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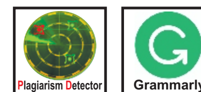

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Biological potential of *Ascophyllum nodosum* extract on rhizobial diversity in nodules of mothbean *Vigna aconitifolia* Jacq. via Amplified Ribosomal DNA Restriction Analysis

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Abstract

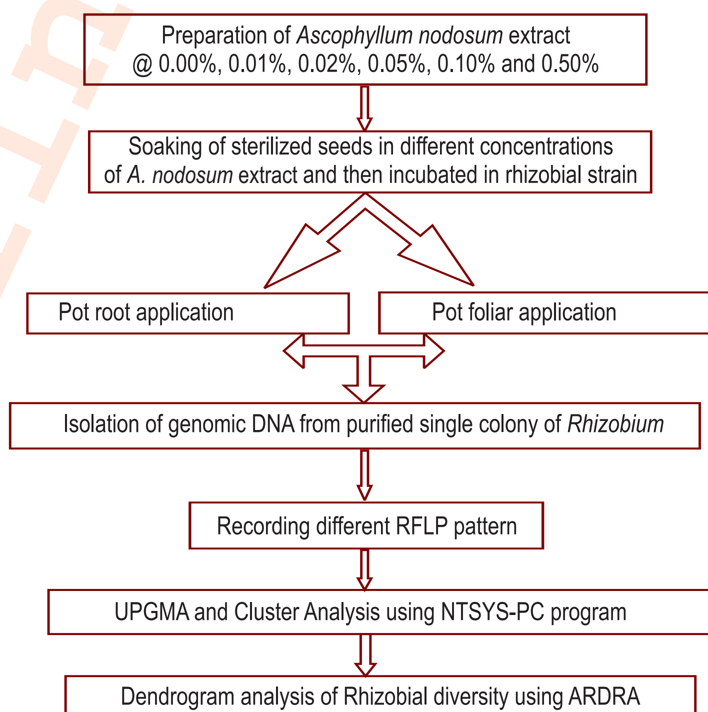
Aim: The aim of this study was to use *Ascophyllum nodosum* for potentially increasing the growth and rhizobial diversity in nodulating rhizobia in *Vigna aconitifolia*.

Methodology: Different concentrations of *Ascophyllum nodosum* extracts (0.01%, 0.02%, 0.05%, 0.10% and 0.50%) were applied via foliar spray and on roots of *Vigna aconitifolia*. Growth characteristics and Amplified Ribosomal DNA Restriction Analysis were conducted to detect the morphological and molecular changes in rhizobial diversity. The restriction profiles thus obtained were used to study the rhizobial communities via Cluster analysis and Dendrogram using NTSYS-PC program and UPGMA constructed.

Results: Roots treated with 0.05% *Ascophyllum nodosum* extract showed best growth of plants. This concentration not only proved best for the aggregation of nodules but also for obtaining enormous rhizobial diversity.

Interpretation: *Ascophyllum nodosum* is a modern, cheap, non-toxic natural biofertilizer and Amplified Ribosomal DNA Restriction Analysis represents a favorable alternative to culture dependent method for assessing rhizobial diversity in nodulating bacteria.

Key words: ARDRA, *Ascophyllum nodosum*, *Rhizobium, Vigna aconitifolia*



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Introduction

Pulses form an important nutritive supplement of human diet in India (Verma *et al.,* 2017a).Being a rich source of protein, it is known as "poor men's meat" because of its low cost as compared to animal proteins (Vietmeyer, 1986). In addition to high protein content, legumes are low in fat content. A good amount of dietary fibres and many micronutrients in legumes contribute to its nutritional properties (Rungruangmaitree *et al.,* 2017). *Vigna acontilifolia* commonly known as moth or Turkish gram is a drought-resistant legume, also growing in arid and semi-arid regions of India and Pakistan. Rajasthan, is the main moth bean producing state and contributes about 86% production in the country. It is also used to reduce fever and the roots used are as narcotic (Chunekar and Pandey, 1998; Watt *et al.,* 1889). More than 1000 accessions of *V. acontilifolia* are available in National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, out of which RMO 40 and RMO 225, cultivars are mainly grown in India.

Most legumes have the capacity to formulate a symbiotic relationship with N_2-fixing soil microorganisms rhizobacteria, broadly known as Rhizobia in the family Rhizobiaceae e.g., *Bradyrhizobium, Rhizobium, Allorhizobium, Mesorhizobium, Azorhizobium,* or *Sinorhizobium* (Velazquez *et al.,* 2010). Rhizobia are Gram-negative, motile, non-sporulating rods (Long, 1996), genetically diverse and physiologically heterogenous group of bacteria (Somasegaran and Hoben, 1994). In the rhizosphere of legumes, rhizobia are very important part of soil micro-flora (Allen and Allen, 1981; Somasegaran and Hoben, 1994). Rhizobia play a significant role in nitrogen acquisition through symbiotic nitrogen fixation in a broad variety of leguminous plants (Fujihara *et al.,* 2002). Sylvie and Patrick (2010) reported that the application of *Rhizobium* in *Phaseolus vulgaris* significantly improved the number of pods per plant, number of seeds per plant, seed weight and seed yield. Leguminous crops are widely distributed; however, in some places the soil lack rhizobial population (Brockwell *et al.,* 1995).

