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A high throughputs and consistent method for the sampling and isolation of Endophytic bacteria allied to high altitude the medicinal plant *Arnebia benthamii* (Wall ex. G. Don)

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A B S T R A C T

Bacterial communities in the root zones are vibrant and play an essential role in medicinal plant microbe interaction and are a major driving force for maintaining the stability of soil ecosystem. The major bottleneck for the studying the interaction lies on sampling methods. Though various methods from time to time have been reported and developed however each method exhibited variation and inconsistency. We recommend here the standardized method after consulting the major developed methods so far across the globe. Primarily we divide the root zone in major bulk and rhizopshere fractions and a definite bacterial gradient around the root zone of the medicinal plant *Arnebia benthamii* (Wall ex. G. Don), a high altitude medicinal herb was observed. We propose to use this method as general basis for soil sampling and enumeration of bacterial diversity from root systems.

1. Introduction

Micro-diversity depicts the intricacy and unevenness among microbes at various hierarchical organizations which includes genes, species, communities, ecosystems etc. Microorganisms are found everywhere but they always act in consortium and this theory is best explained in Great Plate Count Anomaly [1] which states that less than 1% of all microorganisms can be productively cultured on their own. It is understandable now that activity of microorganisms modulates the plant architect and its diverse functions [2, 3] besides improving soil structure and fertility [4].

The diversity of these microbes is being explored via surveys drawing hundreds and thousands of samples [5] and other artificial conditions [6] with swift genetic assessment modus operandi for functional and structural diversity [7, 8]. Even so the imperative confront for us is to unstitch the community diversity and comprehend their dynamics particularly regarding their prospective interactions with allied plants [9] particularly monitoring their mutualism [10]. This entails to have foremost separate scenario regarding the bulk soil and rhizosphere for evaluating their microbial diversity [11].

The slight thin zone of soil surrounding and influenced by plant roots is a hotspot for abundant microorganisms with dominance of bacteria and fungi [12] and is considered as one of the multifaceted ecosystems of biosphere [13]. These microbes acquire huge amounts of rhizodeposits released by plant roots regulating their dynamics and activity on plants [14].

Though number of arguments are in vogue regarding the activity and influence on plants with one school of thought postulating the possible role of stimulating only those

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microbes with beneficial traits over them while other advocated that exudates are passively released as waste products [15]. But these rhizospheric microorganisms are well studied for their beneficial effects on plants like N_2 fixation, PO_4 soluibilisation, biocontrol mechanism, induced systematic resistance etc. Consequently, it signifies that root sphere influences the type and abundance of microbial community and not startlingly the bacterial diversities between bulk and rhizosphere soil will significantly differ [5, 16] and it is of substantial significance to isolate and evaluate each fraction for total bacterial diversity depicting their role and function [2].

Despite the fact that there are uncountable research findings regarding the microbial extraction in the rhizosphere area however full-fledged methodology has not been yet observed perhaps may be due to the rhizosphere zone isolation or other contributing factors like plant roots, edaphic factors etc [16, 17]. In the current study, we are proposing the standardized and reproducible methodology for sampling and isolation of rhizobacterial communities in the bulk and rhizosphere soil. The plant *Arnebia benthamii* wall Jakson selected is a high altitudinal medicinal herb prevailing in North Western Himalayan region. The plant is well suited in the sand loam soil at higher areas above the alpine zone mostly seen at an altitude of 3000-4000 m a.s.l [18, 19]. It is a perennial plant with rhizomes along with the large tiny and strong roots. We believe that the methodology developed will be best suited for the mountainous herbs.

2. Materials and methods

The whole plants along with associated soil samples of the medicinal plant *Arnebia benthamii* (Wall ex. G. Don) were collected from four different sites in the Kashmir Region i.e. Raiyel, Vishenser, Duksum, Sinthon Top during the flowering stage in the month of July-August 2016. All the sites were located at an average altitudinal height of 3500-4500 m above sea level. The average depth up to which soil sample taken was 20-35 cm. All the soil samples were separately processed in Laboratory for isolation of rhizobacteria.

