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Original Article

Isolation and Characterization of Phosphate Solubilizing Micro-Flora from Saffron Rhizosphere of Northwest Himalaya, India

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Abstract
Saffron, a spice derived from the *Crocus sativus* flowers is used as a coloring agent along with its medicinal application. Pampore area in Kashmir valley is famous for saffron cultivation and is one of the major saffron cultivation areas in the world. In the present study we aimed to isolate and characterize the phosphate solubilizing micro-flora from saffron soils of Pampore (9 sites were selected). The site selection was done based on the slope and altitude of the study region i.e. elevated (location with higher altitude but lower slope), sloppy (location with median altitude than elevated and plains but higher slope) and plain (location with lower altitude and lower slope. Four types of arbuscular mycorrhizae were isolated from the soils of saffron fields by Gerdemann & Nicolson technique. The genera isolated were identified as *Glomus*, *Acaulospora*, *Scutellospora* and *Gigaspora*. *Glomus* and *Acaulospora* were pre-dominant. The highest spore population was found in summer (11.3/g soil) as compared to winter due to the congenial temperature in summer. Root colonization was more (79.9 %) in less phosphorus containing soils. The phosphatase activity was high in summer (37 µg*p*-NP/g/ha) than winter and maximum values were found in sloppy areas ascertained to minimum phosphorus in these soils. The phosphorus solubilizing bacteria was maximum in summer (18.5×10^5 CFU/g) than in winter and the maximum values were found in plain soils exhibiting the presence of more arbuscular mycorrhizae. The present study concludes that VAM species can act as good bio-fertilizers in order to improve the production of Saffron in Kashmir Valley by demanding more attention to the microbial population of saffron soils and without the use of chemical fertilizers which pollute our soil as well as our environment.

Conflict of Interest: The author declare no potential conflict of interest related to this research.

Keywords: Saffron soil, Mycorrhizae, Root colonization, Phosphatase.

Introduction

The Kashmir Valley in India is renowned for its high-quality saffron, an enchanting spice with a rich historical background. The term "saffron" finds its origins in the Arabic word "Zafaran," meaning yellow (Kafi et al., 2018; Pandey et al., 2020; Pandita, 2021). Widely cultivated across Mediterranean Europe and Western Asia, saffron serves as a pivotal flavoring and coloring agent in food, as well as playing a crucial role in the dye, perfumery, and flavoring industries. Saffron is also celebrated for its diverse biological properties, including anticancer, antimutagenic, and antioxidant effects. In the Kashmir Himalayas, saffron cultivation takes place in uplands locally known as "wudur." These uplands feature glacio-fluvio-lacustrine deposits at elevations ranging from 1585 to 2300 meters above mean sea level, existing within temperate climatic conditions (Kanth et al., 2008; Kozgar and Jabeen, 2012). The history of saffron cultivation in the Kashmir Valley dates back to 550 A.D., with some accounts attributing its introduction to Persian rulers who brought saffron corms to Kashmir. Alternatively, local belief holds that Sufis Khwaja Masood Wali (r.a) and Sheikh Sharif-u-din Wali (r.a) brought saffron to Kashmir (GIAHS Saffron site report, 2012), claiming it originated from the Takshak spring in Zewan village, Pampore. Saffron thrives on a variety of soils, with optimal growth observed in well-drained clay calcareous soils. The crop requires well-drained sandy and loamy soils for optimal cultivation. While Pulwama is a prominent region for saffron cultivation, other areas, including Srinagar, Budgam, and Kishtiwar districts of Jammu and Kashmir, also participate in its growth. Unfortunately, recent years have seen a decline in both production and productivity, attributed to factors such as diminishing soil health, the unavailability of high-quality corms, and the persistence of traditional agricultural practices. The indiscriminate use of fertilizers has led to environmental contamination, affecting groundwater and disrupting the balance of beneficial micro-organisms in the soil. The decline in mycorrhiza populations, which play a crucial role in nutrient translocation, is also considered a contributing factor to the reduced productivity of saffron.

