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Morphological and Molecular Characterizations of Arbuscular Mycorrhizal Fungi and Their Influence on Soil Physicochemical Properties and Plant Nutrition

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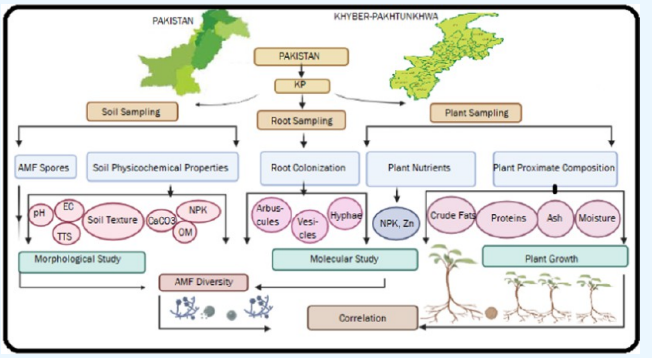
ABSTRACT: Pulses are considered a remarkable and stable source of nutrients, which are being presently extensively cultivated and consumed in different parts of the world. Pulses belong to the family Leguminosae and are a rich source of nutrients such as phosphorus (P) and nitrogen (N) for best growth via symbiotic relationship with bacteria and AMF (arbuscular mycorrhizal fungi). The aim of the current study was evaluating the influence of AMF diversity associated with various pulses (French bean, mung bean, kidney bean, peas, soybean, peanuts, and grams). Furthermore, AMF characterization was done using morphological features of spores and sequencing of the rDNA gene, which confirmed the existence of 10 different AMF taxa. Among the different genera, the genus *Glomus* was observed to be the most dominant with 30% species followed by *Gigaspora* (22%), *Sclerocystis* (12%), *Acaulospora* (8%), *Rhizophagus* and *Septoglomus* (7%), *Diversispora* (5%), and *Claroideoglomus*, *Archaeospora*, and *Ambispora* (3%). Furthermore, soil physicochemical analysis and percentage of AMF colonization results revealed the fact that the phosphorus content (inversely proportional to the AMF diversity) was a determining factor of AMF diversity. The highest amount of available phosphorus (62.825 mg kg^{-1}) in the district Swabi resulted in a low rate of AMF colonization (6.66 ± 11.54%) with a comparatively higher rate of AMF colonization (50.66 ± 1.15%) found in the soil of the district Chitral having a low phosphorus content (17.3 ± 7.6 mg kg^{-1}). Nutrient uptake by pulses including nitrogen (2.4 ± 1.3%), phosphorus (13.5 ± 7.6 mg kg^{-1}), potassium (99.5 ± 25.8 mg kg^{-1}), zinc (1.4 ± 0.5 mg kg^{-1}), moisture (2.3 ± 1.3%), crude fats (5.6 ± 2.8%), ash (4 ± 1.2%), and proteins (13.6 ± 9.01%) determined the fact that AMF species diversity is positively correlated to the plant mineral nutrition. From the current study, it is concluded that AMF inoculation to the soil fields is beneficial to ensure the sustainability and productivity of pulse crops in diverse environmental conditions without polluting the soil.



1. INTRODUCTION

Pulses subsidize economic and nutritional significance following cereals in human diet. The aptitude of pulses to maintain nitrate contents in the soil−crop system is their distinctive and valuable characteristic among other plant species.[1] The United Nations acknowledged the year 2016 as the "International Year of Pulses". It is predicted that the determination of the FAO (Food and Agricultural Organization), United Nations, is educating general public about the beneficial effects of pulses on nutrition and health.[2] Pulses belong to the family "Papilionaceae" and yield edible seeds, a worthy source of minerals including zinc, iron, potassium, magnesium, plant-based protein, dietary fiber, vitamin B, etc.[3] In Pakistan, the mostly grown pulses include common beans (*Phaseolus vulgaris* L.), mung bean (*Vigna radiata* (L.) Wilczek), faba bean (*Vicia faba* L.), moth bean (*Vigna aconitifolia* (Jack) Merechal), chickpea (*Cicer arietinum* L.), pigeon pea (*Cajanus cajan* L. Millsp.), cowpea (*Vigna unguiculata* (L.) Walp.), black gram or mash (*Vigna mungo* L. Hepper), and lentil (*Lens culinaris* Medic.).[4] They are commonly grown in the rain-fed areas having poor soil conditions; however, the goal of higher productivity may be accomplished by means of modified varieties, fertilizers, and micronutrients. Due to the overwhelming occupation of wheat in irrigated areas, more than 80% of the pulses are grown in the Thal and Potohar regions of Pakistan.[5]

In order to justify plant nutrient requirements, most of the smallholder farmers of Pakistan have inadequate competencies

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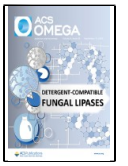

to get inorganic fertilizers.[6] In such circumstances, pulses may be the best option that not only increases soil fertility through biological nitrogen fixation (BNF) but also improves phosphorus (P) acquisition via mycorrhizal associations. Mycorrhizal association is a symbiotic association of some fungal species (including ectomycorrhizae and endomycorrhizae) with plant roots.[7] The most common endomycorrhizal group is the arbuscular mycorrhizal fungi (AMF) that use arbuscules and vesicles for the transportation and storage of plant nutrients.[8] In these mutual relations, the plant offers photosynthates (up to 20%) to the fungus (AMF), while AMF provide the plant with macronutrients, particularly phosphates.[9] AMF show a major concern over plant nutrition; they have a dual role in managing nutrition in soil. For example, below the critical point of Zn (involved in pollen tube formation) concentration, Zn uptake is enhanced by AMF application and above the critical level, Zn translocation to plant shoots is restricted. Soil nutrient application techniques which can also ameliorate AMF are widely promising. This is how AMF play a vital role in developing farming practices toward sustainable agriculture. The significant role of AMF in plant growth is translocating important immovable nutrients such as Cu, Zn, and P and reducing metal toxicity in the host plant.[10,11]

The number of AMF described by morphological description reached 244 in 2013.[12] Numerous AMF taxa show similar morphology during some phases of their growth, which is why both morphological and molecular traits are used in existing cataloging systems of AMF.[13] Focusing on DNA-based techniques, many molecular markers such as AM1, NS31, AML1, AML2, AM2, AM3, ACAU1660F, ARCH1311F, GLOM1310F, GLOM5.8R, GIGA5.8R, ITS1F, ITS4R, and LETC1670F have been designed to explore AMF diversity.[14] The nuclear rRNA genes including a broadly used small subunit of rRNA gene, ITS region, and a part of the large subunit of rRNA gene are considered to be the most frequently used reliable molecular markers.[15] For AMF amplification, numerous primer combinations have been published till now, AM1-NS31 (~550 bp),[2] AML1-AML2 (~800 bp), AM2, and AM3.[16] In order to characterize AMF spores, researchers collected the rhizospheric soil of plants grown in various sites of the Junagadh district, India. After dying with tryphan blue, they observed different types of AMF spores under a scanning electron microscope (SEM). The sizes of spores ranged between 10 and 105 μm. For molecular study, they extracted DNA from the colonized roots in order to observe the gel bands following universal primers pairs: (1) NS1 and NS 4 and (2) AML1 and AML2. For species documentation, they used ACAU1660F-, ARCH1311F-, GLOM1310F-, GLOM5.8R-, GIGA5.8R-, ITS1F-, ITS4R-, and LETC1670F-specific primers.[17] Other researchers evaluated AMF diversity in symbiotic interactions with *Acacia* tree roots in South Western Saudi Arabia. They noticed a considerable change in the colonization rate among species. The roots and rhizospheric soil of *Acacia negrii* were found to have the highest spore density and colonization rate. For molecular investigations, a large subunit of ribosomal DNA of AMF was augmented using a nested-PCR after DNA from plant roots was removed. Sequencing was done on a total of 274 LSU of rDNA-emulated wreckages from the roots of three *Acacia* plants. High AMF diversity was discovered using phylogenetic analysis, particularly in *Acacia tortilis*. AMF was divided into five genera based on the LSU rDNA sequences: *Acaulospora*, *Claroideoglomus*, *Glomus*, *Scutellospora*, and *Gigaspora*. The genus *Scutellospora* was only discovered in the roots of *Accacia tortilis*, although the genus *Glomus* was the most prolific colonizer of all three investigated acacia species. The substantial AMF-Acacia diversity suggested that AMF is crucial to the persistence of *Acacia* species in arid environments.[18] In a recent study, researchers assessed the molecular characterization of AMF, inhabiting the roots of *Carissa edulis* in Lake Victoria basin, Kenya. They collected the root samples from six replicates/spot. AMF diversity was measured via morphological description and sequencing of SSU of rDNA. As a result of morphological and molecular characterization, they identified four AMF genera: *Glomus*, *Gigaspora*, *Scutellospora*, and *Acaulospora*. Only two genera, *Acaulospora* and *Glomus*, were common for both types of analyses. Large quantities of spores in soil and in plant roots confirm strong interrelations of AMF with *Carrisa edulis*.[19] The aim of the current study was to explore the morphological and molecular characterization of AMF colonizing the roots of pulses in various districts of Khyber Pakhtunkhwa, Pakistan. The current study will contribute to the understanding of the associations of AMF populations with pulses and their influences on pulse growth.

2. MATERIALS AND METHODS

2.1. Description of the Research Area. Several field surveys were conducted to collect pulses from different climatic regions of Khyber Pakhtunkhwa (KP), Pakistan. It has a versatile weather and a unique topography. Its climate varies from dry, hot rocky zones in the South to lavish cool green forests in the North. Ecologically, the province can be divided into three regions, i.e., the Southern hottest region and the central moderate and Northern cooler regions (Figure 1). The three climate regions are as follows: northern symbolized by Chitral District, central region symbolized by Nowshera, Charsadda, and Peshawar districts, and southern region symbolized by Dera Ismail Khan and Tank districts.[20]

Figure 1. Ecological regions of Khyber Pakhtunkhwa, Pakistan.

