Characterization of rhizobia from root nodule and rhizosphere of Vicia faba in Algeria

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ABSTRACT

Nitrogen fixation resulting from mutual symbiosis of rhizobia and cultivated legume plants is therefore critical to food security as it directly affects agricultural production. Biological Nitrogen Fixation (BNF) can be an important factor in sustainable agriculture. A collection of 20 isolates from fresh Nodules of the legume plant Vicia faba was isolated from five soil of Algeria. The soil from each region has undergone physical-chemical analysis: (granulometry, total carbon, organic matter, pH, and electrical conductivity, determination of available phosphorus and determination of total nitrogen). A media viz., Glucose-Peptone Agar (GPA), Congo red, Yeast Mannitol Agar (YMA) containing 2% NaCl were employed to make presumptive decisions on the recognition and classification of the isolated bacterial strains. All the isolates were found with poor absorption of dye Congo red and little or no growth on the media of GPA and without altering the pH. Almost all of the isolates exhibit good growth on 2% NaCl, poor growth on GPA, thus confirming the rhizobia. After biochemical tests like catalase test and citrate utilization test isolates were confirmed. The presence of rhizobia on root nodules of leguminous plant.

Key words: Nitrogen fixation, Nodule. Root, Vicia faba.

INTRODUCTION

The annual legume Vicia faba botanically known as Vicia faba L. (Hanelt and Mettin, 1989; Harlan, 1969). The crop is known by many names, most of which refer to a particular subgroup rather than the whole species (Hawtin and Hebblethwait, 1983; Zohary and Hopf, 1973). Faba bean, Fava bean, Broad bean, Horse bean, Windsor bean, Tick beans (small types), Bakela (Ethopia), Boby kurmovje (former USSR), Faveira (Portugal), Ful masri (Sudan), Feve (French) and Yeshil Bakla (Turkey) are the few names used in different parts of world (Hawtin and Hebblethwait, 1983; Naqvi, 1984; Singh et al., 2012, Jing et al., 2016). In Hindi, it is popularly known as Kala Matar and Bakala as well (Singh et al., 2012). It is among the oldest crops in the world, it is third most important feed grain legume after soybean (Glycine max L.) and pea (Pisum sativum L.) area and production (Mihailovic et al., 2005). The area under Vicia faba crop in India is very less and that is why it is still categorized as minor, unutilized or underutilized, less utilized, and still not fully exploited crops. Though, its green pod is mainly used as vegetables, dry cotyledons are one of the excellent and cheap sources of lysine rich protein for poor ones (Bond, 1976; Hawtin and Hebblethwait, 1983; Abdul, 2008).

Vicia faba is also a good source of levadopa (L-dopa), a precursor of dopamine, can be potentially used as medicine for the treatment of Parkinson’s disease (Oplinger, 1982; Vered et al., 1997). It is one of the best crop that can be used as green manure and due to its better nitrogen fixing ability ranging from 130 to 160 kg N/ha (Hoffmann et al., 2007; Horst et al., 2007). Vicia faba cannot only be grown on diverse agro-climatic conditions successfully, but also can be produced on residual soil moisture and relatively more tolerant to biotic and abiotic stress, it is that with minimum input (Singh and Bhatt, 2012). Unfortunately it is grown by poor farmers on poor and marginal land inspite of input responsive crop. In India, faba bean productivity is low as compared to other major vicia faba growing countries (Singh et al., 2012). In India, vicia faba potential is not fully realised.

Vicia faba is a legume capable of fixing nitrogen in an endosymbiotic association with Rhizobium leguminosarum and Biovar vicieae thus improves soil fertility. Rhizobium leguminosarum biovar vicieae, also nodulates pea (Pisum spp.), vetch (Vicia spp.), lentil (Lens spp.), and sweet pea (Lathyrus spp.) (Perret et al., 2000). Among the grain legumes, vicia faba is reported to derive the highest percentage of nitrogen from the atmosphere (McVicar et al., 2005). According to (Somasegaran et al., 1994), the amount of nitrogen fixed by Vicia faba have been 240-325 kg/ha. The ability of Vicia faba to fix the desired amount of nitrogen depends on many factors such as the effectiveness of the symbiont strain, the
genetic variation of the host plant, and other edaphic and environmental factors (Ngwenya et al., 2016). In this context, a collection of strains associated to *Vicia faba* has been isolated from nodules sampled *in natura* in different regions of Algeria in the purpose of phenotypic characterization.