Seaweed extract is a new generation natural organic fertilizer with high nutritive value. It has been found to enhance the germination, nutritional value of seeds of many crops (Verma *et al.,* 2017b). Fertilizers derived from seaweeds are known as Seaweed Liquid Fertilizers. The seaweed extract contains all the essential components beneficial for the growth, development, defence against diseases (Wu and lin, 2000; Verma *et al.,* 2020). Seaweed fertilizer is an effective alternative to chemical fertilizer as it is easily absorbed by plants and has no harmful effects on the ecosystem (Sathya *et al.,* 2010). Jae-Suk Choi RIS *et al.* (2011) reported that methanolic extracts of three species of seaweed could be used as therapeutic agent. Now a day, the most important seaweed that plays a very important role in agriculture is *Ascophyllum nodosum*. *Ascophyllum nodosum* is a brown aquatic alga (Phaeophyceae) that grows along low lying and backwaters area and dominates the rocky intertidal zones of the Atlantic shores of Nova Scotia and New Brunswick, Canada (Ugarte *et al.,* 2010). Application of seaweed extract triggers the growth of important soil microbes and secretion of soil conditioning substances by these microbes. Alam *et al.* (2013) reported the positive effects of *Ascophyllum nodosum* extract on strawberries, as well as benefit to soil microbial community. Seaweed manure increases the moisture holding capacity and soil fertility by adequate supplying trace elements to improving the soil structure (Dhargalkar and Neelam Pereira, 2005). Arun et al. (2014) observed that application of seaweed extract not only improve the growth of *Abelmoschus esculentus* and *Solanum Lycopersicumbut* also improve the microbial diversity. In the present study *Ascophyllum nodosum* Extract (ANE) applied for studying the improvement in growth parameters of *Vigna acontifolia*. Further experiments were conducted to determine the nodules quality and quantity. The molecular changes in rhizobial diversity in nodulating bacterial was also carried out using Amplified Ribosomal DNA Restriction Analysis (ARD RA) and the cluster analysis was done.

Materials and Methods

Experimental design: The *Ascophyllum nodosum* (L.) Le Jolis, (Seaweed) (Trade name: Biovita) extract (ANE) was purchased from PI industries, Rajasthan. Seeds of *Vigna aconitifolia* (RMO 225) were purchased from Rajasthan and the rhizobial strains of *V. acontilifolia* were procured from the Department of Microbiology, CCSHAU, Hisar, India. Two experiments *viz.,* Pot Root Application and Pot Foliar Application were carried out. Five different concentrations of *Ascophyllum nodosum* extract (@ 0.01%, 0.02%, 0.05%, 0.10%, and 0.50%) and control (without extract) were prepared. Plastic pots measuring 30 x 30 cm were filled with 4.0 kg of sterilized river sand, autoclaved at 121°C for 1 hr.

The seeds were surface sterilized with 0.1% mercuric chloride for 3-4 min, washed 5-6 times with sterilized double distilled water. For pot root application, sterilized seeds were soaked in different concentrations of seaweed extract for 12 hrs and then the seeds were inoculated with rhizobial strain (10^9 cells) for 1 hr. Nitrogen free Slogar's solution was added every second evening alternating with water. A 5 ml of each seaweed extract was applied at a regular interval of 15 days to the roots *V. acontilifolia*. Whereas, for pot foliar application sterilized seeds were soaked in rhizobial strain directly without soaking in the seaweed extract and grown in similar manner as pot root application, except 5.0 ml of different concentration of *A. nodosum* extract were sprayed to foliar parts of plants. Each treatment was carried out in triplicate and three plants per pot were kept for further study. The number of nodules per plant in each treatment was recorded after uprooting the plants on day 80.

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Isolation of rhizobia: The roots from different ANE treated plants from PRA and PFA experiments were harvested. The nodules from roots were washed thoroughly, surface sterilized with 0.1% $HgCl_2$ (2-3 minutes) and dipped in 95% ethanol solution and then finally washed (6-7 times) with sterilized distilled water. The surface sterilized nodules were crushed with sterilized glass rod in minimal amount of sterilized water. A loopful of milky suspension obtained by crushing the nodules were streaked on sterilized Yeast Extract Mannitol Agar (Fred *et al.,* 1932) medium plates. The plates were then incubated at 30 ºC for 24 hr. *Rhizobium* cultures were streaked repeatedly on fresh YEMA plates to get a single purified colony. Finally, the purified single rhizobial isolates were cultured on fresh YEMA. After that the rhizobia were streaked on Tryptone Yeast Agar (Bringer, 1974) medium and then inoculated on TY broth for 24 hrs for further molecular studies.