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2.2. Root and Soil Sampling. Rhizospheric soil and roots of pulses were collected during Rabi and Kharif seasons of 2019 and 2020. Four replicate (R) samples/field were collected from some selected districts of KP, Pakistan. For each replicate, four plants along with their root system and rhizospheric soil were taken at the depth of 10−30 cm. Part of the samples (rhizospheric soil and plant roots) were kept at 4 °C until carrying out the microscopic analysis, while part of the roots were kept at −80 °C until the molecular study was achieved.

2.3. AMF Colonization Status, Spore Extraction, and Identification. AMF colonization and spore extraction were carried out in Lab No. 1 of Bacha Khan University Charsadda, Pakistan. The AMF colonization status was assessed by studying root fragments using the Phillips & Hayman technique.[21] The sampled root sections of about 10−20 mm were taken and flooded in an already prepared solution of 10% KOH (potassium hydroxide) solution at 85 °C for about 50 min. The root sections were then bleached with hydrogen dioxide (H_2O_2) for three minutes and were then acidified with 1% of hydrochloric acid (HCl). In the next step, the root sections were tarnished by treating them with fuchsine acid (0.05%) for 90 min at 60 °C. Occurrences of AMF vegetative structures such as arbuscules and vesicles were assessed using the following formula: mycorrhizal frequency (AMF F%) = (N − NO)/N × 100. Similarly, AMF spores (present in the rhizospheric soil of pulses) were extracted by Gerdemann and Nicolson.[22] About 100 g of soil was taken and sieved through a series of meshes having 1000, 100, and 32 μm sieve diameters. The recovered AMF spores were sited in Petri plates and were observed under a stereomicroscope (40× magnification) (Figure 2). The average of spores was assessed as per 0.1 kg of soil.

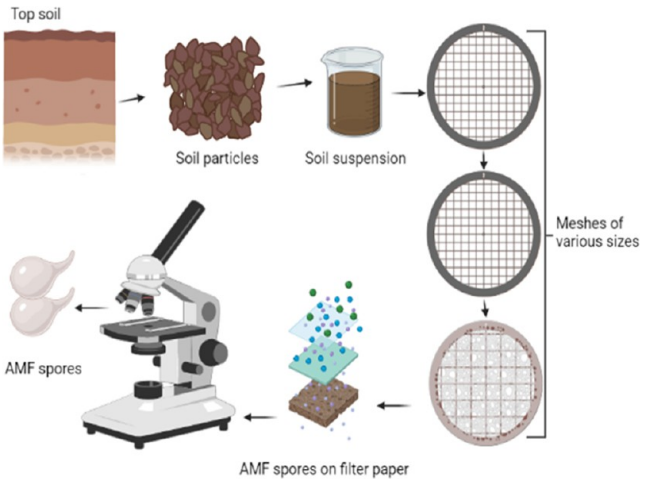

Figure 2. Showing the overall process of AMF spore extraction and identification.

Detailed observation was done for each type of spore and spore shape, color, size, etc. was documented. Further morphotype credentials were based on identification and descriptions criteria prevailing by the International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal-Fungi (INVAM) and on the images available in various studies. For each type of soil samples, species assortment was carried out by using the Shannon−Wiener variety guide. The root colonization and AMF spore imaging facility was provided by The International Center for Chemical and Biological Sciences, Karachi, Pakistan.

2.4. DNA Extraction. The process of DNA extraction was carried out in the Decode Genomics research training and diagnostic center, Lahore, Pakistan (Figure 3). AMF-colonized roots of pulses were used for DNA extraction, using standard protocol. About 1 g of root segments was taken and chopped with the help of a grout and crusher. The samples were vortexed and incubated for 10−15 min at 65 °C after adding 400 μL of activator protein 1 buffer and 4 μL of RNase A. In the next step, 130 μL of P3 buffer was added and mixed properly by pipetting. The samples were then centrifuged for 5 min at 14,000 rpm and autoclaved, without troubling the cell bits. About 1.5 vol of AW1 buffer was supplemented and shaken well. About 550 μL of the mixture was then transferred into a miniature tube. The mixture was then centrifuged for 1 min at 6000*g* (8000 rpm), and the upper layer was cast off. The collection tube was then reused. The spin column was placed into a new gathering hose, and 500 μL of AW2 buffer solution was mixed and centrifuged at 8000 revolutions per minute for 60 s. The upper layer was discarded, the miniature tube was reassembled in the same gathering conduit, and 500 μL of AW2 buffer was added and centrifuged at 14,000 rpm. The upper layer was discarded and centrifuged for 2 min at 20,000*g* (14,000 rpm). The spin column was carefully removed from the gathering cylinder evading the chances of mixing. The spin column was transferred to a new separator tube, and 35−100 μL of elution buffer (AE) was added to elute the DNA and was centrifuged for 60 s. Again, 35−100 μL of AE buffer was added again, left for 300 s at room temperature, and again centrifuged for 60 s.[23]

2.5. PCR Amplification and Agarose Gel Electrophoresis. Amplification and gel electrophoresis were carried out in the Decode Genomics research training and diagnostic center, Lahore, Pakistan. A standard solution of primers was prepared at 0.1 μmol/mL. Similarly, the working solution of primers was prepared at 0.01 μmol/mL. Both solutions were kept at room temperature. (a) First PCR: an absolute volume of 0.025 mL of the following combination was made for the first polymerase chain reaction: 0.01 mL of Master Mix, 0.0005 mL of 10 μM of each forward (AML1) and reverse (AML2) primer, and 0.013 mL of molecular grade water. (b) Second PCR (Nested): an absolute volume of 0.025 mL of the following combination was made for the second PCR: 0.01 mL of Master Mix (2), 0.0005 mL of each left and right primer, and 0.013 mL of PCR grade water. (c) ITS rDNA amplification: An absolute volume of 0.025 mL of the following combination was made for the first polymerase chain reaction: 0.01 mL of Master Mix, 0.0005 mL of 10 μM of each left and right primer, and 0.013 mL of molecular-grade water. The DNA extract was then checked by 1% agarose gel electrophoresis. The primers were provided by Macrogen Company, South Korean.

2.6. Phylogenetic and Statistical Analysis. The phylogeny was inferred by using the neighbor joining method. Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA 11) software, while all the other statistical analyses, i.e., Shannon−Wiener diversity index (for spore diversity) was calculated by using Microsoft Excel and Pearson correlation (correlation between AMF diversity and soil physicochemical, plant elemental and proximate analysis) was carried out by using Statistical Product

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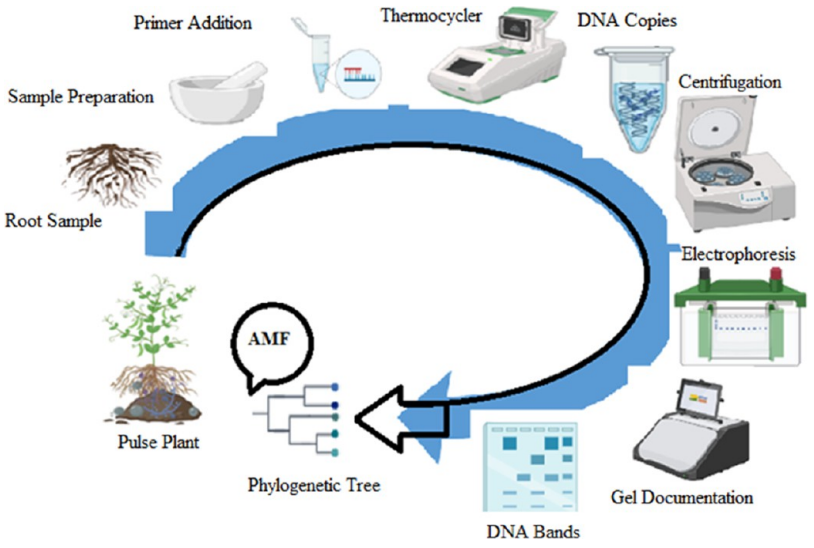

Figure 3. Showing the overall process of DNA extraction and molecular identification of AMF.

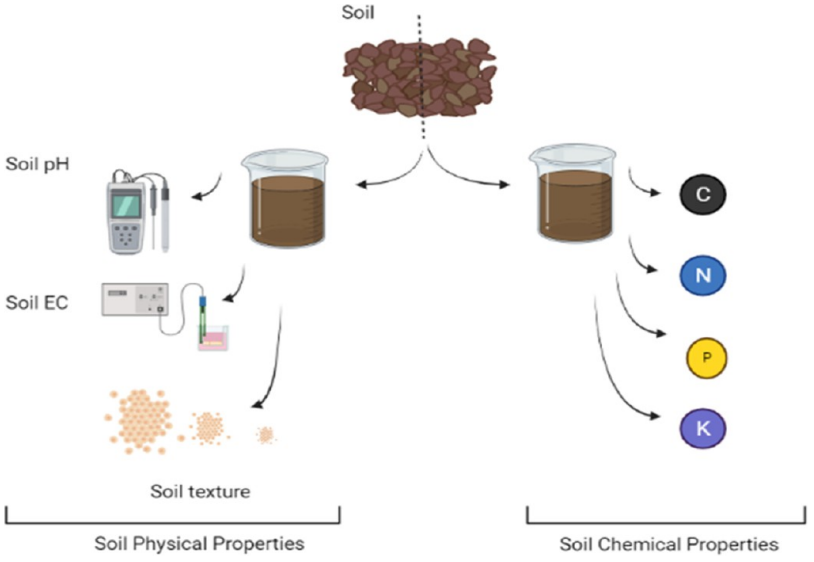

Figure 4. Diagrammatic presentation of the analyzed soil physicochemical properties.

and Service Solutions (SPSS) software. The SPSS function "Pearson correlation coefficient and interpretation" was used.

