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Full Length Research Paper

Isolation and characterization of *Rhizobium sp*. from a wild legume from BGSBU campus of District Rajouri, J&K

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Rhizobium **plays an important role in agriculture by fixing nitrogen. It is symbiotic bacterium and leads to the formation of root nodules in legume plants. In this study, we isolated the *Rhizobium sp*. from the root nodules of *Vicia sativa* and characterized them by means of various biochemical tests. We isolated the *Rhizobium* sp. on YEMA. These bacteria were rod shaped, Gram negative, motile, acid and mucous producing. Strain was positive for methyl red and showed catalase, citricase, urease, nitrate reductase, oxidase, amylase and β-hemolytic activity. The strain also utilized D-glucose and sucrose as a carbon source but not lactose. They were found to be temperature and salt tolerant. Farmers of the region can be made aware to cultivate *Vicia sativa* like leguminous plants as forage crops and to use cost effective biofertilizers which can improve their socio-economic status as well.**

Key words: Rhizobium, bromothymol blue, biofertilizers, *Vicia sativa,* Nitrogen.

INTRODUCTION

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined or fixed forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms interacting with leguminous plants, fix aerobic nitrogen into soil (Franche *et al.,* 2009). Among plant-microbe interactions, legume–*Rhizobium* interactions are unique because they supply 80-90% of total nitrogen requirement of legumes. It involves a complex interaction among host, microbial symbiont and environment. Among nitrogen fixing systems, legume-rhizobium symbiosis is one of the most promising and the bacterial species of *Rhizobium* complex are very important (Sprent, 2001).Rather *Rhizobium* is the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (rhizobium as biofertilizer). *Rhizibium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers *et al*., 2003). The legume-rhizobium interaction is the result of specific recognition of the host legume by *Rhizobium*. Various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (Olivares *et al.,* 1984). It also protects the cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman et al., 1978). In addition, *Rhizobium* strains

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secrete growth hormones like indole acetic acid (IAA), which shows positive influence on plant growth and also plays an important role in the formation and development of root nodules (Nutman,1977).
The present study was carried out to isolate and characterize *Rhizobium* sp morphologically and biochemically and to prepare cost effective biofertilizer from the established cultures of rhizobia and to create awareness among farmers to cultivate leguminous (crop rotation) plants and use biofertilizers for better agricultural and economic growth.

Materials and Methods

Site of Legume Used in the Study:

The present study was conducted during 2014 at School of Biosciences and Biotechnology, BGSB University; Rajouri (N. 33° 23' 39", E. 74° 20' 26") is a part of the J&K region (Map 1), with many ecotypes of N -fixing legumes, which could be used for the improved production of several agro-systems. The average temperature is 32°C in summer and about 6°C in winter with 10.32mm rainfall. Some of the characteristics of experimental soil sites are given as

Soil texture pH

Sandy loam 9.1

Isolation of *Rhizobium* sp. from *Vicia sativa* roots: The fresh and plump root nodules of *Vicia sativa* plant were collected from the field (Figure 1). The collected nodules were surface sterilized with 75% and 0.1% ethanol and mercuric chloride respectively and then washed thoroughly with distilled water. *Rhizobium* sp. were obtained by streaking the crushed root nodules on

YEMA (Yeast Extract Mannitol Agar pH 6.8-7.0) media plates and incubated at 30ºC (Aneja 2003). After one day of incubation at 30ºC, *Rhizobium* colonies were obtained. Pure isolates were used for further analysis.

Growth of test organism on selective medium:

The test organism was inoculated on selective medium (YEM+Bromothiomol blue) and then incubated at 30 ºC for 24 hrs.

Colony Morphology:

The colony morphology of isolates was examined on YEM agar plates. After an incubation of 1 day at 30 ºC, individual colonies were characterized based on their colour, shape, appearance, colony diameter, transparency and margin (Dubey and Maheshwari, 2007).

Gram's Reaction:

Gram staining was performed to check out whether the test organism was Gram positive or Gram negative.