Isolation of Genomic DNA: Genomic DNA was isolated following the method of Ausubel *et al.* (2002) with some modification. Bacterial strains isolated from all the treatments were cultured in TY medium for 48 hr. 1.5 ml of each culture was centrifuged for 5 min at 12,000 rpm till a compact pellet was formed. The pellet was resuspended in 570 µl TE buffer, 30 µl of 10 % SDS and 4 µl of 20 mg ml^{-1} proteinase K. All components were added to make a final volume of 100 µg ml^{-1} proteinase K in 0.5 % SDS. After proper mixing, these were again incubated for 3 hr at 37 ºC in a water bath mixed with 100 µl NaCl. To this mixture, CTAB/NaCl solution was added and mixed thoroughly and incubated for 10 min at 65°C in a water bath. Chloroform/isoamyl alcohol (24:1) of approximately equal volume was added and spinned at 12,000 rpm for 10 min.

To equal volume of supernatant, phenol/ chloroform/ isoamyl alcohol (25:24:1) was added. In a fresh tube, the aqueous phase was taken and the supernatant was mixed with two third volume of isopropanol (kept at 4°C) to precipitate the nucleic acids. The tube was shaken to and forth till a stringy white DNA precipitate was clearly visible and tubes were incubated overnight at -4°C. The following DNA was pellet by centrifugation for 15 min at rpm 12,000. After discarding the supernatant, the pellet of each treatment was washed with 200 µl of 70 % ethanol (kept at -4°C) and centrifuged at 12,000 rpm for 5 min. Pellets from each sample were dissolved by tapping in TE buffer, incubated for 10 min at 65°C, if required tapping was done again. Finally, the DNA present in the buffer solution was stored in a deep freezer.

Quantification of Genomic DNA: Genomic DNA was quantified by reading the absorbance at 260 nm and 280 nm wavelength. The amount of DNA was calculated by using the relationship that O.D. of 1.0 corresponds to 50 µg ml^{-1}. The purity of DNA was checked by measuring the ratio of A260/A280; 1.5 - 1.8 values is for pure DNA. Purity of DNA was also checked by observing bands on 0.8 % agarose gel.

Amplification of 16S rDNA sequences by Amplified Ribosomal DNA Restriction Analysis: Amplification of 16S rDNA sequences was carried out by polymerase chain reaction using a thermal cycler MJ Research, USA (Lukow *et al.,* 2000). The forward primer BAC 27F (5'- AGA GTT TGA TCC TGG CTC AG - 3') and reverse primer 1378R (5'- CGG TGT GTA CAA GGC CCG GGA ACG - 3') allow the amplification of 16S rDNA gene sequence that present in rhizobial DNA. The genomic DNA was cut with individual restriction enzyme (*Hae III* and *Msp I*) separately. The fragments thus obtained were separated on 2% agarose gel. Size of amplified 16S rDNA fragments was analyzed by using 100-1000 bp DNA ladder (medium range, Quigen) and different RFLP pattern were recorded.

Scoring and analyses: The size of each obtained band was compared with the standard marker (100-1000 bp DNA ladder) and the profiles of the isolates were prepared. Only reproducible bands were scored and analyzed. Matrix was prepared from 0-1 on the basis of presence or absence of a particular band. By following Sim Qual Coefficient, Similarity matrices were made and analyzed by UPGMA (Unweighted Pair Grouping with Mathematic Average) cluster analysis using NTSYS-PC program (version 2.1: Exeter Software, Setauket, N.Y.) (Rohlf, 1998). UPGMA Dendrograms were constructed from the genetic similarity between different *rhizobia*.

Results and Discussion

Rhizobium interaction plays an active role in plant growth. This experiment was conducted to understand the role of bioactive components of *Ascophyllum nodosum* extract on the growth and nodule formation. *Ascophyllum nodosum* extract was applied by two different methods namely, pot root application and pot foliar application to explore the possibility of application process to mitigate the logistic costs.The growth was recorded up to 75 days, at an interval of 15 days in both pot foliar application and pot root application of 0.00%, 0.01%, 0.02%, 0.05% and 0.10% and 0.50% of *Ascophyllum nodosum* extract. It was observed that root application of extracts showed significant growth and increase in leaf size (Fig. 1A, B). The 0.05% of *Ascophyllum nodosum* extract was the best for growth as well as for procuring nodules(Fig. 2A, B). This concentration was superior nodule quality in both foliar and root application of *Ascophyllum nodosum* extract experiments. The findings of this study is agreement with the previous studies which showed that the application of seaweed extract increases the growth of soil microbes and soil fertility which was attributed to the bioactive compounds present in the seaweed extract (Alam *et al.,* 2013). *Ascophyllum nodosum* positively affected the nodule formation in legume, Alfalfa (Khan *et al.,* 2012; Khan *et al.,* 2013; Zhai, 2012). Verma *et al.* (2019) observed that tyrosinase inhibition activity and photosynthetic pigments accumulation also increased in 0.05% *Ascophyllum nodosum* extract treated sprouts of *Vigna aconitifolia*. Higher concentrations of *Ascophyllum nodosum*