2.7. Soil Sampling, Processing, and Soil Chemical Characterization. Weeds, stones, and pebbles were carefully removed from the area with hands wearing gloves. Rhizospheric soil samples were collected by taking away a lump of about 20 cm deepness. The soil samples were carefully transported to the laboratory in zip locks and were preserved till further analysis. The overall procedure is given in Figure 4. All the chemicals and instruments required for soil physicochemical analyses were provided by BKUC and Agricultural Research Institute, Tarnab Peshawar, Pakistan.

2.7.1. Soil pH and Electrical Conductivity. Soil pH was determined using a 1:2 soil/water method as described by Kalra.[24] For this purpose, 0.02 kg of the sampled soil was taken and mixed with 0.04 L of purified water. The mixture was agitated continuously for about 30 min with a stirrer and was left undisturbed for 60 min. In order to detect the pH values, electrodes were inserted into the mixture. The electrode was washed out with purified water before going to the next reading. Similarly, for measuring electrical conductivity, the mixture was prepared by following the Kalra and Maynard method.[25] Then, an electrical conductivity meter was immersed in the mixture in order to note the electrical conductivity values.

2.7.2. Soil Texture. In order to determine the soil texture, a sediment jar test method was used, as described by Sammins.[26] The vessel was filled with 1/3 of soil sample, by leaving some space at the top, for introducing clean water. The vessel was covered with a cap and was shaken vigorously, after introducing one tablespoon of ground dishwashing detergent. The vessel kept undisturbed for about two days (48 h). After 2 days, the height of each layer was measured with a ruler. By using the following formulae, the percentage of each sheet was calculated:

$$\%X = [(\text{height of } X)/(\text{total height})] \times 100\%$$

$$\%Y = [(\text{height of } Y)/(\text{total height})] \times 100\%$$

$$\%Z = [(\text{height of } Z)/(\text{total height})] \times 100\%$$

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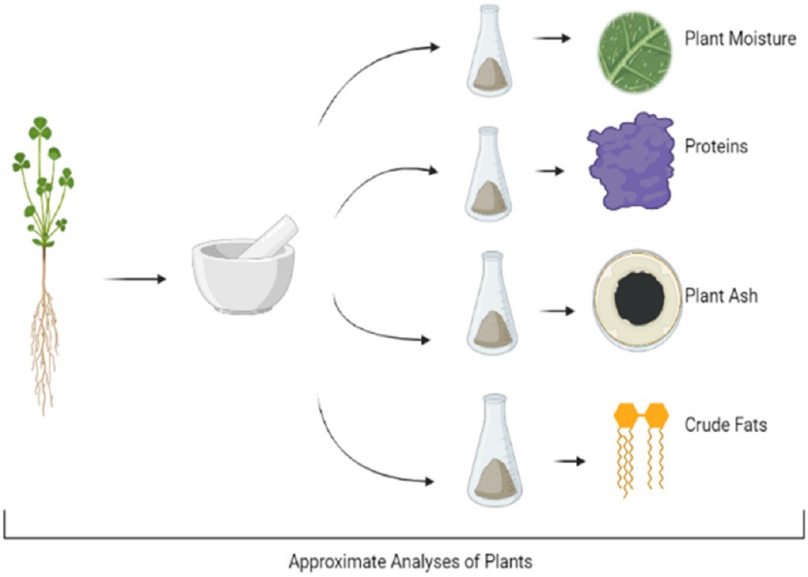

Figure 5. Diagrammatic presentation of the analyzed plant proximate analysis.

where X = layer of sand, Y = layer of silt, and Z = layer of clay. At the end, the soil texture triangle was used for the assessment of the soil type.

2.7.3. Organic Carbon. By using the Walkey−Black dichromate method, the amount of carbon (organic) was evaluated. For this purpose, one gram of finely powdered soil sample was taken into a 500 mL conical flask. Then, 20 mL of con. H_2SO_4 (sulfuric acid) and 10 mL of potassium dichromate were supplemented, and all the materials were continuously shaken for about 60 s; after that, all the contents were subjected to established. After 30 min, a mixture prepared by 0.2 L of purified water, 0.01 L of H_3PO_4 (phosphoric acid), and 0.001 L of $C_{12}H_{11}N$ (diphenylamine) indicator was poured. The mixture was then titrated against the standardized solution of $(NH_4)_2$ $Fe(SO_4)_2$ (ferrous ammonium sulfate) till the tint of the mixture was transformed to avocado color by using the Walkey−Black dichromate method; the amount of carbon (organic) was evaluated.[27]

2.7.4. Estimation of Nitrogen. The contents of available nitrogen were assessed by the alkaline permanganate method as described by Subbaiah and Asija. About 0.02 kg of the sampled soil was taken into a flask. Then, 10 mL of each $KMnO_4$ (0.32%) and NaOH (2.5%) was added respectively. 25 mL of 0.02 N sulfuric acid was taken with few drops of methyl red indicator. The contents of the flask were distilled in order to collect about 30 mL of the distillate into the known excess of 0.02 N sulfuric acid. The excess of the sulfuric acid was titrated against 0.02 N potassium hydroxide till the pink color was changed to yellow. After doing the whole procedure, the volume of the consumed 0.02 N sulfuric acid by ammonia and percentage of nitrogen present in the given soil sample were calculated.[28]

2.7.5. Estimation of Phosphorous. By following Olsen's method, the amount of available phosphorus was assessed. In a 250 mL conical flask, about 0.005 kg of sampled soil was taken and was dissolved into 100 mL of 0.5 M sodium bicarbonate solution. One teaspoonful of carbon black was added and the mixture was shaken thoroughly for about 30 min. The muddle was then passed through a Whatman filter paper (No. 40). Then, 10 mL of the mixture was taken into a volumetric flask and a droplet of para nitrophenol ($C_6H_5NO_3$) indicator was supplemented by keeping acidic pH (3.0), with 4 N of hydrochloric acid (HCl). The standardized arc was established with the equal amount of sodium bicarbonate ($NaHCO_3$) involved. The constant color strength was noted photometrically after 5 min. The phosphorus amount was assessed as kg hac-1 of the soil.[29]

2.7.6. Estimation of Potassium. Levels of available K in the sampled soil were assessed by following the flame photometric method as described by Jankowski and Freiser; about 0.005 kg of oven-dried sampled soil was taken into a flask (150 mL) and was dissolved into a 25 mL of One-N solution of ammonium acetate ($C_2H_7NO_2$). The mixture was then strained immediately by using Whattman filter paper (No.1). About 5 mL of the mixture was diluted with 25 mL of purified water. The watery extract was atomized to a flame photometer to ascertain the extent of potassium in kg/hectare of sampled soil.[30]

2.8. Stuff/Powder Preparation for Proximate Analyses. Plant vegetative parts were shade-dried and ground into fine powder. The powdered contents were then subjected to different standard protocols described[31] in order to detect protein, carbohydrate, crude fiber, crude fat, ash, and moisture contents (Figure 5). All the chemicals and instruments required for plant proximate analyses were provided by Agricultural Research Institute, Tarnab Peshawar, Pakistan.

2.8.1. Detection of Moisture. By using a gravimetric method as described by AOAC (The Association of Official Analytical Chemist's), 5.0 g of powdered materials was taken and subjected to oven drying at 105 °C for 3 h. The material was then kept in a desiccator, cooled, and reweighed. The reweighed materials were then kept in an oven for further drying. The three practices drying, cooling, and weighing were repeated again and again after each hour. When no further reductions in weight were observed, by using the following formula, the moisture content percentage was detected:

$$\text{moisture content } (\%) = \frac{W_2 - W_3}{W_2 - W_1} \times \frac{100}{1} \quad (1)$$

where W_1 is the weight of empty moisture, W_2 is the weight of the empty can plus the sample already dried, and W_3 is the weight of the can plus weight of the dried sample.

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2.8.2. Detection of Proteins. The protein content was detected via the Kjeldahl method, modified by Barbano et al.[32] For protein detection, first of all, the total nitrogen content was worked out. In order to obtain the protein content value, the value of total nitrogen was crossed with 6.25 factor. The overall procedure was that 0.5 g of sample material was taken in a digestion flask and dissolved into 10 mL of concentrated sulfuric acid (H_2SO_4). The solution was then heated, and a selenium tablet was added as a catalyst. After heating the mixture, the solution was poured into a volumetric flask and was thinned to 100 mL for further analyses. About 10 mL of the mixture was then shifted to the Kjeldahl distillation apparatus, and 10 mL of 45% NaOH solution was added to it. The mixture was condensed into 10 mL of 40% boric acid having three drops of mixed indicator (bromo-cressol-green/methyl-red). About 50 mL of extracts was recovered and was titrated against 0.02 N of EDTA solution up to the appearance of bright red color by using the below-mentioned formulae (i, ii); the nitrogen content was determined, resulting in determination of the protein content:

$$N_2\ (\%) = \frac{100}{W} \times \frac{N \times 14}{1000} \times \frac{V_t}{V_a} \times T \times B \quad (2)$$

where W is the weight of sample material = 0.5 g, N is the normality of sulfuric acid = 0.02 N, V_t is the total volume (100 mL), V_a is the analyzed volume (10 mL), T is the sample titer rate, and B is the blank titer rate.

$$\text{protein}\ (\%) = N_2\ (\%) \times 6.25 \quad (3)$$

2.8.3. Detection of Ash. Determination of ash was carried out by a furnace incineration gravimetric method.[33] About 5.0 g of powdered sample was taken into an already weighed ceramic crucible. The powdered plant material was burnt at 550 °C to ash by using a softening furnace. After complete burning, the ash was subjected to cooling in a desiccator and reweighed. Ash weight was calculated by difference as a % of the mass of the plant material analyzed:

$$\text{ash}\ (\%) = \frac{W_2 - W_1}{\text{weight of sample}} \times \frac{100}{1} \quad (4)$$

where W_1 is the weight (in g) of the unfilled container and W_2 is the weight (in g) of the unfilled container + ash.