Motility test:

The test was performed to determine the ability of test organism to move. YEM with triphenyl tetrezolium was stab cultured and incubated at 30°C for 24 hrs. of incubation

Biochemical characterization of Isolates:

Methyl red test:

The test was performed to determine whether the test organism is acid fermenters or butanediol fermenters. The test organism was inoculated in MR broth (peptone, glucose, potassium phosphate, pH-6.9) and the uninoculated broth was kept as control. Incubate the cultures at 30ºC for 24 hrs. And then add 5 drops of methyl red indicator into the cultures and observe them for change color.

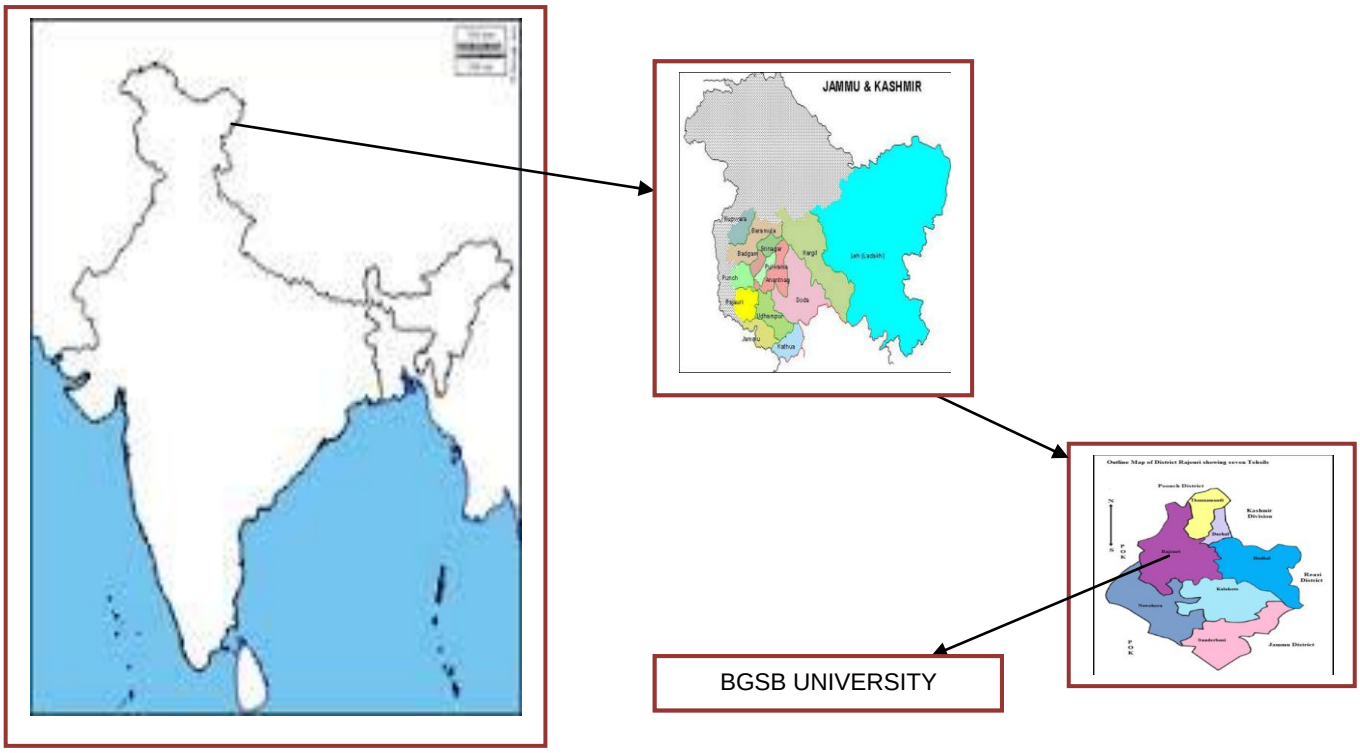
Catalase test:

The test was performed to determine the capability of test organism to utilize oxygen to produce hydrogen peroxide. Hydrogen peroxide is toxic. Organisms which produce Catalase converts hydrogen peroxide to water and oxygen. The colony of test organism was picked and transferred on a glass slide in a drop of water while as on other side of glass slide the drop of water was kept as such(not mixed with bacterial colony). Few drops of 3% hydrogen peroxide were placed over the two drops on two sides of glass slide and both the drops were observed for the appearance of bubbles.