Vesicular arbuscular fungi play a crucial role as abundant symbionts in plant roots within terrestrial ecosystems, serving as a vital link between biotic and abiotic components (Neill et al., 1991; Soka and Ritchie, 2014; Jamiolkowska et al., 2018). The formation of Vesicular Arbuscular Mycorrhiza (VAM) represents a symbiotic association between plant roots and fungi, aiding in nutrient uptake and enhancing plant growth by modifying the rhizosphere soil while increasing soil volume (Marschner & Dell, 1994; Abiala et al., 2013; Pierre et al., 2014). Consequently, VAM association often leads to an augmented uptake of immobile nutrient elements, particularly phosphorus, due to the expanded soil volume (Castelli et al., 2014).

Saffron, characterized by inefficient photosynthesis during its vegetative growth in extreme climatic conditions particularly during winter when the temperatures are below sub-zero, benefits from VAM inoculation in saffron fields. This inoculation results in increased fresh and dry matter weight of corms, promoting overall saffron plant growth (Shaub et al., 2016). Moreover, VAM enhances the production of replacement corms and reduces the occurrence of fungal diseases (Caser et al., 2018). Aymani et al. (2019) observed varying root mycorrhizal frequencies in the saffron rhizosphere, highlighting the pertinent interaction between saffron roots and native endomycorrhizal populations under field conditions. This interaction involves diverse mechanisms such as the modulation of water transport, nutrient acquisition, and stimulation of root phosphates exudation, aiding the plant in mitigating environmental stress, especially under drought conditions.

Arbuscular mycorrhizal fungi (AMF) play a crucial role in sustainable farming systems, particularly in periods of low nutrient availability and when nutrients are bound to organic matter and soil particles. Due to their efficiency in nutrient acquisition, AMF are utilized as "bio-fertilizers," influencing the growth, development, and survival of plant species while impacting plant secondary succession and community structure. Phosphorus, a vital soil element occurring in both inorganic and organic compounds, is released into the soil by various micro-organisms through processes like solubilization and mineralization (Hilda & Fraga, 1999). Considering the significant roles of arbuscular mycorrhizae, phosphorus, and the importance of saffron, this study was undertaken to isolate and characterize the phosphate solubilizing micro-flora from saffron rhizosphere of Northwest Himalaya, India.

Materials and Methods

Study Area:

District Pulwama in Kashmir Himalayas lie between the geographical coordinates of 33º 40′-34º 01′ N latitude and 74º 40'-75º 00'E longitude and the Pampore region falls within geographical coordinates of 33º 57′-34º 01′ N and 74º 40'-74º 58'E. The altitude of the study area varies from 1800 to 3500 m above mean sea level (m, asl). The study area experiences temperate type of climate with moderately hot summer and cold winter. Most of the precipitation in the region is recorded in the form of snow. The high altitudes of districts remain covered with snow during winter and spring season. The average annual precipitation recorded in the district from 1901 to 2018 is 681 mm. The maximum precipitation is recorded in the month of March (101 mm) and minimum in the month of November (20 mm) (Jeelani et al., 2021). The highest temperature is recorded in the month of June (24ºC) and lowest in the month of January (1.6 ºC). District Pulwama is drained by Rambiara, Romshi, Sasara and Aripul streams before confluence with Jhelum River. The study area is covered by forests towards higher altitudes and dense apple orchards and agricultural fields in plains. In Pulwama the maximum area is covered by agriculture (paddy fields, 345 km^2) in which 37.85 km^2 under Saffron cultivation, followed by forests (114 km^2), wasteland (119 km^2), horticulture (apple orchards 54 km^2) and built-up land (39 km^2).

Soil sampling and Laboratory analysis:

Soil sampling campaigns were carried out in October 2021 and 9 sites [(including: Meej, Rajwos, Samboora, Ladhoo, Shaar, Konibal, Lethpora, Chandhara and Dussu (Fig. 1)] from the Saffron growing areas of Pampore were selected and divided on the basis of altitude and slope as elevated, sloppy and plain with the help of clinometer and Global Positioning System (GPS). From each village three saffron fields were randomly chosen and from each field three rhizospheric soil samples (containing plant roots also) were collected at a depth of 0-15 cm using soil auger. Stratified random sampling was preferred due to large number of saffron fields in this region. A portion of soil samples was analysed for the physico-chemical parameters and the remaining samples were used for isolation of VAM spores. The root samples were washed thoroughly with tap water to remove adhered soil particles. Then roots were cut into small pieces of about 1 cm, treated with KOH solution and 2% HCl, then assessed for colonisation of arbuscular mycorrhiza and root colonisation was calculated by following formula (Philips & Hayman,1970):

$$\text{Colonisation (\%)} = \frac{\text{No. of root segments colonised with VAM}}{\text{Total no. of root segments observed}} \times 100$$