2.8.4. Detection of Crude Fats. Contents of crude fats were worked out via a gravimetric method as described by Kirk and Sawyer.[34] About 5 g of powdered plant sample was taken, enveloped in a Whatman filter paper, and placed in a thimble. The thimble was put in a Soxlet reflux flask shifted to a flask (of known weight), having 200 mL of petroleum ether. The top of the reflux flask was connected to a water condenser. The petroleum ether was boiled, let to vaporize, and then condensed into the reflux flask. Petroleum ether was added to the thimble sample until the reflux flask occupied and tapped over, collecting its oil content in a boiling flask. The practice was allowed to continue constantly for about four hours, after which the defatted material was removed, and the petroleum ether was recovered and the oil extract was collected in the flask. The flask having oil content was desiccated in an oven at 60 °C for 30 min. The oil extract was cooled and weighed. The weight of fat (oil extract) was detected as a difference and calculated as a weight percent of the plant sample analyzed:

$$\text{fat}\ (\%) = \frac{W_2 - W_1}{\text{weight of sample}} \times \frac{100}{1} \quad (5)$$

where W_1 is the weight (in g) of the unfilled flask and W_2 is the weight (in g) of the unfilled flask + fats (oil extracts).

3. RESULTS

3.1. Studied Sites and Pulse Crops. AMF diversity was randomly checked in 10 Districts (Charsadda, Mardan, Swabi, Chitral, Dir, Bunner, Haripur, Mansehra, Karak, and Abbottabad) of agroecological importance (Figure 6). Detailed visits were done during Rabi and Kharif seasons of 2019 and 2020 in order to collect different pulse crop plants along with rhizospheric soil. The pulses included French bean (*Phaseolus vulgaris* L.), mung bean (*Vigna radiata* (L.) R. Wilczek), kidney bean (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), soybean (*Glycine max* (L.) Merr.), peanuts (*Arachis hypogaea* L.), and grams (*Cicer arietinum* L.).

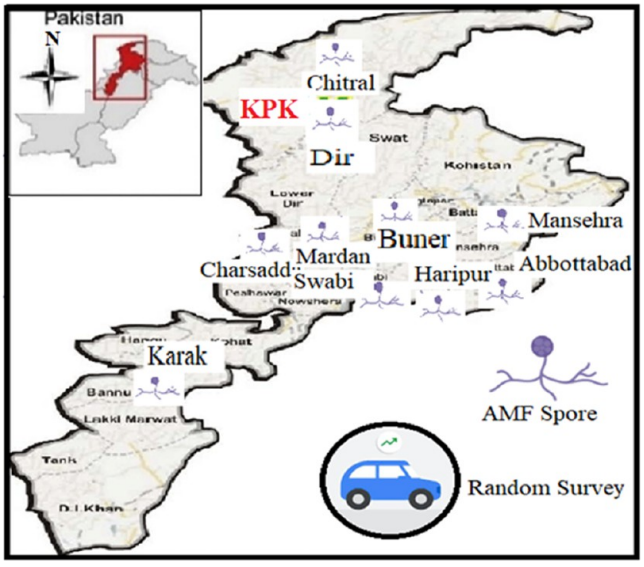

Figure 6. Geographic locations of the surveyed Districts of the Khyber Pakhtunkhwa province of Pakistan.

3.2. Degree of Colonization in Roots. The presence of AMF was noted in roots in only 36 samples of the total 40 collected samples from 10 sites (Figure 7A–F). Mycorrhizal enhancement regarding percent root colonization in samples collected from various districts was ranked as Chitral, Mansehra > Dir > Abbottabad > Haripur > Bunner > Charsadda > Karak > Mardan > Swabi (Tables 1 and 2, and Figure 8).

Data given in Tables 1 and 2 showing highly significant ($p < 0.05$) AMF colonization rate, i.e., external hyphae (40.66%), internal hyphae (43.33%), arbuscules (14%), vesicles (50.66%), and spore number, were recorded in the soil of Chitral. Chitral was followed by Mansehra > Dir Upper > Abbottabad > Haripur > Bunner > Charsadda > Karak > Mardan. The lowest rate of colonization in terms of external hyphae (6.66%) was recorded in the root samples of the Swabi district.

3.3. AMF Spore Identification. A total of 21 AMF morphotypes were collected from the rhizosphere of surveyed pulses. On the basis of morphological characterization, they were kept under the following genera, viz., *Glomus*, *Gigaspora*,

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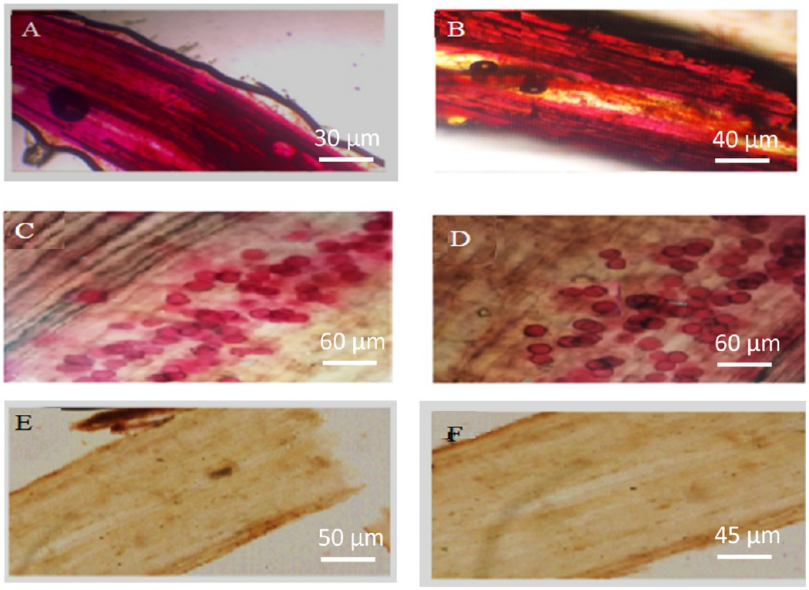

Figure 7. (AMF width). (A). External and cortical hyphae with spores (20−40 μm), (B). Cortical hyphae with spores (10−20 μm), (C, D). AMF vesicles (40−0 μm), (E, F) with no colonization.

Table 1. AMF External, Internal Hyphae, Arbuscules, and Vesicles Observed in the Plant's Roots, Collected from Four Sites of Each District[a]

district name	external hyphae (%)	internal hyphae (%)	arbuscules (%)	vesicles (%)	spores count/100 g of soil
Chitral	40.66 ± 9.01a*	44 ± 6.92a*	14 ± 6.37a*	50.66 ± 1.15a*	35.25 ± 15.6a*
Mansehra	40 ± 10a	43.33 ± 5.77a	13.33 ± 5.94a	50 ± 0a	34 ± 13.7a
Dir Upper	36.66 ± 1.54a	37.33 ± 5.77b	7.33 ± 6.42b	36.66 ± 5.77b	32 ± 12a
Abbottabad	33.33 ± 5.77ab	33.33 ± 11.54c	6.66 ± 5.77b	35.66 ± 5.77b	32 ± 12a
Haripur	24 ± 5.29bc	24.33 ± 6.65d	4 ± 6.08c	25 ± 5.29c	30.75 ± 11.5a
Bunner	20.66 ± 10.06cd	23.33 ± 5.77de	4 ± 6.92c	24 ± 5.30c	30 ± 10a
Charsadda	20 ± 10cd	20 ± 0ef	3.33 ± 6.35c	22.33 ± 5.77cd	29 ± 9.9a
Karak	13.33 ± 11.54de	16.66 ± 5.77fg	3.33 ± 0c	20 ± 0d	25.75 ± 9.7ab
Mardan	6.66 ± 5.77e	13.33 ± 5.77g	2.22 ± 0c	10 ± 10e	24.25 ± 9.8ab
Swabi	6.66 ± 11.54e	-	-	-	14.5 ± 9.5b

[a]Key: Mean of four ± standard error mean;.

Table 2. Mean Square Values of AMF External, Internal Hyphae, Arbuscules, and Vesicles Observed in the Plant's Roots, Collected from Four Sites of Each District

SOV	DF	external hyphae	internal hyphae	arbuscules	vesicles	spores
districts	9	676.8[a]	788.5[a]	85.4[a]	1047.6[a]	1315.5[a]
sites/district	3	26.5	0.2	0.67	10	132.1
error	27	14.1	2.3	0.74	1.1	739.9
total	39					

[a]Represents the significance at $p \leq 0.05$; DF = Degree of Freedom.

Sclerocystis, *Rhizophagus*, *Septoglomus*, *Acaulospora*, *Diversispora*, *Claroideoglomus*, *Archaeospora*, and *Ambispora*. The genus *Glomus* was the leading genus having a species percentage of 30%, followed by *Gigaspora* (22%) > *Sclerocystis* (12%) > *Rhizophagus* (7%), *Septoglomus* (7%), *Acaulospora* (7%) > *Diversispora* (5%) > *Claroideoglomus* (1%), *Archaeospora* (1%), and *Ambispora* (1%). Details of identified families, genera, and species are given in Figure 9A,B.

It was concluded from our findings that each habitat was similar with the species of the following three AMF genera: *Glomus*, *Gigaspora, and Sclerocystis* species (Figures 10−12). The reports exposed the fact that AMF "*Glomus*" was the most prevailing genus in all the assessed soil samples of different environments. *Glomus* was followed by *Gigaspora* and *Sclerocystis* in terms of percentage (%) of species diversity.