Citrate test:
This test is based on the ability of an organism to utilize citrate as sole carbon source and ammonia as sole nitrogen source. Use of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH. The Simmon's citrate agar (blue colored) slants were inoculated with test organism and incubated at 30ºC for 24 hrs and observe the slants for color change and growth.

Urease test:

Urea is degraded into ammonia and CO_2. Urease production can be easily detected due to the production



Map 1. Showing the region of the legume used in the study.

of ammonia which raises the pH of the medium and it can be demonstrated by the following reaction:

$NH_2 + CO + NH_2 \longrightarrow 2NH_3 + CO_2$

Urea broth medium (yeast extract, KH_2PO_4, K_2HPO_4, phenol red) was inoculated with test organism and incubated at 30ºC for 24- 48 hrs.

Nitrate test:

This test was performed to determine the ability of test organism to produce nitrate reductase which can reduce nitrate to nitrite. The addition of potassium iodide to a solution of a nitrite, followed by acidification with dilute sulphuric acid, results in the liberation of iodine, which may be identified by the blue colour produced with starch solution

$2\ NO_2^- + 2\ I^- + 4\ H^+ \longrightarrow I_2 + 2\ NO \uparrow + 2\ H_2O$

I_2+Starch $\longrightarrow$ Blue Color

The nitrate broth (potassium nitrate, peptone, beef extract) was inoculated with test organism and incubated at 30ºC for 24 hrs. Then few drops of nitrate reagent (zinc chloride, starch, potassium iodide) and 1-2 drops of sulphuric acid (1:3; acid: water) to the culture tubes and observe for color change.

Oxidase test:

A dye dimethyl-p-phenylediamine hydrochloride donates electron to cytochrome C becomes oxidized and produce a color. Strips coated with the dye were used to perform this test. A strip was coated with the bacterial culture cultivated in basal medium and the strip was observed for color change.

Growth on blood agar:

YEMA was enriched with blood (5ml of blood for 100 ml of medium). Blood was added to the medium when the medium was at 40ºC inside laminar cabinet and then poured in petriplates.

Test organism was inoculated on blood agar plates and incubated at 30ºC for 24 hr. The plates were then observed for the presence of clear zones.

Starch Hydrolysis (Amylase test):

The test was performed to determine capability of test organism to use starch as carbon source (de Oliveira *et al.*, 2007). In this test, starch agar is inoculated with the species in question. After incubation at an appropriate temperature, iodine is added to the surface of the agar. Iodine turns blue-black in the presence of starch. Absence of the blue-black color indicates that starch is no longer present in the medium. Bacteria which show a clear zone around the growth produce the exoenzyme amylase which cleaves the starch into di- and monosaccharides (Bird *et al* 1954). Starch agar medium

Colony characters	Observations
Shape	Circular
Elevation	Convex
Margin	Entire
Surface	Smooth
Opacity	semi translucent
Texture	Gummy
Color	Creamish
Size	2-3mm diameter

Table 1. Showing the morphological characteristics of the isolated microorganism.

Test performed	Results
Gram's Reaction	Gram-Ve
Motility test	Positive
Methyl red test	Positive
Catalase test	Positive
Citrate test	Negative
Urease test	Positive
Nitrate test	Positive
Oxidase test	Positive
Hemolysis	β
Starch Hydrolysis	Positive
Sugar formation	
Glucose	Positive
Sucrose	Positive
Lactose	Negative

Table 2. Showing the biochemical character of the isolated organisms.

were inoculated with test organism and incubated at 30ºC temperature for 24 hours. After incubation iodine solution was added in starch plate to determine capability of test organism to use starch.