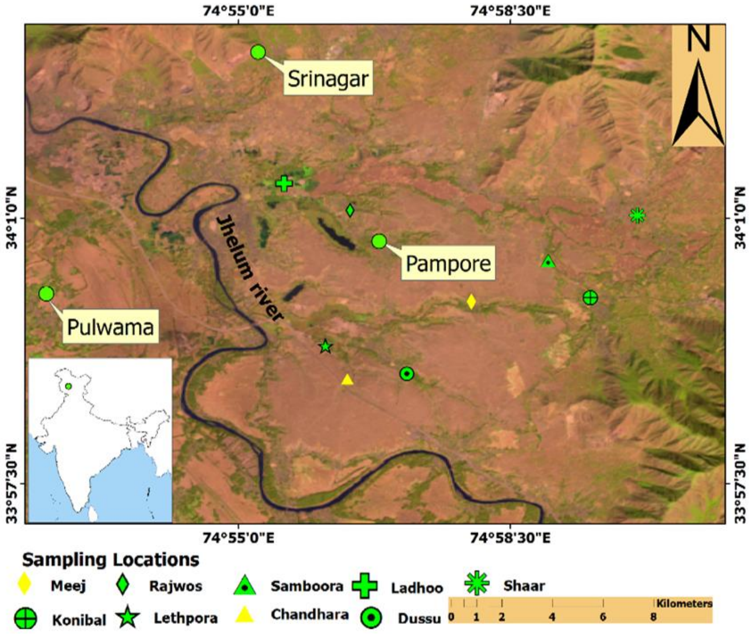

Fig 1. Study area cum sampling sites in Pampore area of Pulwama. The base map was prepared using Landsat TM imagery of October 2022. The Image has been download from Earth Explorer (https://earthexplorer.usgs.gov/).

Soil phosphatase activity was determined by Tabatabai & Bremner (1969) and phosphate solubilizing bacterial count was determined by Aneja (2001). Statistical analysis of data was carried out using IBM SPSS statistics 26, Microsoft Excel 2021, Grapher 18.3.4 and Origin Pro 2021.

Isolation and identification of arbuscular mycorrhizal fungal spores:
Wet sieving and decanting method (Gerdemann & Nicolson, 1963) was done to isolate the fungal spores from rhizospheric soil samples. The spores were counted under microscope and spore population was the expressed in terms of number of spores per gram of dry soil. Identification of spores up to generic level was based on spore size, spore colour and wall layers using the descriptions provided by Schenck et al.,1990.

Results and Discussion

Isolation and identification of arbuscular mycorrhizal spores:
Mycorrhizal fungi are known for their positive contributions in preserving plant diversity, promoting plant growth, enhancing crop production, and maintaining the stability of the ecosystem. Arbuscular mycorrhizal fungi can make phosphorus more available to the plants. Plants acquire greater resistance to both biotic and abiotic stresses due to the presence of these mycorrhiza. *Glomus* sp. (Fig. 2a), *Acaulospora* sp. (Fig. 2b), *Gigaspora* sp. (Fig. 2c) and *Scutellospora* sp. (Fig. 2d) were isolated and spore density was expressed as total number of spores per gram rhizosphere soil.

Count and morphological characteristics of arbuscular mycorrhizal spores:
Arbuscular mycorrhizal fungal spores were isolated from the soil samples collected from the Saffron fields of Pampore, Kashmir. Spore count revealed that count is maximum in summer and minimum in winter due to the influence of various factors like temperature, host fungus relationship and spore density which tends to decrease during root growth but increases during root inactivity or senescence. Similar observations were recorded by Muthukumar et al. (2003). Spores pertaining to four genera *viz Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora* were isolated from rhizospheric Saffron soils of Pampore and were identified on the basis of

morphological traits and identification was confirmed following Shenck & Perez (1990) and Redecker et al. (2000). Spores of *Glomus* were of yellow brown in colour, possess sub-globose to globose shape and were about 40-120 µm in size. Spores of *Acaulospora* were light orange to yellowish brown, possess sub globose to globose shape, 150-210µm in diameter and consists of spore wall with three layers. Spores of *Gigaspora* are yellow in colour, possess sub globose shape, 200-300 µm in diameter and consists of spore wall with two layers. Spores of *Scutellospora* are orange brown to dark orange brown in colour, possess globose shape and are 40-120 µm in diameter. Our work matched with the findings of Koske & Gemma (1990).