**MATERIALS AND METHODS**

**Sample collection and preparation:** The plant samples of *Vicia faba* were collected for the isolation of rhizobia. Fresh and healthy root nodules were selected from each plant for the present study. The selected nodules were usually light brown or pinkish in color, which indicates that an active nitrogen fixation had been established between the nodule bacteria and the legume plant. Besides collection of nodule from leguminous plant, the rhizospheric soil around the plant was also collected. The nodules are disinfected in ethanol at 95% for 5 to 10 seconds to break the surface tension and then transferred to 3 per cent solution of hydrogen peroxide and soaked for 2-3 minutes (Vincent 1970). Nodules were then rinsed in five changes of sterile distilled water using sterile forceps for transferring.

**Studies of physicochemical parameters of different soil samples:** Soil samples from five city of Algeria were collected. The soil samples were taken at the depth of 20 cm from the soil surface from different places of the city in the year 2015. The soil samples were preserved in polythene bags for further analysis. In laboratory these samples were analyzed to measure various chemical parameters by standard methods. Soil pH was determined in distilled water at a soil : solution ratio of 1 : 1 using Practitronic M.V 88 pH electrometer (Peech, 1965). Organic carbon was determined using the wet combustion method of Walkley and Black (1934) while the Bray and Kurtz (1945) method was used to measure available phosphorus. Total nitrogen was determined by the distillation and titration method of Brennner (1965). The cation exchange capacity (CEC) of the soils was determined by extraction of the exchangeable bases using neutral ammonium acetate (NH4OAc, pH 7.0) and an aliquot was used to determined Mg, K and Na. Soil texture was determined based on particle size analysis using the modified Bouyoucos hydrometer method as describe by Day (1965).

**Isolation of root nodule bacteria:** The first step of the isolation process was to crush the sterile nodules with a blunt tipped forceps in a large drop of sterile water in a petri dish (Somasegaran and Hoben 1994). Using yeast extract mannitol selective culture media (YEM) (Handley et al., 1998; Castro et al., 2003; Kucuk et al., 2006) the nitrogen fixing bacteria isolated directly from the root nodules of the host plant or from the soil (Geniaux et al., 1993), After 4 to 5 days of incubation at 28°C, the colonies obtained were purified by successive sub culturing, examined by photon microscope to check their purity, and then isolates were stored at -80°C with glycerol (1:1 v/v) in eppendorf based on the method (Vincent 1970).

**Maintenance of cultures:** The isolates were sub-cultured on YMA slants. Growth was observed at 30°C and then the slants were kept at 4°C. Periodic subcultures from these stock cultures were performed at a 15 days interval. For long time storage, the isolates were streaked on YMA slants and after incubation at the same temperature; sterile glycerol was added on the media and then stored at room temperature.

**Morphological Characteristics of the Isolates**

**Colony characteristics:** The colony characteristics (i.e. shape, size, color, opacity, elevation, edge, margin of the bacterial colony and their growth rate) were determined by observing the colonies on YMA plates after growth at 30°C. Microscopic observation of the isolates was done using Gram staining technique as described by (Arora, 2003).

**Cultural and Metabolic Characteristics**

**Presumptive tests:** Strain of rhizobia can be described according to their growth on the solid and liquid media. The size, shape, color, texture of the colonies and their growth on different media and their ability to alter the pH of the media are generally stable characteristics use in defining strains.

**Congo Red test:** The purity of the rhizobial isolates was detected by adding Congo red in YMA media (Somasegaran et al., 1994). Most rhizobia absorb the dye only weakly whereas contaminants including Agrobacteria, will absorb strongly.