Sugar Fermentation Test:

The test was performed to determine the capability of test organism to use various carbohydrate (glucose, sucrose and lactose) sources as media for growth. When a sugar is fermented it results in the acid formation which is detected by the pH indictors (one color at acidic pH and another color at alkaline pH). Nutrient broth (sugar, yeast extract, $MgSO_4$, K_2HPO_4, NaCl, (NH_4) H_2PO_4, bromothymol blue (pH-6-yellow and pH-7.6-blue)) with three different kinds of sugars, that is, glucose, sucrose and lactose (in three separate tubes) were inoculated with test organism and incubated at 30ºC for 24 hours. Three sugar broths were then examined for any color change.

The biochemical traits evaluated comprised temperature tolerance and salt tolerance.

Temperature Tolerance:

Tolerance to high temperatures was tested by typing in YEM broth and incubating at 30, 40, and 50ºC for 10 min. and then inoculating and incubating at 30ºC for 24 hr. on YEMA plates.

NaCl Variation Assay:

Rhizobium cultures were grown on YEMA medium having different concentrations of sodium chloride - 1%, 2% and 3% of NaCl.

RESULTS AND DISCUSSION

Colonies of *Rhizobium* were observed on YEMA medium after incubation for 24 hrs. at 30°C. Raised, gummy and smooth margin colonies with cremish appearance were observed. (Figure 2) (table 1) Microscopic observation of these isolates showed them to be gram negative rods. (Figure 3

) The YEM medium was enriched with BTB @ (25 µg /ml) to selectively identify *Rhizobium* sp. as quoted by (Vincent, 1970). The inoculated media was incubated at 30°C for 24 hrs. After incubation moist and gummy colonies were observed and surrounding medium plate was yellow due to acid production by the *Rhizobium* sp. (Figure 4)

To test for motility, the YEMA (semi-solid; agar@0.4%)containing 2,3,5-triphenyl-2H-tetrazolium chloride @ (0.08%) was stab inoculated and incubated for 24 hr. at 30°C. TTC in its oxidized form is colorless. As bacteria grow in the presence of TTC, the dye is absorbed into the bacterial cells where it is reduced to the insoluble red-colored pigment formazan (MacFaddin *et al,* 1972). A positive motility test is indicated by a red turbid area (hazy appearance) extending away from the line of inoculation. (Figure 5)

On addition of methyl red (pH indicator) the MR medium remained red indicating that the test organism is acid producer. Therefore the test organism is MR positive. (Figure 6)

Oxygen bubbles were released when 3% H_2O_2 was added to a drop containing the test bacteria while no oxygen bubbles were observed when H_2O_2 was added to a drop of distilled water indicating that the test organism is Catalase positive.

No color change was observed in the Simmon's citrate agar (blue colored) after incubation confirming that the test organism is citrase (citrate test) negative. (Figure 7)

Urea broth had turned pink after incubation suggesting alkaline nature of medium because of ammonia production indicating that the test organism is urease positive. (Figure 8)

Figure 1. *Vicia sativa*.

Figure 2. *Rhizobium* on YEMA.

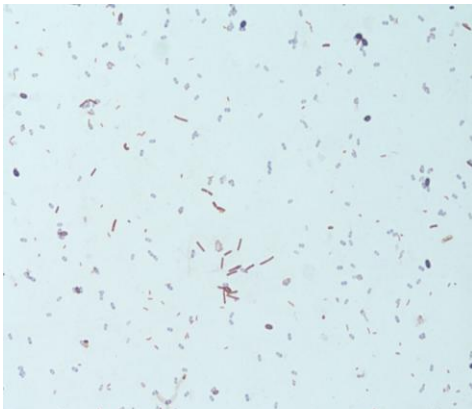


Figure 3. Grams Reactions.

