	Short rods	Chains	Gram positive	*Streptomyces* sp.-3

The roots of this bulbous plant species were detected for the presence of Arbuscular Mycorrhizal (AM) infection and extreme infection was observed with Arum type colonization. The hyphae were entering the cortical cells of root and profuse rectangular vesicles were also observed. Different endomycorrhizal AM spores of *Glomus* species *i.e. Glomus mosseae*, *Glomus macrocarpum* were isolated. AM spores of *Sclerocystis coccogenum*, *Sclerocystis sinuosus*, *Gigaspora gigantean* and *Acaulospora bireticulata* were also observed in the rhizosphere of this plant species. The observation that this plant is heavily infected with Arbuscular Mycorrhizae cannot be considered ubiquitous as *Zephyranthes durmmondii*, another ornamental plant of the same genus has been recorded with the absence of AM association (Muthukumar & Udaiyan 1994).

The rhizosphere of this plant species is found to harbour large number of microbes which indicates its probable importance to help in bioremediation as microbes help to alter the mobility and bioavailabity of heavy metals. Previously, *Zephyranthes candida* another species of the same genus has been recorded to accumulate moderate-high concentrations of Cr (Chromium) and other heavy metals (Yuan *et al.* 2016). Also, the interaction of microbial communities of the rhizosphere with bulbous roots can be attributed to the presence of valuable alkaloids (Perry *et al.* 2007). The phytocompounds such as Lycoriside 2 and Lycorine- lO-/3-D-glucoside 4 are accumulated by some plants of Amaryllidaceae family which are responsible to reject the vast majority of micro-organisms and phanerogamic parasites colonization in the root system (Ghosal *et al.* 1985). Since, *Z. rosea* is recorded with high rhizospheric microbial diversity, it may be concluded that these two Lycorine compounds may be absent in its roots as compared to *Z. durmmondii*, which is reported with absence of AM association (Muthukumar & Udaiyan 1994) earlier and such studies can be taken up in near future to see the microbial interaction in accumulation/production of phytocompounds with bulbous root system of plants.

CONCLUSION

The study indicates that the target plant species is profoundly associated with endomycorrhizal fungi and other microbes *i.e.* Fungi, Bacteria and Actinomycetes and the this microbial association and interaction can be attributed to the presence of some toxic alkaloids in the bulbous roots of the target plant as per the literature review and thus, this area needs to be further explored. Also, the plant exhibit future scope in the area of bioremediation and which can be further studied.

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