Root colonization studies of arbuscular mycorrhizal fungi:

Data on root colonization reveals that the root colonisation in winter is more than in summer (Table 1, Fig. 3). This seasonal trend in root colonization by mycorrhizae may be due to the different phenological trends especially vegetative and flowering stages. Another reason for the maximum root colonization in winter is that due to the vegetative phase of Saffron in winter because vesicular arbuscular mycorrhizal association is well established and functional when the plants require higher nutrient supply to support their metabolic activities. Similar trends were also found by Beena et al. (2000), Kennedy et al. (2002), Bouamri et al. (2014) and Ghosh and Verma (2015) in different crops.

Soil phosphatase activity:

The soil phosphatase activity of Saffron soils was recorded higher in plain areas as compared to elevated and sloppy areas (Table 1). The reason for this may be the minimum phosphorus present in the plain soils. Similar findings were also observed by Dick and Tabatabai (1993) and Chen et al. (1996). High phosphatase activity was observed in summer as compared to winter due to high temperature. Similar findings were observed by Sardans et al. (2006).

Phosphorus solubilising bacteria:

Data pertaining to phosphorus solubilising bacteria reveals that population of phosphorus solubilising bacteria was maximum during summer season than winter (Table 1, Fig.4 a, b). Among the three strata's, the maximum mean value for phosphorus solubilising bacteria was found in plain areas. The decrease in number of PSB in winter is due to the presence of less carbon and unfavourable temperature. In summer, the value of PSB is increased due to the presence of nitrogen in large quantities which they utilise as nitrate or nitrite which in turn greatly influences the activity of phosphorus solubilisation. Highest population of AM fungi also increase the population of phosphorus solubilising bacteria. Similar findings were also reported by Kumar et al. (2002), Habte & Osrio (2012).

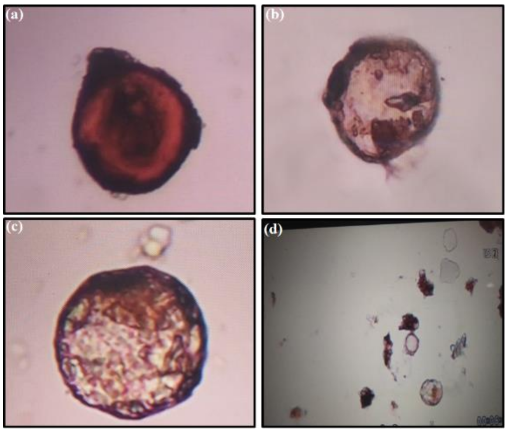

Fig 2. Spores of VAM isolated from Saffron soils of Pampore area (a) Glomus (b) Acaulospora (c) Gigaspora (d) Scutellospora

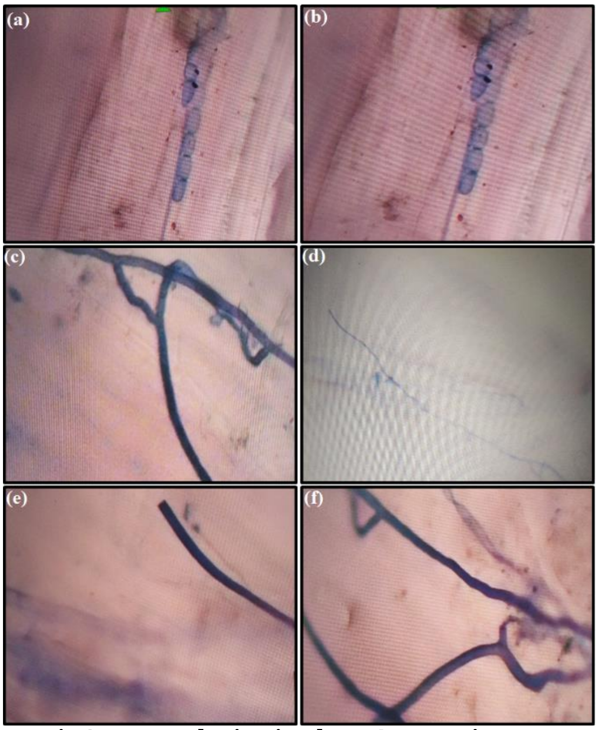

Fig 3. Root colonization by VAM species.