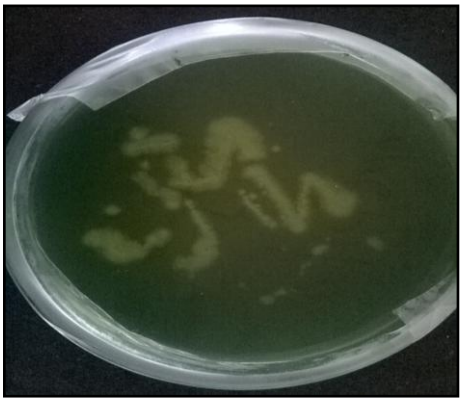


Figure 4. *Rhizobium* on selective media.

Figure 5. Motility test for *Rhizobium.*

Figure 6. Methyl red test.

After the addition of Nitrate test reagent to overnight incubated nitrate broth (inoculated with test organism), blue color appeared which indicated nitrite is produced and our test organism is nitrate reductase positive. (Figure 9)

When overnight cultivated culture of test organism was spread over the Oxidase strips, the color of the strips changed to blackish purple confirming that the test organism is positive for Oxidase. (Figure 10).

When Rhizobia were cultivated on blood agar it resulted

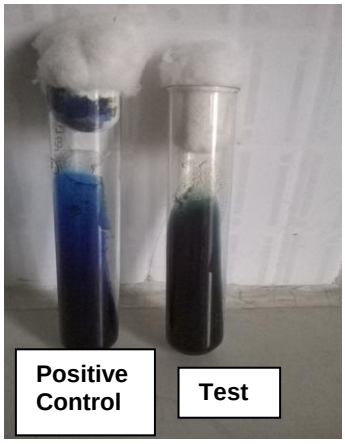


Figure 7. Citrate test.

Figure 8. Urease test.

Figure 9. Nitrate test.

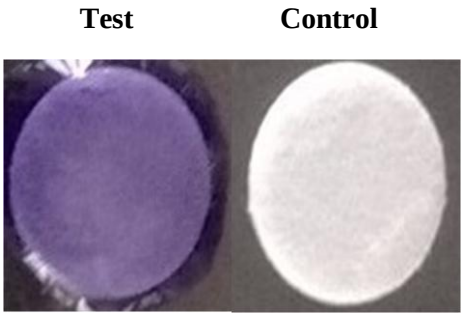


Figure 10. oxidase test.

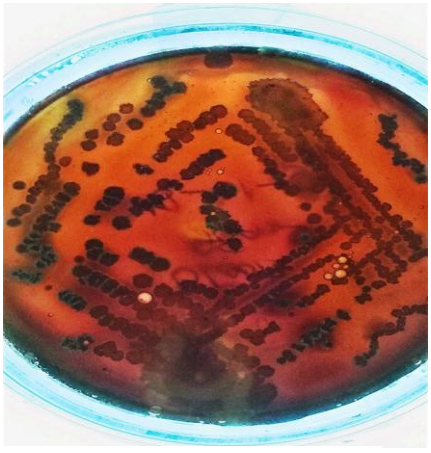


Figure 11. β-Hemolysis.

Figure 12. Starch hydrolysis test.