3.4. Molecular Characterization of AMF. The genera of the AMF families *Glomeraceae*, *Claroideoglomeraceae*, *Gigasporaceae*, *Acaulosporaceae*, and *Diversisporaceae* were amplified by using the primer pair AM1 and NS31 (Figure S1). The new primer pair AML1 (ATC AAC TTT CGA TGG TAG GAT AG) and AML2 (GAA CCC AAA CAC TTT GGT TTC) was used to amplify the members of the family *Glomeraceae* and the family *Claroideo glomeraceae* (Figure S2). Similarly, the members of the family *Acaulosporaceae* were detected by using ACAU1660 (TGA GAC TCG GAT CGG) (Figure S3), with ARCH1311 (TGC TAA ATA GCC AGC CTG Y) for

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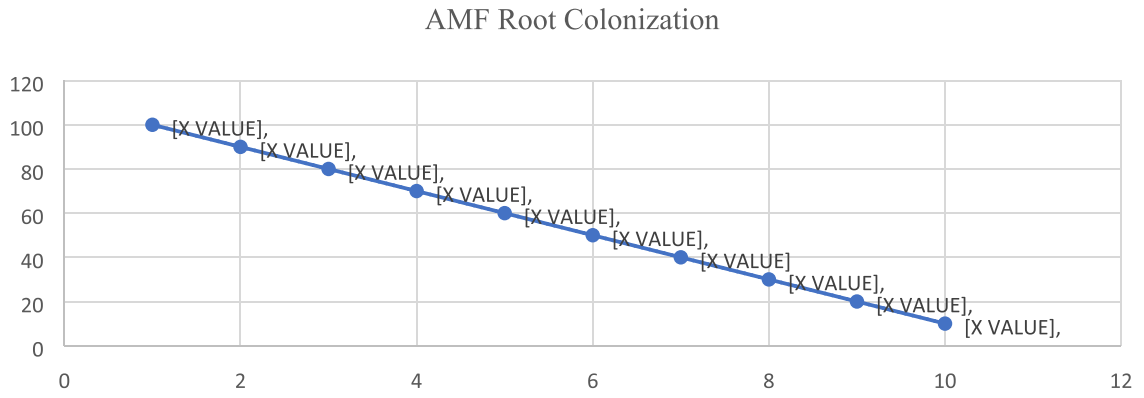

Figure 8. District-wise percentage of AMF colonization in roots of various pulses collected from the research area.

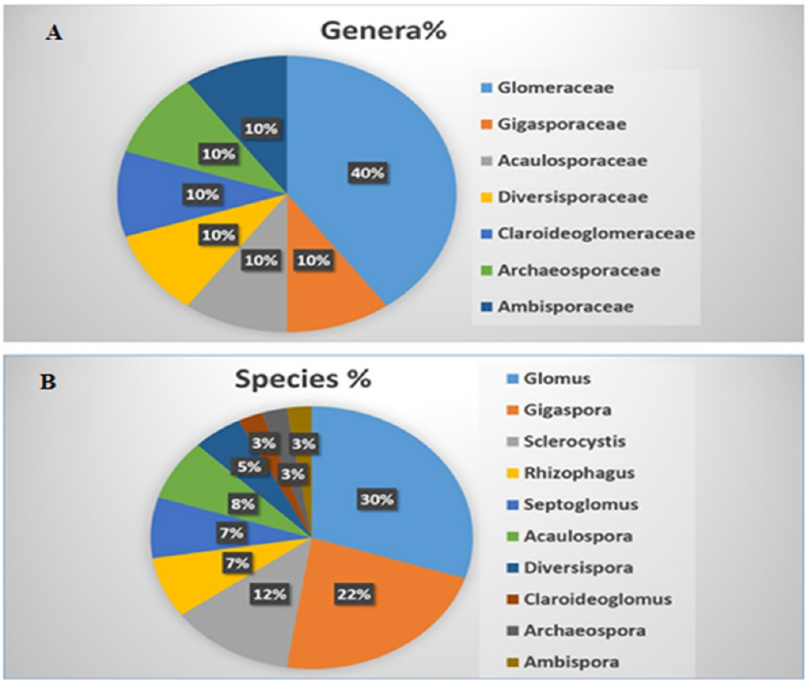

Figure 9. (A, B) Percentage of recovered AMF genera and species in each AMF family.

the family Archaeosporaceae (Figure S4) and with LETC1670 (GAT CGG CGA TCG GTG AGT) for the family *Diversisporaceae* (Figure S5). Primer ITS4 was used as a reverse primer with ACAU1660, ARCH1311, and LETC1670. The choice of the appropriate primer set is most decisive to study AMF diversity. In order to characterize natural AMF diversity, a set of primers would be predictable to amplify majority of AM fungi. In the present study, we used two mostly used primers, i.e., NS31/AM1 and AML1/AML2, for genus amplification. The primer NS31/AM1 covering 500 bp of SSU of the rDNA-gene has been extensively used in the molecule-based study of AMF. Keeping in view the above-mentioned facts, primers for species identification were designed and used to amplify various sections of the rDNA gene (Table S1).

3.5. Phylogeny of AMF Taxa. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the neighbor joining tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11. In the present study, the molecular range of AMF allied to the rhizosphere of pulses sampled from various localities of the KP province of Pakistan was evaluated by examining large subunit ribosomal DNA gene taken out from AMF hyphae inhabiting the roots of pulse crops. After alignment, our sequences could be grouped in 21 OTUs according to the 1000 bootstrap value identity level (Figure 13).

Results of the phylogenetic tree revealed that the total 21 OUTs can be divided into three clades. Clade 1 consists of 11 OTUs, and there is 0.0 or 0.1 base difference with each other. Only the Rhizophagus fasciculate has 0.2 base difference with the members of its own clade but a large difference of 0.8 base with clade 2. Similarly, in clade 2, the members have 0.1 or 0.2 base difference with each other. Clade 3 has 1.3 base difference with clades 1 and 2.

3.6. Soil Physicochemical Characterization and Its Relationship with AMF Spore Diversity. The soil pH values ranged from 7.5 to 8.2, and significantly ($p < 0.05$) higher pH (8.2) was recorded in the soil samples of Chitral,

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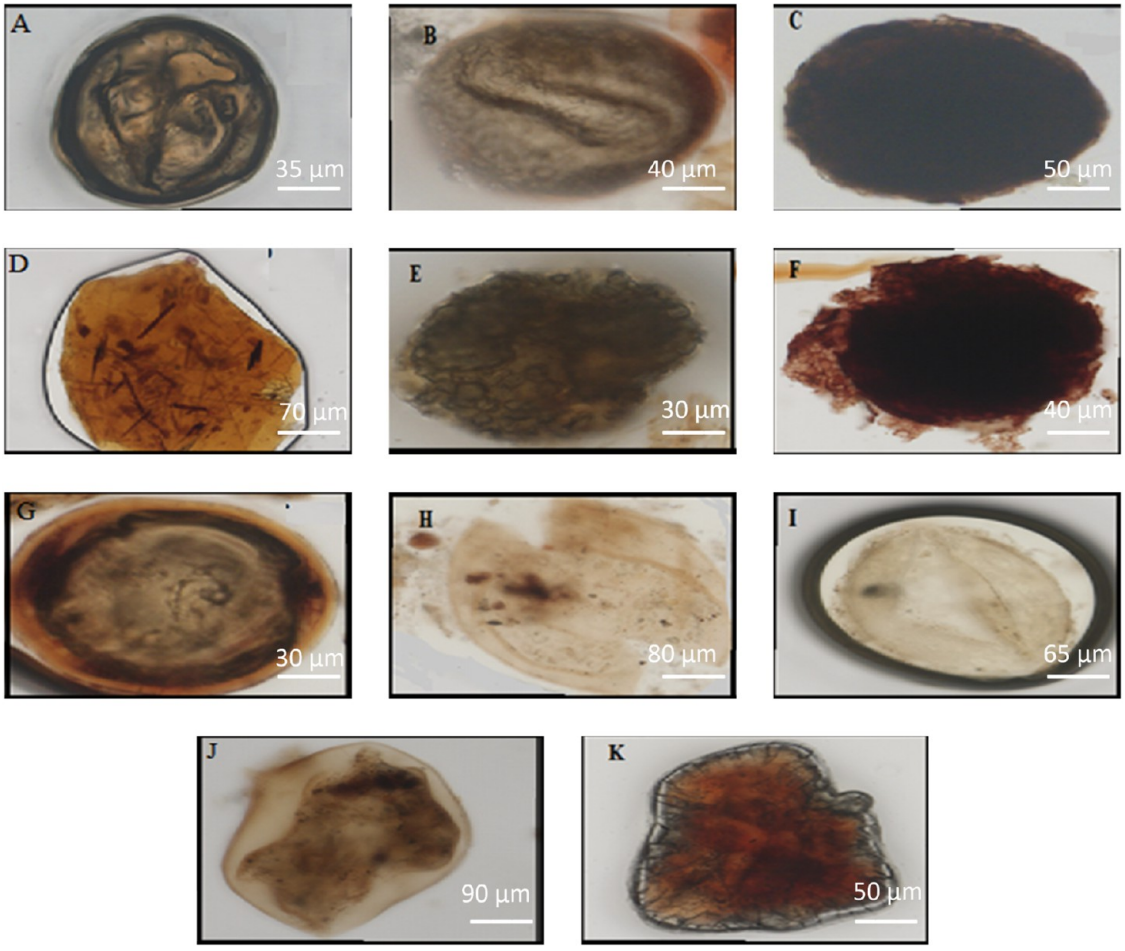

Figure 10. (A). *G. constrictum* (B). *G. fasciculatum* (C). *G. mosseae* (D). *G. lacteum* (E). *G. aggregatum* (F). *G. macrocarpum* (G). *G. deserticola* (H). *G. lamellosum* (I). *G. intraradices* (J). *G. clarum* (K). *G. versiforme*.

