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IDENTIFICATION OF VAM SPORES (*GLOMUS AGGREGATUM* AND *GLOMUS MOSSEAE*) BASED ON CTAB METHOD IN THE RHIZOSPHERE SOIL OF QUINOA (*CHENOPODIUM QUINOA* WILLD.)

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ABSTRACT

The bio-fertilizer properties of plant growth promoting bacteria are frequently ascribed to their ability to increase the bioavailability. Rhizosphere microflora like VAM shown incensement in the growth and yield of some agricultural crops. It is the necessary and primary step to identify the presence of VAM in the rhizosphere soil of the higher plants. So, the standardization of DNA isolation is basic requirement for any further research to be carried out. We report here modified CTAB technique for isolation of genomic DNA from two cultivars of Quinoa (INIA-431, INIA – 427) (*Chenopodium quinoa* Willd.) We got very good yield of DNA samples from the rhizosphere soil of Quinoa. Genomic DNA isolated by modified CTAB method was pure; the highest level of purity was obtained from two cultivars of Quinoa. The two cultivars of Quinoa (INIA - 431, INIA – 427) gave good yield of DNA from the established modified CTAB protocol. Identification of spores (*Glomus aggregatum* and *Glomus mosseae*) based on CTAB method in the Rhizosphere soil of Quinoa (*Chenopodium quinoa* Willd.)

Keywords: Bio-fertilizer, Rhizosphere microflora, VAM, CTAB technique, Quinoa (*Chenopodium quinoa* Willd.) *Glomus aggregatum, Glomus mosseae*

INTRODUCTION

VAM fungi are eco friendly bio-fertilizers which enrich the soils and increases the efficiency of plants in phosphate utilization by formation of dense root clusters (Koide *et al*., 2004). Mycorrhizae show a symbiotic association with all terrestrial plants (Miransari, 2011). Nutrient uptake and seed production and quality in ground nut cultivars. (Khirood DOLEY*, Paramjit Kaur JITE, 2012) explained the role of VAM fungi in absorption of phosphorus and other nutrients which are immobile and present at very low concentrations in soils, but with a significant impact on plant growth and nutrient uptake in mycorrhizal plants than non mycorrhizal plants. VAM mycorrhizae increased seed yield and nutrient values in many cereal crop species like rice, barley, oat, wheat, sorghum, maize and in sunflower. VAM fungi may alter the morphological and physiological properties of root structure in host plant.

Optimization of DNA isolation protocol for genetic characterization is the primary and necessary step in field of molecular biology (Tan and Yiap, 2009). DNA isolation was the first and foremost step in molecular biotechnology. In this step, the process of extraction and purification of nucleic acids is a complicated, time-consuming, labor-intensive, and limited in terms of overall through put. The extraction of DNA is a high quality and yield has lead to the development of a variety of protocols, however the fundamentals of DNA extraction remain the same. Firstly DNA must be purified from cellular material in a manner that prevents degradation, for this even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow it for multiple end uses. After that protein digestion and action of detergents during the extraction process destroys the plasma membrane and the nuclear membrane surrounding the DNA. EDTA in extraction buffer is added to prevent DNA from degradation, EDTA chelate the Mg^{2+} needed for enzymes that degrade DNA. For removal of polysaccharides higher concentration of Cetyl Trimethyl Ammonium Bromide (CTAB) is added (Channarayappa, 2007). A range of methods is available to assess the quality of the isolated DNA which include gel electrophoresis, spectrometric analysis, restriction digestion, PCR amplification and chromatographic techniques (Varma *et al*., 2007).

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight. Quantification of nucleic acids is commonly done in molecular biology to determine the concentrations of DNA or RNA present in a mixture (Channarayappa, 2007). Spectrophotometers are commonly used to determine the concentration of DNA in a solution. It is possible to use UV-spectrophotometer to estimate the purity of a solution of nucleic acids. This method involves measuring the absorbance of the solution at two wavelengths, usually 260 and 280 nm, calculating the ratio of the two absorbance: an

characteristic of pure DNA with 1.8 of A260/A280 ratio is considered pure (Nieman and Poulsen, 1963). In this research, we developed a modified CTAB technique for genomic DNA isolation from two cultivars of Quinoa (INIA - 431, INIA – 427) (*Chenopodium quinoa* Willd.) The modifications in standard CTAB protocol are made such that good yield of DNA from two cultivars of Quinoa (INIA - 431, INIA – 427) (*Chenopodium quinoa* Willd.)

MATERIALS AND METHODS

Sample collection: VAM fungi were collected by wet and decanting method (Gerdemann and Nicolson 1963), 100g rhizosphere soil samples were taken in 500 ml beaker with sufficient quantity of water and stirred thoroughly to make soil suspension. Soil suspension was passed through sieves of different sizes (450 µm, 250 µm, 106 µm, 75 µm and 53 µm) which were kept one below the other in ascending order.