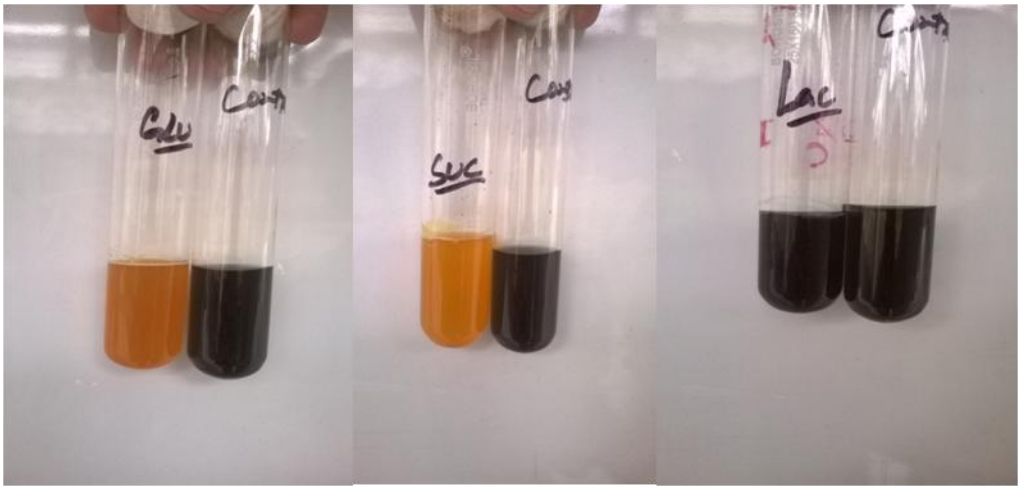


Figure 13. Sugar fermentation by *Rhizobium.*

in the formation of clear zones around the colonies showing β hemolysis. (Figure11)

Iodine and starch makes a complex of blue color. While iodine does not react with any product of starch degradation and hence no color is formed in such cases. On flooding the inoculated plates with iodine solution, clear zones around the colonies were observed while blue color appears on no growth areas Therefore positive results were obtained from the starch hydrolysis assay. This indicates that the isolates have the potential to hydrolyze starch present in the medium. (Figure 12)

After inoculation and 24 hours incubation, yellow colour was observed in tubes containing glucose and sucrose as carbon source while no color change was observed in tube containing lactose as carbon source confirming that the test organism has the ability to ferment glucose and sucrose but not lactose. (Figure13)

From the above observations (table 1 and 2) we could conclude that the test organism was *Rhizobium sp.*

After incubating the bacteria at different temperature that is 30°C, 40°C &50°Cfor 10 min. and then cultivating under normal conditions , we observed that bacteria which were incubated at 30°C and 40°C for 10 min. were able to grow well while the bacteria which were incubated at 50°C for 10 min. showed minimal growth. So we could conclude that the isolated strain of *Rhizobium* were tolerant to high temperature.

When the isolated strain of *Rhizobium* was inoculated on YEMA plates having 1%, 2% and 3% NaCl and then incubated at 30°C for 24 hr., growth was observed on all the three concentrations on NaCl. Thus we could conclude that the isolated strains of bacteria are tolerant to high salt (NaCl) concentrations. But according to Hashem *et al.,* (1998) salt stress may decrease the efficiency of the Rhizobium-legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function.

The isolated, pure culture of *Rhizobium* was used to prepare the biofertilizer according to method described by (Baljinder Singh, et al, 2008). Charcoal was used as inert carrier (for adsorption of bacterial cells) so that inoculants can easily be handled, packed, stored, transported, used and afforded by farmers; however the field trials are yet to be conducted. Also according to, G.S. Nagananda, *et al*, 2010, seeds coated with charcoal-mixed biofertilizer yielded better growth than those coated without charcoal.

As *Vicia sativa* is growing as a weed in the said region, it can thus be concluded that the soil of this region is apt for crop rotation of leguminous plants and biofertilizers as well. But through our personal experience, we noticed that farmers of this region do go for crop rotation (maiz in summer and wheat in winter) but they neither go for the cultivation of leguminous crops nor for mixed crop cultivation. So, there is an immediate need to educate the farmers of this region to cultivate *Vicia sativa* like leguminous plants as forage crops or to cultivate leguminous plants during mixed crop cultivation which would not only feed their cattle but would also act as a source of green manure. Simultaneously, we need to encourage them to use biofertilizers instead of chemical fertilizers not only because the biofertilizers are cost effective but because biofertilizers are effective in promoting healthy soil and plant life, that is, overall environment is healthier, as air and water quality are inextricably linked to soil quality. And the most important of all, following such practices can improve their socio-economical status which is a need of hour in remote area like Rajouri, J&K.

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