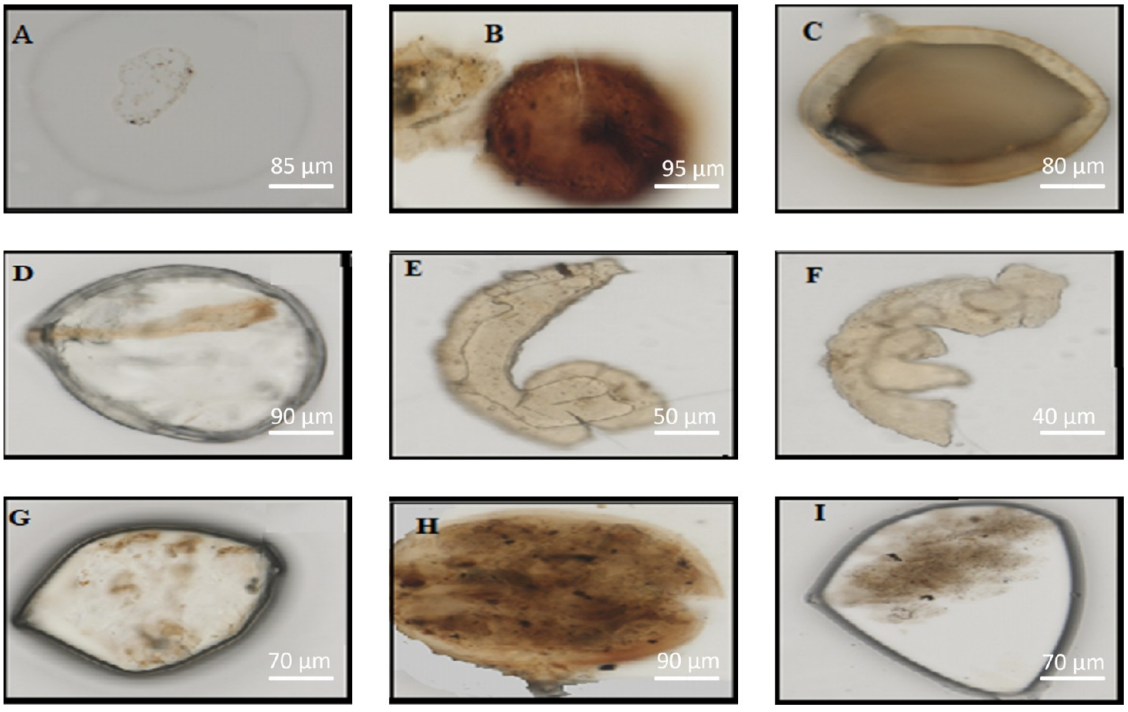

Figure 11. (A). *G. margarita* (B). *G. albida* (C). G. species. (D). G. species (E). G. species (F). G. species (G). G. species (H). G. species (I). G. species.

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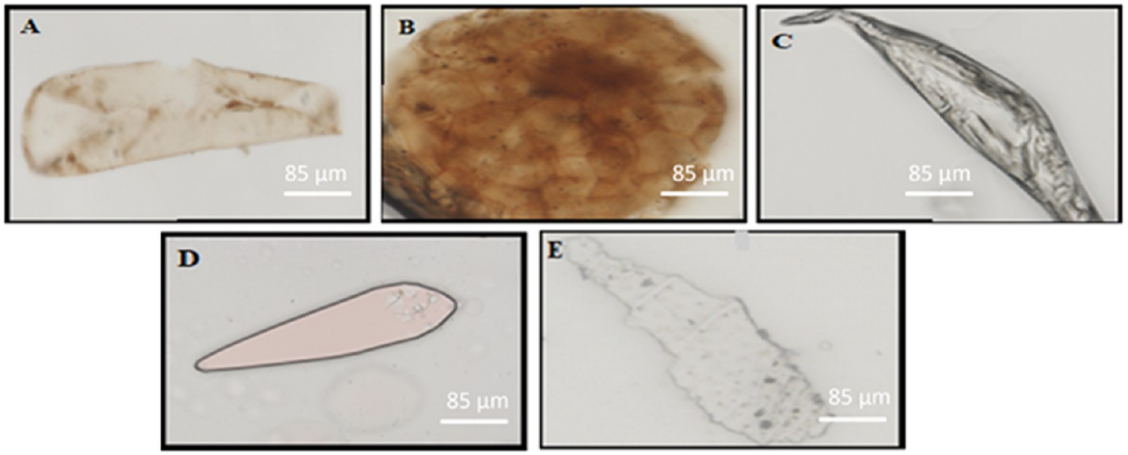

Figure 12. (A). *Sclerocystis pakistanika* (B). *Sclerocystis sinuosum* (C). S. species (D). S. species (E). S. species.

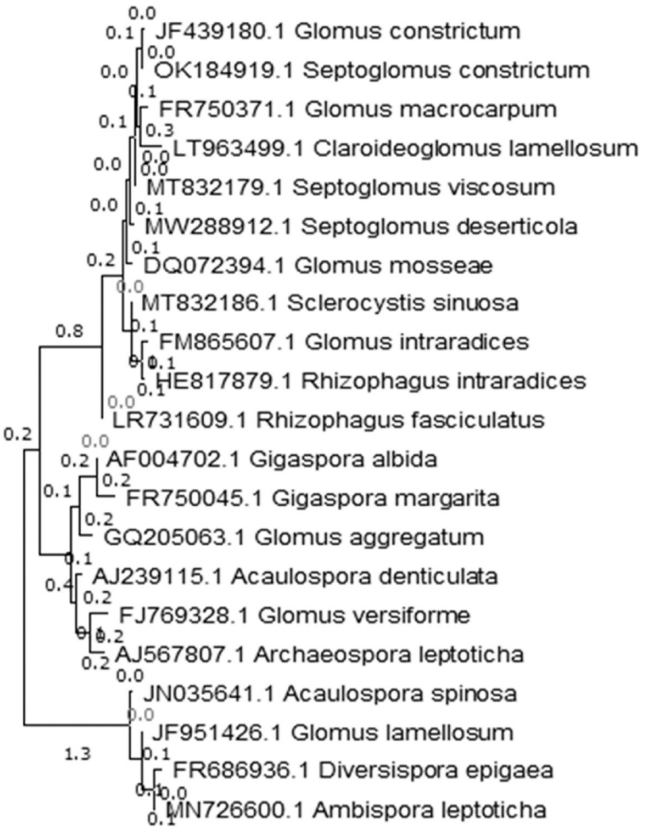

Figure 13. Neighbor joining tree of 21 AMF taxa recovered from rhizospheric soil of pulses of various study sites.

Mansehra, Dir Upper, Abbottabad, Bunner, Charsadda, and Haripur. Similarly, values for electrical conductivity (EC) ranged from 0.2×10^{-3} to 0.5×10^{-3}. A significantly ($p < 0.05$) high value (0.5×10^{-3}) of EC was recorded in the soil samples of Abbottabad. The texture tensile strength (TTS) values ranged between 0.005 and 0.3%, and a significantly ($p < 0.05$) high value (0.3%) of TTS was recorded in the soil samples of Charsadda. All the collected soil samples were slightly alkaline and have normal soluble salts and chemical reactions. Significantly ($p < 0.05$) high clay (23%), silt (61.5%), organic matter (1.3%), and potassium (142.5 mg kg^{-1}) contents were recorded in the soil samples of Chitral. A significantly ($p < 0.05$) high sand (60.3%) content was recorded in the soil of Bunner. Nitrogen (N) levels varied from 0.003 to 0.06 (%), and a significantly ($p < 0.05$) high N content (0.06%) was recorded in the soil sample of Haripur. The phosphorus (P) content was also fluctuated with a significant variation of 1.5−8 (mg kg^{-1}). Significantly ($p < 0.05$) high P (62 mg kg^{-1}) and calcium carbonate (8%) contents were recorded in the soil samples of Swabi (Tables 3 and 4).

Pearson correlation values exposed the fact that there is a significantly positive correlation of AMF diversity with soil pH, silt contents, organic matter, and nitrogen at $p < 0.01$. There is a significantly negative correlation of AMF diversity with calcium carbonate ($CaCO_3$) and available phosphorus (AP) at $p < 0.01$ and $p < 0.05$ levels, respectively. Data showing the Pearson correlation are given in the Supporting Information file.

3.7. Plant Elemental and Proximate Characteristics and Their Relationships with AMF Diversity. Significantly ($p < 0.05$) high amounts of nitrogen (11.4%), phosphorus (34.8 mg kg^{-1}), potassium (114.7 mg kg^{-1}), and zinc (1.9 mg kg^{-1}) were recorded in plant samples collected from Chitral (Tables 5 and 6). Significantly ($p < 0.05$) high amounts of moisture (6.5%), crude fats (5.9%), ash (12.2%), and proteins (71.09%) were recorded in plant samples collected from Chitral (Tables 7 and 8).

The Pearson correlation results revealed that there is a significantly positive correlation between AMF diversity and plant total nitrogen (TN), total potassium (TK), and zinc (Zn) at the $p < 0.05$ level, while there is a significantly positive correlation between AMF diversity and available phosphorus (AP) at the $p < 0.01$ level. Pearson's correlation data of plant elemental properties and AMF diversity are given in the Supporting Information file.

The Pearson correlation results revealed that there is a significantly positive correlation between AMF diversity and plant moisture, ash, and crude fats at the $p < 0.01$ level, while there is a significantly positive correlation between AMF diversity and plant protein content ($p < 0.05$) level. Pearson's correlation data of plant proximate properties and AMF diversity are given in the Supporting Information file.