The overall texture of soil collected was sandy loam. The soils were instantly transferred into Ziploc bags to avoid excessive dehydration during transport and were stored afterward at 4^0C. Each soil samples were separated into Rhizosphere (R) and Bulk soil fractions. The bulk soil was vigorously shaken by hand for 10-15 min or shaking was stopped when all non adhered soil particles are detached from roots. The 10/100 g of bulk soil was suspended in 100/1000 ml 0.85 % NaCl solution. However the best way to collect the rhizosphere soil was by immersing of hand shaken roots in 500 -1000 ml autoclaved saline water (0.85% NaCl) supplemented with 0.03 % Tween-20 for around 15 min. Both the Bulk and rhizosphere soil suspension fractions were incubated in orbital shaker at 3000 rpm, 10 min, 25 ^{0}C followed by centrifugation (4000 rpm, 10 min) the supernatant was collected in fresh sterile tubes and was subsequently filtered through whatman's filter paper (No 42) to eliminate any residuals in suspension.

2.1 Enumeration of bacterial diversity

Serial dilution of collected supernatant of each fraction was carried out to enumerate the bacterial strains from both the fractions. 1 ml of supernatant was transferred into 9 ml sterile saline water until reaching 10^{-5}. Spread plate technique was followed for CFU (colony forming unit) determination. Generally numerous fastidious nutrient agar media are recommended however R2A agar medium (*Hi media*) was found sturdy and best for growth of all types of bacteria. The number of cfu was obtained by spreading 100 µl of each dilution onto R2A agar plates supplemented with cyclohexamide (100 mg/l). All the agar plates were kept in bacteriological incubator at 27 ^{0}C for 24-48 hrs followed by colony counting on colony counter. The whole process is depicted in a flow chart (Fig1).

2.2 Statistical analysis

The number of CFU obtained were subjected to statistical analysis by SPSS using Dunnet's multiple range test at a significance level $P<0.05$.

3. Results

The soil samples from 4 different sampling sites (S1, D2, V3, R4) were screened for the bacterial richness of their bulk and rhizosphere part in the above revealed protocol. The estimated diversity was expressed as cfu/g soil (dw). Gentle shaking of roots processed the rhizosphere sample and 1 g of soil was suspended in 9 ml autoclaved water and shaken for 1 hr. The dilution range 10^{-3} was best enumerable and viable for counting and obtaining bacterial colonies in all soil fractions. Preferably saline water (0.85% NaCl) is used for dilution and isolation was added with Tween and is often encountered. Tween 20 is a mild nonionic surfactant and is highly hydrophobic as well as with emulsifying effect and generally acting as wetting agent, dispersant and solubiliser. The Bulk soil values ranges from 1.9 x 10^6 (S_1) to 1.05 x 10^6 (R_4) cfu/g dw (Fig 2). The bacterial density was found significantly higher in rhizosphere soils and definite gradient was observed. Similarly as recorded for Bulk soil, the values of colonies in rhizosphere ranges between 3.7 x 10^6 (S_1) to 2.5 x 10^6 (R_4) (Fig 2) and the results obtained are statistically significant at $P<0.05$. The bacterial density was almost obtained is almost similar which ranges from 10^6-10^8 for bulk and for rhizosphere it ranges 1.3 x 10^8-6.2 x 10^8 cfu/g dw soil (Fig 2).

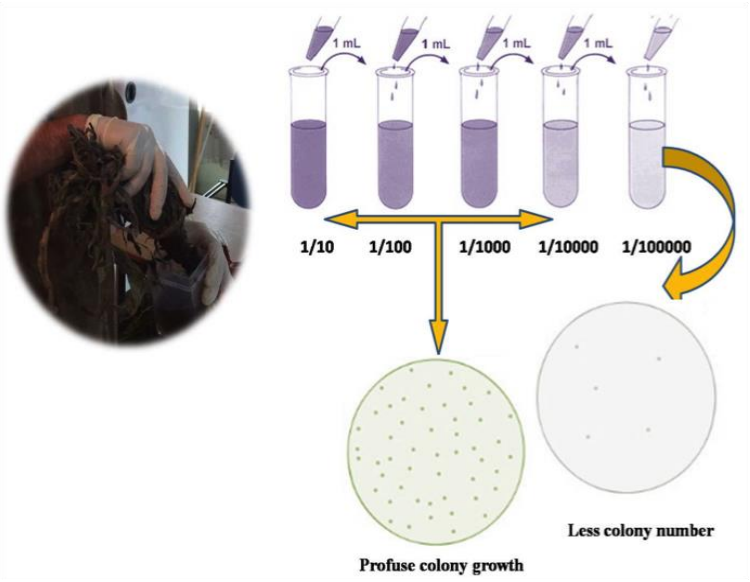

Fig1. Diagrammatic representation of developed protocol of isolation and enumeration of rhizobacteria.

