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Screening of Potential Bioinoculants from leaves of the weed *Parthenium hysterophorus*

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ABSTRACT

The present invention relates to the field of microbiology and agricultural biotechnology. In particular, the invention relates to the isolation and screening of potential phyllosphere microorganisms from Parthenium weed for their bioinoculant activity and their ability to enhance the germination of brinjal and chili seeds. The invention provides a novel method for isolating and screening microorganisms from the phyllosphere of Parthenium, a noxious weed. The method involves collecting Parthenium leaves from various locations, surfacesterilizing them, and plating the resulting washes on different media. The resulting colonies are then screened for their ability to fix nitrogen, solubilize phosphate, and produce plant growth-promoting substances. The invention has identified several potential bioinoculants from the phyllosphere of *Parthenium*, which has the potential to improve the growth and yield of various crops. These microorganisms have also been tested for their ability to enhance the germination of brinjal and chili seeds. The results of these tests have shown that the cultured microorganisms from Parthenium can significantly improve the germination rate and growth of both brinjal and chili seeds under *in vitro* conditions. The invention is particularly useful for developing sustainable agricultural practices that reduce the use of synthetic fertilizers and pesticides. It offers a cost-effective and environmentally friendly solution for enhancing crop productivity, soil health, and seed germination. In summary, the present invention provides a method for isolating and screening potential bioinoculants from the phyllosphere of *Parthenium*, whichcan be used to improve crop productivity, soil health, and seed germination. Itrepresents a significant advance in the field of agricultural biotechnology andhas significant commercialization potential.

INTRODUCTION

An abundant and diverse community of microorganisms naturally exists on the surface of above-ground parts of plants, known as the phyllosphere. The phyllosphere can be subdivided into the caulosphere (stems), phylloplane (leaves), anthosphere (flowers), and carposphere (fruits). The phyllosphere is one of the most prevalent microbial habitats on earth and bacteria are by far the most abundant and persistent phyllosphere organisms, with a typical cell density of 10^6–10^7 cells cm^{-2} .Phyllosphere microbial community studies to date have mainly focused on plant species such as *Arabidopsis thaliana* (thalecress), *Lactuca sativa* (lettuce), *Glycine max* (soy bean), *Trifolium repens* (white clover), and *Oryza sativa* (rice) and the greatest microbial diversity has been described using metagenomic tools (1). Broadly, leaf microbial communities mainly comprise bacteria belonging to the phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. Further, Proteobacteria species have been reported to comprise about half of the phyllosphere community suggesting that, at higher taxonomic levels, phyllosphere bacterial communities are similar across various host plant species. Increased knowledge of plant–microbe interactions enables a better understanding of their role during natural plant growth and development, and this knowledge can be translated into improved agricultural biomass production and microbe-assisted phytotechnologies (2). In this study, the bacterial phylloplane community of *Parthenium hysterophorus* is explored using culture-dependent and -independent techniques. This weed is considered to be a cause of allergic respiratory problems, contact dermatitis, mutagenicity in human and livestock. Crop production is drastically reduced owing to its allelopathy (3).

To enhance our understanding about the diversity and function of microbial communities living in the phylloplane, culture-independent approaches are indispensable. Nevertheless, one of the key challenges for microbiologists remains to develop strategies to culture the vast diversity of microorganisms. There has been a recent resurgence in the application of classical culture techniques to interrogate the microbial world, with particular success in environments such as the human gut. In general, a wide diversity of cultured bacteria may be retrieved by

increasing the diversity of growth media used to include complex media rich in macro- and micronutrients, and custom media formulations that are more oligotrophic. This includes growth media with low concentrations of mineral salts , the addition of (host) plant extracts , separated preparation of growth medium components , and the use of a range of solidifying agents (4). Monitoring for colony formation over extended incubation periods is also useful. Once a collection of bacterial isolates is obtained and maintained in the laboratory their functional characteristics can be evaluated, including plant growth-promoting (PGP) potential through the biosynthesis of PGP hormones and production of specific enzymes.

Phyllosphere is an abode for different kinds of microorganisms. Recent developments in the advancements of molecular and computational tools, high-throughput screening procedures, and amalgamation of omics techniques have significantly enhanced the understanding of phyllosphere- associated microbial communities in relation to their structural, functional, and ecological properties (5). Several research findings indicated that phyllosphere microbiome has played an important role in sustaining crop growth and health management by regulating plant physiological processes under ever-changing environmental conditions (6).