4. DISCUSSION

4.1. AMF Colonization Status and Spore Diversity in Various Habitats. The results given in Tables 1 and 2 revealed that plant roots collected from Chitral have a

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Table 3. Soil Physicochemical Properties and AMF Spore Diversity Collected from Various Districts of the Khyber Pakhtunkhwa Province[a]

district name	spore count/ 100 g of soil	pH	EC × 10^{-3}	TTS (%)	clay (%)	silt (%)	sand (%)	$CaCO_3$ (%)	OM (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})
Chitral	35.25 ± 15.6a	8.2 ± 4.4a	0.3 ± 0.1ab	0.07 ± 0.02c	23 ± 13.6a	61.5 ± 25.8a	35 ± 23.9b	1.5 ± 1.02c	1.3 ± 0.5a	0.04 ± 0.01ab	5.17 ± 2.8g	142.5 ± 62.4a
Mansehra	34 ± 13.7a	8.2 ± 4.4a	0.3 ± 0.1ab	0.1 ± 0.02c	18 ± 7.cd	60 ± 25.8a	25.3 ± 13.6c	1.6 ± 1.02c	0.8 ± 0.3b	0.04 ± 0.01ab	10.45 ± 5.8e	101.7 ± 62.4e
Dir Upper	32 ± 12a	8.2 ± 4.4a	0.3 ± 0.1ab	0.2 ± 0.02b	21.5 ± 13.6ab	60.5 ± 25.8a	25.5 ± 13.6c	1.9 ± 0.5c	0.8 ± 0.3b	0.04 ± 0.01ab	12.57 ± 9.01cd	107 ± 62.4d
Abbottabad	32 ± 12a	8.2 ± 4.4a	0.5 ± 0.2a	0.09 ± 0.02c	21.3 ± 11.6ab	60.3 ± 25.8a	24.5 ± 13.6cd	2.06 ± 1.3c	1.2 ± 0.5a	0.05 ± 0.01ab	14.24 ± 9.01c	115.5 ± 62.4c
Haripur	30.75 ± 11.5a	8.0 ± 4.4a	0.3 ± 0.01ab	0.08 ± 0.02c	19.5 ± 9.01cd	55.5 ± 25.8b	32 ± 23.9b	2.8 ± 1.3bc	0.8 ± 0.3b	0.06 ± 0.01a	11.6 ± 0.6de	136.3 ± 62.4b
Bunner	30 ± 10a	8.2 ± 4.4a	0.3 ± 0.1ab	0.07 ± 0.02c	19 ± 9.01bcd	55.5 ± 25.8b	60.3 ± 25.8a	2.2 ± 1.3bc	0.9 ± 0.01b	0.04 ± 0.01ab	13.45 ± 7.6cd	102.5 ± 62.4e
Charsadda	29 ± 9.9a	8.2 ± 4.4a	0.4 ± 0.1ab	0.3 ± 0.02a	19 ± 9.01bcd	54.3 ± 25.8b	25.5 ± 13.6c	2.36 ± 1.26bc	0.8 ± 0.3b	0.04 ± 0.01ab	8.27 ± 4.4f	82 ± 25.8f
Karak	25.75 ± 9.7ab	7.9 ± 3.7a	0.2 ± 0.02b	0.07 ± 0.02c	17 ± 7.6cd	46.5 ± 25.8c	22 ± 13.6de	4.13 ± 0.8b	0.7 ± 0.3b	0.04 ± 0.01ab	17.05 ± 7.6b	76.7 ± 25.8g
Mardan	24.25 ± 9.8ab	7.8 ± 3.7a	0.2 ± 0.1b	0.07 ± 0.02c	18 ± 7.6cd	42 ± 23.9d	21.5 ± 13.6de	6.7 ± 3.7ab	0.7 ± 0.3b	0.04 ± 0.01ab	17.3 ± 7.6b	76.7 ± 25.8g
Swabi	14.5 ± 9.5b	7.5 ± 3.7a	0.2 ± 0.1b	0.005 ± 0.02c	16.5 ± 7.6d	23.3 ± 13.6e	19.3 ± 9.02e	8 ± 4.4a	0.06 ± 0.3c	0.03 ± 0.01b	62.8 ± 25.8a	42.2 ± 23.9h

[a]Table Key: EC = electrical conductivity, TTS = texture tensile strength, $CaCO_3$ = Calcium carbonate, OM = organic matter, N = nitrogen, P = phosphorus, and K = potassium.

Table 4. Mean Square Values for the Soil Physicochemical Properties and AMF Spore Diversity Collected from Various Districts of the Khyber Pakhtunkhwa Province

SOV	DF	pH	EC × 10^{-3}	TTS (%)	clay (%)	silt (%)	sand (%)	$CaCO_3$ (%)	OM (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})
district	9	0.2329	0.03956[a]	0.02792[a]	17.447[a]	568.5[a]	570[a]	20.579[a]	0.4388[a]	2.489 × 10^{-31}[a]	1077.8[a]	3620[a]
sites/district	3	0.4667	0.002	0.00265	0.333	1.8	1.2	0.969	0.0176	4.00 × 10^{-5}	0.5	5
error	27	0.9852	0.00719	0.00127	1.222	1.5	1.5	0.563	0.0062	7.70 × 10^{-5}	0.7	1
total	39											

[a]Represents the significance at $p \leq 0.05$; DF = Degree of Freedom.

Table 5. Relationship of AMF Spore Diversity with the Elemental Properties of Various Sites of Khyber Pakhtunkhwa

district name	spore count/100 g of soil	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Zn (mg kg^{-1})
Chitral	35.25 ± 15.6a	11.4 ± 6.7a	34.8 ± 18.6a	114.7 ± 62.4a	1.9 ± 1.3a
Mansehra	34 ± 13.7a	8.7 ± 4.4b	23.9 ± 13.6b	102.3 ± 62.4b	1.7 ± 1.3ab
Dir Upper	32 ± 12a	2.4 ± 1.3c	21.4 ± 13.6c	103.5 ± 62.4b	1.7 ± 0.5ab
Abbottabad	32 ± 12a	2.4 ± 1.3c	18.6 ± 8.3d	104.7 ± 62.4b	1.6 ± 1.02bc
Haripur	30.75 ± 11.5a	2 ± 1.3cd	16.7 ± 7.6de	99.5 ± 25.8c	1.6 ± 0.5bc
Bunner	30 ± 10a	2.4 ± 1.3c	16.2 ± 7.6de	97.5 ± 25.8c	1.6 ± 0.5bc
Charsadda	29 ± 9.9a	1.9 ± 1.3cd	14.8 ± 9.01ef	97 ± 25.8c	1.5 ± 1.02bc
Karak	25.75 ± 9.7ab	1.5 ± 0.5cd	14.3 ± 9.01ef	94 ± 25.8d	1.4 ± 0.5c
Mardan	24.25 ± 9.8ab	1.3 ± 0.5cd	13.5 ± 7.6f	74.5 ± 25.8e	1.4 ± 0.5c
Swabi	14.5 ± 9.5b	0.8 ± 0.01d	13.1 ± 7.6f	69.3 ± 25.8f	0.05 ± 0.01d

Table 6. Mean Square Values of Plant Elemental Properties and AMF Spore Diversity of Various Sites of Khyber Pakhtunkhwa

SOV	DF	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Zn (mg kg^{-1})
district	9	50.67[a]	176.34[a]	762.2[a]	1.05[a]
rep	3	0.07	1.13	0.9	0.006
error	27	0.37	0.91	1.2	0.009
total	39				

[a]Represents the significance at $p \leq 0.05$; DF = Degree of Freedom.

significantly ($p < 0.05$) higher rate of colonization of external hyphae (40.66%), internal hyphae (43.33%), arbuscules (14%), and vesicles (50.66%). The rate of colonization was almost equivalent in the root samples of Chitral and Mansehra, while the root samples of Swabi have a significantly ($p < 0.05$) lower rate of colonization of only external hyphae (6.66%). It may be due to the fact that the soil of the district contained a higher content of phosphorus (P). Our results are supported by the findings of Hui et al.[35] and Li et al.[36] They found a significantly negative correlation between the mycorrhizal colonization ratio and available phosphorus.

Intensity of AMF settlement can be resolved by the manifestation of hyphae (external and internal) and main vegetative structures such as arbuscules and vesicles. Our results revealed that all the above-mentioned forms were existed, but the percentage of vesicles was significantly ($p < 0.05$) higher and common at all. It may be due to the fact that

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Table 7. Pulse Crop Proximate Properties and AMF Spore Diversity Collected during Survey of Khyber Pakhtunkhwa

district name	spore count/100 g of soil	moisture (%)	crude fats (%)	ash (%)	proteins (%)
Chitral	35.25 ± 15.6a	6.5 ± 3.7a	5.9 ± 3.8a	12.2 ± 0.4a	71.09 ± 25.8a
Mansehra	34 ± 13.7a	2.3 ± 1.3b	5.6 ± 2.8ab	10.5 ± 5.8a	62.4 ± 23.9b
Dir Upper	32 ± 12a	2.3 ± 1.04b	4.2 ± 0.8abc	6.6 ± 4.9b	15.0 ± 5.1c
Abbottabad	32 ± 12a	1.6 ± 0.8bc	3.8 ± 2.2bcd	5.6 ± 4.8bc	15.2 ± 7.6c
Haripur	30.75 ± 11.5a	1.6 ± 0.3bc	3.5 ± 2.8cde	4.3 ± 1.5cd	12.5 ± 0.5cd
Bunner	30 ± 10a	1.5 ± 1.02bc	2.7 ± 0.2cde	4 ± 1.2cd	13.6 ± 9.01cd
Charsadda	29 ± 9.9a	1.25 ± 0.5cd	2.5 ± 3.5cde	3.7 ± 1.5d	12.04 ± 0.6d
Karak	25.75 ± 9.7ab	1.05 ± 0.1cd	2 ± 0.8de	2.5 ± 1.4d	9.06 ± 4.4e
Mardan	24.25 ± 9.8ab	0.4 ± 0.2de	1.7 ± 0.9e	2.5 ± 0.2d	6.6 ± 0.5ef
Swabi	14.5 ± 9.5b	0.1 ± 0.01e	1.7 ± 0.2e	2.6±1.1d	5.4 ± 3.2f