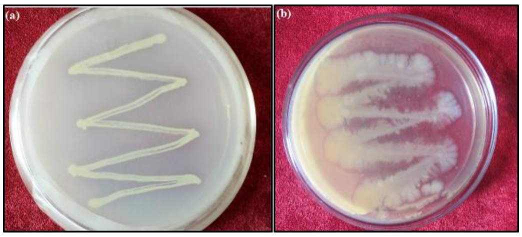

Fig 4. Phosphorus solubilising bacteria from Saffron soils of Pampore.

Table 1. Statistical summary of microbial and enzymatic characteristics of Saffron soil of Pampore.

Site physiography	Location	Summer				Winter			
		Spore count	Root colonisation (%)	Phosphatase (µgp-NP/g/ha)	PSB (CFU×10^5/g soil)	Spore count	Root colonisation (%)	Phosphatase (µgp-NP/g/ha)	PSB (CFU×10^5/g soil)
Elevated	Lethpora	7.6±1.52	39.95±0.50	34.65±1.85	15.80±0.60	3.66±1.58	75.92±0.03	15.50±0.29	8.66±0.41
	Dussu	8.0±1.10	37.04±0.16	35.52±0.43	17.00±0.20	4.33±0.57	76.85±0.01	16.47±0.24	9.16±0.20
	Samboora	7.0±1.76	37.77±0.30	35.28±0.41	17.00±0.52	3.33±1.52	77.93±0.05	16.76±0.14	9.33±0.30
Mean ± S.d		**7.53±1.46**	**38.25±0.32**	**35.15±0.89**	**16.60±0.44**	**3.77±1.22**	**76.90±0.03**	**16.24±0.22**	**9.05±0.30**
Sloppy	Meej	6.0±1.02	33.06 ±0.31	28.31±0.25	12.57±0.35	3.0±1.0	69.57±0.52	10.66±0.27	6.68±0.39
	Konibal	6.0±1.02	34.70±0.29	29..80±0.23	14.13±0.30	3.0±1.0	70.99±0.21	11.30±0.28	7.60±0.40
	Chandhara	5.6±0.57	35.41±0.40	31.38±0.92	14.20±0.52	1.60±0.57	72.08±0.15	12.26±0.16	7.70±0.43
Mean ± S.d		**5.86±1.02**	**34.39±0.03**	**29.83±0.46**	**13.63±0.39**	**2.53±0.85**	**70.88±0.29**	**11.40±0.23**	**7.32±0.40**
Plain	Ladhoo	12.0±1.19	38.31±0.08	35.08±2.12	17.83±0.20	5.33± 0.57	78.43±0.10	21.61±0.28	9.40±0.40
	Shaar	11.0±1.13	38.61±0.14	36.87±0.20	18.50±0.30	4.33±1.50	79.10±0.53	22.25±0.11	9.40±0.20
	Rajwos	11.0±1.16	39.19±0.24	39.22±0.40	19.20±0.17	5.33±1.52	79.75±0.02	24.34±0.24	11.0±0.20
Mean ± S.d		**11.33±1.16**	**38.70±0.15**	**37.05±0.90**	**18.51±0.22**	**4.99±1.19**	**79.09±0.21**	**22.73±0.21**	**9.93±0.26**

Conclusion:

This research aimed to isolate and characterize phosphate-solubilizing microorganisms from the saffron rhizosphere in the Northwest Himalaya region of India. The identified genera included *Glomus, Acaulospora, Scutellospora*, and *Gigaspora,* with *Glomus* and *Acaulospora* being predominant. The highest spore population was observed in the summer season compared to winter, attributed to the favourable temperature conditions during summer. Root colonization was more prominent in soils with lower phosphorus content. Phosphatase activity reached its peak in summer compared to winter, with maximum values noted in sloped areas where phosphorus levels were minimal. The population of phosphorus-solubilizing bacteria was highest in summer compared to winter, and peak values were identified in plain soils, indicating a higher presence of arbuscular mycorrhizae. This study concludes that various vesicular arbuscular mycorrhiza (VAM) species can serve as effective bio-fertilizers, potentially enhancing saffron production in the Kashmir Valley. Emphasizing the microbial population of saffron soils is crucial for optimizing these bio-fertilization strategies.

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