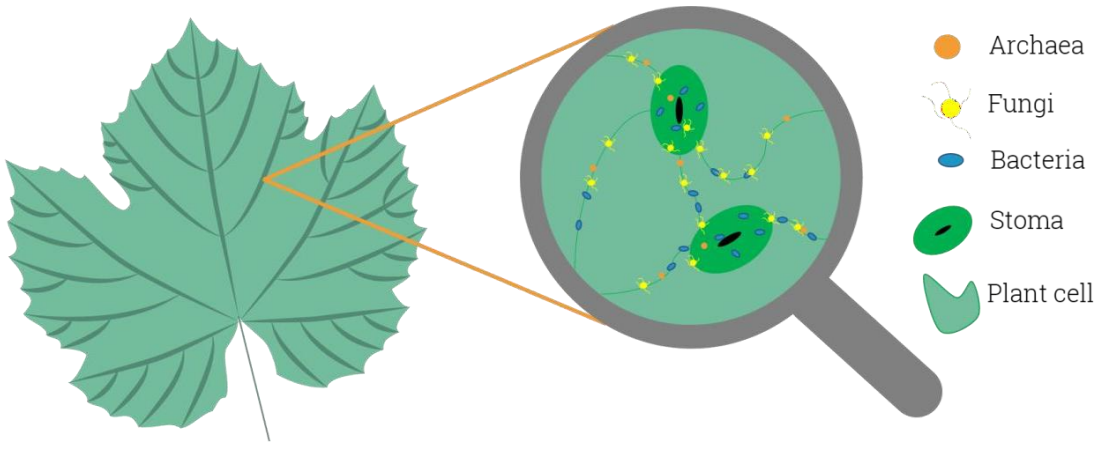
Cultivation-independent studies have revealed that a few bacterial phylapredominate in the phyllosphere of different plants and that plant factors are involved in shaping these phyllosphere communities, which feature specific adaptations and exhibit multipartite relationships both with host plants and among community members (7). Insights into the underlying structural principlesof indigenous microbial phyllosphere populations will help us to develop a deeper understanding of the phyllosphere microbiota and will haveapplications in the promotion of plant growth and plant protection(8).

MATERIALS AND METHODS

Sample collection and microbial isolation:
The fresh and healthy leaves *Parthenium* were collected from the premises Sri Shakthi institute of Engineering and Technology and put separately into sterile bags then taken back to laboratory in less than 2 hours for isolation of epiphytic and endophytic phyllosphere microorganisms.To analyze epiphytic microflora, leaf washings were used for the isolation. A leaf sample (was shaken for about 1h in 100 ml of sterile distilled water. An aliquot of 1ml from leaf wash was plated on LB Agar, 20.0 for LB Medium (g/L): Peptone, 5.0; Beef Extract, 3.0; Agar, 15.0, wasused for bacterial isolation. For endophytic microflora, leaves of each weed were washed through in running water followed by surface-sterilization in 70% ethyl alcohol (1 min), 2.6% NaClO2 (3 min), and 70% EtOH (1 min). Sterile leaves were ground in blender with 100ml of sterile distilled water toform a homogenized leaf solution mixture.Leaf mixture (1 ml) was then plated onLB Medium for fungal bacterial isolation, respectively. The Petri dishes were incubated for 3-4 days at 25- 28°C for the fungal colony count. Bacterial colonies were counted after 24 hours at 37°C and purified for further identification.

Morphological taxonomy of bacterial isolates Isolated fungal specieswere plated onto Petri dishes and incubated for 5 days at 25- 28°C in darkness to observe the colonies' morphology and measure their diameters.

Bacterial strains were identified including pigment, colony form, elevation, margin, texture and opacity (Smibert and Krieg, 1981). In addition, bacterial strains were tested with respect to Gram reaction andbiochemical characteristics (Holt et al., 1994).



Additionally, for each growth medium, representative bacterial isolates were purified such that their PGP potential could be evaluated. This study highlights the usefulness of high-throughput sequencing to evaluate the diversity of bacterial communities present on growth media in comparison touncultured samples from the original environment.

The information obtained can guide targeted single-colony isolation, focusing on growth conditions that favour certain taxa thereby increasing the likeliness to isolate previouslyuncultured or underrepresented bacterial species.

Molecular Characterization of Bacterial Isolates

Culture DNA was obtained using the lysozyme-SDS-phenol/chloroform method (Maniatis et al., 1982). DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. The extracted DNA was treated with DNase-free RNase at a final concentration of 0.2 mg/ml at 37°C for 15 min, followed by a second phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation. Finally, the DNA pellet was re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), stored at -20°C, and used as template DNA in PCR to amplify the 16S rRNA for phylogenetic analysis.