Table 8. Mean Square Value of Proximate Properties and AMF Spore Diversity Collected during the Survey of Khyber Pakhtunkhwa

SOV	DF	moisture (%)	crude fats (%)	ash (%)	proteins (%)
district	9	12.64[a]	9.3[a]	46.41[a]	2256.3[a]
rep	3	0.123	1.043	1.93	1.8
error	27	0.138	0.48	0.4	1.3
total	39				

[a]Represents the significance at $p \leq 0.05$; DF = Degree of Freedom.

the development of vesicles safeguards AMF symbiotic association with the plant host as observed by Kalamulla et al.[37] Based on root observation, the mycorrhizal structures, i.e., arbuscules, were not reported except in few soil samples; this may be accredited to the statement that the likelihood of the existence of AMF arbuscules becomes scarcer with the growing age of the plant root. It is find out that AMF arbuscules become transmuted into a bumpy structure with the growing age of a plant and become problematic to extricate them anymore, as observed by Wipf et al.[38]

AMF spore diversity was found in the soil samples of Chitral highly significant. Our results are supported that majority of AMF have little host specificity by Muneer et al.[39] Sporulation in AMF is an extremely carbon-demanding process; this may clarify why the sum of AMF spores in the vicinity of plant roots fluctuates among plant types and for the same plant species among locations, as observed by Kemmelmeier et al.[40] Our results are also aligned with those of Bertola et al.,[41] who found that in the uppermost soil horizon, AMF persistence is intense because of the abundantly found organic matter. It also enlightens how the disruption activities such as grazing lead to the deterioration of soil, hence resulting in diminishment of the spore density, as observed by Von Dudinszky et al.[42]

4.2. AMF Molecular Characterization. In previous studies, the primer pair AML1/AML2 has been found to be more specific to the AMF phylum "Glomeromycota" and observed to be responsible for better coverage across "Glomeromycota", including various groups of AMF, Glomus Group B (Claroideoglomus), Glomus group C (Diversisporaceae), Paraglomeraceae, and Archaeosporaceae, as observed by Stefani et al.[43] It is find out that the primer pair AML1−AML2 had much coverage capability over the LSU rDNA gene, which is an appropriate molecular indicator to resolve taxonomy and phylogeny within the AMF phylum "Glomeromycota", as observed by Lee et al.[44] The degree of variable sections, i.e., D1−D2 of LSU rDNA, permitted not only the analysis of AMF diversity but also the strategy of taxon-discriminating primers, which can be used to monitor AMF in the roots of plants, as observed by Kolarikova et al.[45] In the present study, 21 sequences were amplified, which indicated that these primers were suitable for field-collected AMF inhabiting the roots of pulses.

The identified sum of OTUs (operational taxonomic units) is a customary conjecture of the sum of AMF morphs presented by Vandenkoornhuyse et al.[46] Our detected estimation of 21 OTUs inhabiting the roots of various pulses was far away from our expectancy because only about one hundred and ninety AMF types have been well defined worldwide. The sophisticated figure of AMF revealed by the present study may be due to the subsequent motives: (1) extraordinary potential of colonization of AMF morphs in a variety of habitats and (2) the usage of great root masses (1 g including root fragments of four sites from the same field) for DNA extraction. By and large, extra root biomass resulted in an additional type sequence of AMF. It has been put forward that AMF lushness surges with the rise in sample size by Sweeney et al.[47] The phylogenetic tree analysis results revealed that all the sequences fell within the AMF phylum Glomeromycota. Our results showed that although the genus *Glomus* species, i.e., *Glomus lamellosum* is the member of the genus *Glomus*, it was grouped in clade 3 (having a 1.3 base difference with clade 1 and clade 2); it may be due to the reason that in this species, only the 18s gene region was targeted due to the lack of sequence data in the NCBI database. In several reports, the complete gene or the 28s was targeted, which is considered to be the most variable region of the gene (D1−D2 region of LSU rDNA) by Delavaux et al.[48] This region is far more variable than any region of the SSU rDNA and is known to provide information to estimate higher level phylogenetic relationships by Kumar.[49]

4.3. Soil Physicochemical Properties and AMF Diversity. The results revealed (Tables 3 and 4) that there was no significant difference in the pH of rhizospheric soil of each pulse crop and AMF species (*Glomus*, *Gigaspora*, and Sclerocystis). Furthermore, it was investigated that the AMF genus *Glomus* was the most prevalent in all the assessed soil samples. Our results correlate with those of Liu et al.,[50] who found that neutral or slightly basic pH favors AMF diversity. Also, it was supported by the findings of Klichowska et al.[51] that soil pH is the determining factor in shaping the AMF community and there is a significantly positive correlation between soil microbiota and soil pH.[52] Pearson's correlation test revealed that the soil silt content is significantly positive correlated with AMF diversity. Similarly, the phosphorus content showed a significantly negative correlation with AMF diversity. Our results agreed with the findings of Xiao et al. and Silva-Flores et al.[53,54] that in the soil structure, the soil silt

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content (%) is considered to be the strongest predictor, showing significant influences on the AMF community composition as compared to clay and sand contents. Similarly, AMF spore diversity decreases with increasing soil P levels. Soil samples of the Swabi site showed a significantly higher P content as compared to the other sites. In general, soil P contents and AMF spore population are inversely interrelated to each other. It might be accredited to the fact that little availability of P in the soil results in the exudation of few assured chemicals from the plant roots, resulting in the enhancement of AMF establishment and germination of spore, but such exudations are not possible when the phosphorus level is elevated as stated by Lu et al.[55] In the current study, AMF diversity indices were negatively correlated with phosphorus. It is find out that species richness and AMF diversity associated with cowpea are negatively correlated to P levels, as observed by Sarr et al.[56] There is a strongly positive correlation existing between AMF spore diversity and organic matter and nitrogen levels, as observed by Alimi et al.[57] A negative correlation was demonstrated between calcium carbonate and spore density and diversity. Levels of calcium carbonate significantly reduce mycorrhizal symbiosis and influence germination, elongation, and AMF sporulation as found by Palta et al.[58] and Posada et al.[59]

4.4. Plant Elemental and Proximate Characteristics and Their Relationships with AMF Diversity. Results of elemental properties and AMF density correlation described in Tables 5 and 6 showed that plant nutrition levels increase in the presence of AMF species. Our results are supported by Crossay et al. and Sarah et al.[60−62] that the plant nutrient (TN, TK, P, and Zn) level was significantly increased by using a mixture of various natural AMF inocula. Furthermore, the results of Pearson's correlation test revealed that AMF spore diversity is significantly positively correlated to plant nutrients (TN, TK, P, and Zn). Our findings of the proximate (moisture, ash, crude fats, and proteins) composition revealed the fact that AMF diversity and plant proximate characteristics were parallel (Tables 7 and 8). Our results are supported by Naz et al.,[63] who found that AMF inoculation significantly improved the proximate composition in "chickpea". AMF not only helped determine the primary and secondary metabolic pathways in pea plants but also improved the host response against pathogen attacks as investigated by Sistani et al.[64] In the case of mung bean, the average total soluble protein contents were significantly higher as compared to the control (T0), described by Kaur and Kumar.[65]

5. CONCLUSIONS AND FUTURE PERSPECTIVE

The current study revealed different AMF morphotypes in various pulse crop fields of Khyber Pakhtunkhwa, Pakistan, which signify that there is a great diversity of AMF in the pulse crop agroecosystems in the study areas. Furthermore, the morphological and molecular characterization of AMF species was conducted for their proper identification. The current study concluded that soil phosphorus contents contributed more to the colonization and distribution of AMF species as compared to other different soil chemical properties. Plant proximate and elemental analysis results revealed the enhanced quantity of protein, crude fat, moisture, ash, nitrogen, phosphorus, potassium, and zinc contents in AMF-colonized plants. Results obtained in the current study constitute solid scientific evidence for growing pulses on a large scale in the province Khyber Pakhtunkhwa, Pakistan, without polluting soil compared to synthetic fertilizers; thus, it is a way toward sustainable agriculture. In future, we recommend the stable use of AMF in agriculture to carry out large-scale multilocation field trials. In addition, farmers are encouraged to prepare and extract their own AMF inocula from local or native soils, and their application as a biofertilizer produced higher response than that of synthetic fertilizers in agronomic parameters, yielding, and productivity; thus, synthetic fertilizers can be abandoned. This makes the biofertilization technology more likely to be affordable and ecofriendly for farmers, including those in developing countries who need their cropping system to be as highly sustainable as possible. Modern molecular techniques are already available for these studies, and it is the responsibility of microbial ecologists and agronomists to take up these challenges, as their contribution could help lead to practical solutions to the problems of producing more food in a sustainable way.

■ ASSOCIATED CONTENT

Data Availability Statement

The data are contained within the article.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02489.

Gel images, tabulated presentation of primers, soil physicochemical properties, and plant elemental and proximate properties (PDF)

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Author Contributions

All authors contributed to the study conception and design. S.N. and T.Y. contributed to the methodology of the study. J.I., B.A.A., S.I., I.A., S.E., M.F.E., and T.Y. performed formal analysis and investigation of the study. S.N., T.Y., I.A., and J.I. wrote the original draft of the article. S.N. and J.I. reviewed and edited the article. T.Y. supervised the research work performed.

Notes

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