**Growth on Glucose- peptone agar:** Glucose–peptone media was used to differentiate rhizobia, which usually shows little or no growth on the media without altering the pH of the media. Contaminants like Agrobacteria, shows massive growth on the media with a distinct change in pH. The strains growth on basal medium of YMA, 2% NaCl was added to check the purity of the isolates. As 2% NaCl is inhibitory for most rhizobial isolates it can serve as an identification tool. Differentiation between fast and slow growers on the Bromothymol blue media : Yeast Mannitogl Agar (YMA) media incorporated with bromothymol blue was used to distinguish fast-(acid producing) growing strains from slow (non acid producing or alkali producing) growing rhizobia (Somasegaran et al., 1994). In this medium, the fast growers require 48 hours to produce an acidic reaction by turning the color of the media yellow from green, whereas the slow growers take >96 hours to produce alkaline endpoints with or without changing the color of the media from green to blue.

**Symbiotic characterization (test of nodulation):** The seeds of *Vicia faba* disinfected with absolute ethanol 5 to 10 seconds and then in sodium hypochlorite at 12° for 3 minutes, followed thoroughly by rinse with sterile distilled water and putting to germinate on Petrie Dishes containing water agar (0.8 %) in the dark at 28°C. After 4 to 5 days, the root let obtained were transferred into disinfected plastic pots containing 150g of sterile sand.
The seedlings were inoculated with 1 ml of bacterial culture containing (108 bacteria/ml). All plants were watered 3 times per week in alternation with a free nitrogen nutrient solution. The nodulation test was conducted in triplicate forms. The treated plants were stored in a house of culture with 16/8h of light/darkness and 28/20°C day/night.

Biochemical Characteristics

To confirm whether the isolates were rhizobia or not, they were incubated in different media for each physiochemical test and then incubated depending upon their growth rate at 30°C. The presence of the enzyme catalase in the rhizobial isolates was examined by suspending one loopful of organism in a drop of 3% H2O2 on a glass slide. This test was performed as per standard procedure (Cappuccino et al., 1992). Production of bubbles indicates a positive result. Citrate utilization test: Citrate utilization by the isolates was observed by the growth on slants of Simmon’s Citrate Agar. A distinct change in color from green to blue refers to a positive test.

RESULTS AND DISCUSSION

Physico-chemical characteristics of soils: The fertility of the soil depends on the concentration of N, P, K, organic and inorganic materials and water (Brar et al., 2017). Conductivity. The physicochemical properties such as moisture content, specific gravity Nitrogen as a fertilizer required for the growth of plant. Potassium is used for flowering purpose and phosphate is used for growth of roots in plants (Kanimozhi et al., 2011). The results of the soils analyzes of the five studied sites (Table 1) showed that pH of soil samples shows variation 8 to 8.9, the above 7.5 value of pH shows basic nature.

The Conductivity study of the five soil shows variation in conductivity values between 0.07 mhos to 1.01 mhos this value suggest normal soil. Percentage of carbon varies from 0.38 to 0.49 also shows normal soil. Percentage of N varies from 0.38 to 0.046, Percentage of P varies from 0.026 to 0.04. Percentage of N and P are also in normal range. The five samples with a clay loam texture. These 5 types of soils are classified among the balanced textures (Gadrat et al., 1997).

Identification and characterization Staining properties:

On YEMA, Twelve isolates were fast growing (appearance of colonies 4 to 5 days after incubation), the colonies are circular, convex, translucent. In contrast, the other 8 isolates showed a slow growth on YEMA (appearance of colonies after 5 days of incubation). All isolates are Gram negative. The strains do not absorb the Congo red as it has been described by Jordan [1984] for the rhizobia. On the other hand 60% of the isolates have acidified the YMA + bromothymol blue after 24 h incubation (the indicator turned yellow).

All the isolates, showed poor absorption of dye Congo red, little or no growth on the media of GPA without altering the PH. This fact give further evidence for purity of the Rhizobial isolates (Somasegaran et al., 1994). High salt concentrations, like 2% NaCl, act as an inhibitor for Rhizobial growth, some common bacterial contaminant e.g. Agrobacterium shows considerable growth on similar salt concentration. But almost all of the isolates exhibit growth on 2% NaCl. This was in line with findings of Dubey et al., (2010). Poor growth on GPA can be explained as such that, rhizobia do not prefer the peptone as a source of nitrogen, vitamin or growth factor or amino acids.