RESULTS

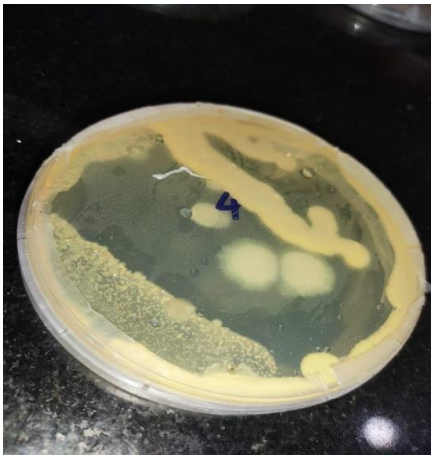


Figure 1: Microbial colonies Isolatedfrom *Parthenium*

We isolated bacteria from the phylloplane of ***Parthenium hysterophorus*** , a widespread evergreen weed, using growth media: Nutrient Agar, Nutrient Broth. We also included a comparison with the uncultured phylloplane, which we showed to be dominated by Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. The in vitro plant growth promotion (PGP) profile of the isolates obtained in this study indicates that previously uncultured bacteria from the phylloplane may have potential applications in phytoremediation and other plant-based biotechnologies.

Characteristics	10^1 dilution	10^2 dilution	10^3 dilution	10^4 dilution	No dilution
Colony characteristics	Cocci cluster	Cocci cluster	Single cocci	Cocci	Cocci
Cell morphology	Creamy, white	Creamy, dense, white	creamy	Creamy, dense, white	Creamy, dense, white
Shape	Circular, irregular	Irregular	Circular	Circular, irregular	Irregular
Size	Medium	Large	Small, tiny	Medium	Large
Margin	Wavy	Mixed boundary	Distinguishable boundary	Distinguishable boundary	Wavy
Growth on nutrient agar	Transparent colonies, thin, spotted pinpoint colonies	Thick colonies	Thick, single, pinpoint colonies. Round and small	Thick, mass (the lawn ofcolonies), small pinhole colonies	Thick, lawn of colonies

Invitro Germination Study

The results of these tests have shown that the cultured microorganisms from *Parthenium* phyllosphere can significantly improve the germination and plant growth of both varieties of brinjal and chilli seeds under *in vitro* conditions.

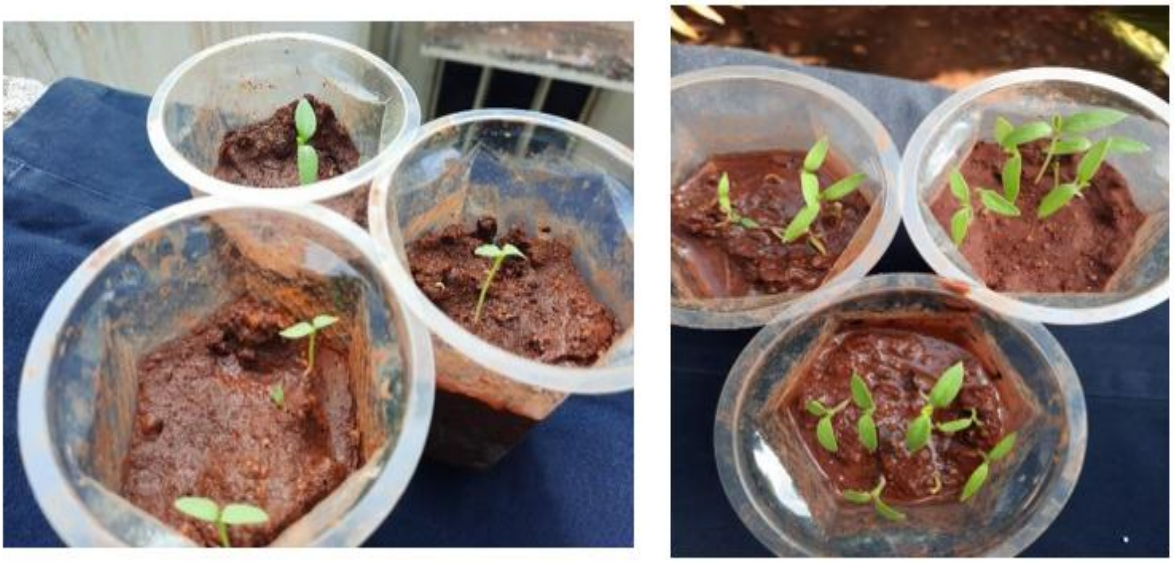


Figure 2: Germination of BrinjalCO2 seeds treated with isolates

CONCLUSION

This study gives first insights into the total bacterial community of the ***Parthenium hysterophorus***, including an evaluation of its culturability using different growth media. We further provide a collection of bacterial isolates underrepresented in current databases, including the characterization of **PGP** (plant growth promoting) profiles. Here we highlight the potential of simple strategies to obtain higher microbial diversity from environmental samples and the use of high- throughput sequencing to guide isolate selection from a variety of growth media. The present invention provides a method for isolating and screening of potential bioinoculants from the phyllosphere of *Parthenium*, which can be used to improve crop productivity, soil health and seed germination. It represents a significant advance in the field of agricultural biotechnology and has significant commercialization potential.

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Declarations:
Conflict of interest**:** The authors report no conflicts of interest.
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Ethical Clearance: Nil

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