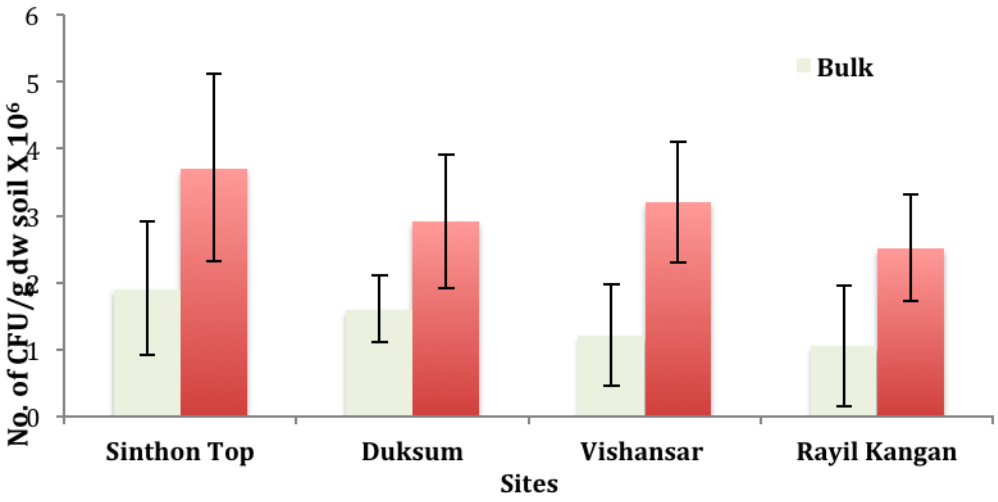

Fig 2. Enumeration of bacterial diversity from rhizosphere and bulk soil from the roots of A. benthamii from four sites

4. Discussion

Though the values of culturable colonies didn't varies with in different sites but there was a significant gradient between the bulk and rhizosphere soil fractions. Different strategies for soil sample collections have been suggested from time to time [13, 20] however recent methods encountered are by Barillot et al [21] who divided the root zone of *A.thaliana* into bulk, rhizosphere and rhizoplan fractions and also shaking of roots was validated in this protocol. Another topical method used only bulk and rhizosphere fractions and used sonication technique for isolation from *Glycine max* [17].

The rhizosphere sample was processed by gentle shaking of roots and 1 g of soil was suspended in 9 ml autoclaved water and shaken for 1 hr [2, 3]. Preferably saline water (0.85% NaCl) is used for dilution and isolation was added with Tween and is often encountered [3, 22]. Though there are numerous reports depicting use of buffers particularly phosphate buffer saline (PBS) however it is not preferred due to interference of PO_4^- with soil chemistry [23].

Tween 20 is a mild non ionic surfactant and is highly hydrophobic as well as with emulsifying effect and generally acting as wetting agent, dispersant and solubiliser. The major annotations asserted from above results is a definite bacterial diversity gradient moving from bulk to rhizosphere and viability of protocol for isolation of bacterial strains from any soil fractions particularly associated with herbaceous mountainous plants in loam sand soil. Our results are in consistent with Ofek et al. [24] who divided the root zone into bulk, rhizosphere and rhizoplan fractions of *A. thaliana.* Various general purpose nutrient media TSA agar media [21], LB agar media [13, 25], R2A gar media, Pikoskay's gar media[26] are generally recommended for isolation of rhizopsecfic or soil bacteria, however R2A was highly recommended amongst the other media used [27].

R2A is considered as low nutrient media and in combination with low temperature and long incubation time stimulates the growth of stressed and fastidious bacteria [10, 28].Though views of authors are not common on the use of solution for soil suspension [17, 28] but the widely accepted point is the shaking step which only depends upon the complete removal of adhered soil [15, 16].

5. Conclusion

The standardization of sampling protocol will ease the study of bacterial diversity particularly in rhizosphere region and above developed method will solve the all hindrance for studying plant microbe interactions. The method is validated for sandy loam soils and surely will work on clay soils as well and surely is in agreement with previous studies developed from time to time.

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Conflict of interest

Authors declares no conflict of interest

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

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