Symbiotic characters: All the strains were able to nodulate their host plant Vicia faba. While no nodule was observed on the not inoculated roots seedlings. The average number of nodules varies from 4 to 16 per plant. The more infective strains are F141 and 243 with 19 nodules formed by plant (Fig.1). The strains F228 and F207 are the less infective. The efficiencies relating of strains nodulant Vicia faba after describing two months of inoculation obtained varies from 50% to 90% (Figure.2). The most important values were obtained with the strains F141 and 243 with 77 and 72% of

Table 1: Chemical properties of the soils used for this study.

<table>
<thead>
<tr>
<th>N° of soil samples</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
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<td>8</td>
<td>7.5</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Totale N (g kg⁻¹)</td>
<td>0.041</td>
<td>0.038</td>
<td>0.045</td>
<td>0.046</td>
<td>0.043</td>
</tr>
<tr>
<td>CEC (cmol kg⁻¹)</td>
<td>0.08</td>
<td>0.21</td>
<td>0.87</td>
<td>1.01</td>
<td>0.07</td>
</tr>
<tr>
<td>P (mg kg⁻¹)</td>
<td>0.034</td>
<td>0.026</td>
<td>0.023</td>
<td>0.028</td>
<td>0.031</td>
</tr>
<tr>
<td>Organic carbon (g Kg⁻¹)</td>
<td>0.41</td>
<td>0.46</td>
<td>0.38</td>
<td>0.43</td>
<td>0.49</td>
</tr>
<tr>
<td>Texture</td>
<td>clay loam</td>
<td>clay loam</td>
<td>clay loam</td>
<td>clay loam</td>
<td>clay loam</td>
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Fig 1: Infectivity of strains nodulant Vicia faba.
efficiencies. In general, the strains were isolated very efficient with 78% of strains present are may more than 60%.

**Biochemical characteristics of the isolates**: All the isolates were tested for selective biochemical tests (Mahana et al., 2000). The 20 strains also reported catalase activity, 15 isolates from nodules and rhizosphere showed a catalase positive and citrate negative. So in catalase test bubbles were produced and in citrate test. Algeria has made impressive progress in improving the production of rice and other crops during the past few years. However, there are still much lacking in nutrition. We are mainly concerned with carbohydrate and protein intake. A vast majority of the poor in the world cannot afford animal or fish proteins, and thus have to depend on cheap and easily available vegetable protein – mainly pulses and legumes, which are known as “poor man’s beef”. Research on the rhizobia has revealed a lot about the role of biological nitrogen fixation in the field of agriculture. Rhizobia by diminishing input of the fertilizer in the field and positively influencing plant crop growth, contribute to the development of the sustainable agriculture, which is necessary for the agriculture based, under developed country like Algeria. The present study is expected to reveal the diversity of these Rhizobial strains native to Algeria, to some extent, especially with the agronomically and ecologically interesting pulse legume. This study showed the presence of rhizobia on root nodules of leguminous plant. Not only the leguminous plants but also the rhizosphere contain rhizobia which help in soil fertilization. Further studies are recommended to identify additional characteristics of rhizobia, and to assess biological nitrogen fixation.

**CONCLUSION**

In this work, we were interested in the rhizobia nodulating *Vicia faba* grown in five regions of Algeria. A collection of 20 strains isolated from fresh nodules has been established. These isolates have been authenticated by seedling inoculation of *Vicia faba* growing in jars containing autoclaved sand. *Vicia faba* is one of the best crops among the grain legume with more than 7.0 grain yield potential with addition of atmospheric nitrogen to soil as added advantages. Being so incredible crop, there is need to make *Vicia faba* more acceptable to all party. Despite all good qualities, the major bottleneck is anti-nutritional element, taste and aroma. Availability of zero tannin cultivar is boon for expansion of it area and inclusion in the daily diet especially the population depends upon vegetable protein.

**REFERENCES**