Genomic DNA isolation from VAM Spore

Genomic DNA was isolated by modified procedure of CTAB method as described by Murray and Thompson (1980). Pure culture of spores (*Glomus aggregatum* and *Glomus mosseae*) was mixed with 40% sucrose water and centrifuged at 5000 rpm for 2 'min' taken supernatant for DNA isolation. DNA solution was purified with the standard phenol: chloroform method. DNA solution was mixed gently with phenol: chloroform (1:1) and centrifuged at 5000 rpm for 10 'min' at room temperature. The aqueous phase was separated and mixed with an equal volume of chloroform, mixed gently and centrifuged at 5000 rpm for 10 'min' at room temperature. The aqueous phase was separated and mixed with two volumes of absolute ethanol and incubated at -200C for 20 'min'. The DNA pellet was spooled out with a glass hook; washed with 70% aqueous ethanol. The DNA pellet was air dried for 20 'min' and dissolved in an appropriate volume of sterile TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at 4C. The concentration of DNA was determined spectrophotometrically and the quality of DNA was checked by agarose gel electrophoresis.

Primers were obtained from Bioartist, Hyderabad India (Table.1), and used to amplify DNA of two varieties of VAM spores

Table 1 : List of the primers used for 26S rRNA sequencing in *Glomeromycetes*

S. No	Primer	Sequence
1	1311	5'-TGC TAA ATA GCT AGG CTG Y- 3'
2	1310	5'-AGC TAG GCT TAA CAT TGT TA-3'
3	5.8 r	5'-TCC GTT GTT GAA AGT GAT C-3'
4	1670	5'- GAT CGG CGA TCG GTG AGT-3'
5	LSU 0061	5'- AAA TTG TTG AAA GGG AAA CG-3'
6	LSU 0805	5' CAT AGT TCA CCA TCT TTC GG- 3'

PCR reaction

1.PCR reaction were carried out in 50 µl reaction made of 40 ng of DNA as template, 1xPCR buffer, 250 µm of each dNTP, 5 Pico moles of primer and 1 unit of taq DNA polymerase. A 40 cycles PCR reaction is set with all the above mixture programme is set with 95^0C of initial denaturation for 5 'min' followed by 40 cycles of 94^0C of denaturation for 1 'min', 50^0C of annealing temperature for 1 minute, 72^0C of extension for 1 minute and finally final extension at 72^0C for 10 'min'. Primers were used for *Glomus aggregatum* (LSU 0061 Primer) and *Glomus mossae* (LSU 0805 primer). The amplified product is resolved in 1.5% Agarose gel stained with ethidium bromide in 0.5xTBE buffer with 50 bp gene ladder at one end of the gel.

RESULT AND DISCUSSION

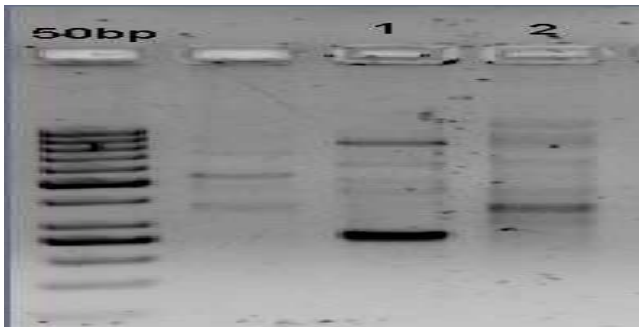

Fig. 2 : Agarose gel electrophoresis of 26S rRNA Amplicons

1. *Glomus aggregatum* (LSU 0061 Primer) (150bp)
2. *Glomus mosseae* (LSU 0805 primer) (120bp)

Sequence of *Glomus aggregatum*

LOCUS DEFINITION Assembly of LSU 0061, and LSU 0805

SOURCE known
ORGANISM known
BASE COUNT 102 A 126 C 149 G 115 T

ORIGIN

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attaccgatt ttagcggaca tgatctctga tcatggtctc gcgaaaacat tgtatttaaa
accccactct tataaataga atcatattat attgtatata aataaataaa gatcactttc
aacaacggat ctcttggctc tcgcatcgat gaagaacgta gcgaagtgcg ataagtaatg
tgaattgcag aattccgtga atcatcgaat ctttgaacgc aaattgcact ctctggcaac
ccggggagta tgcctgtttg agggtcagtg ttaataaaaa tcggtgcgtt gcaaattttt
ttgtgatgtt tccggagttt gagttatctt aattaactct tctggggttt ttaagaggct
taaaattgac ctttttgtg cattttaga cgtacataaa tttttttta ttcgtccatc
ttaatgccaa aatctaatag atgcgaccat atcatgtggt ttcgtgtcca taaatttttc
atgatttgac ctcaaatcag gtaggaacac ccgctgtaat ttcctgataa ggatagcttc
tcgctcttca acgaggaatc cctagtaagc acaagtcatc agcttgtgtt gattacgtcc
ctgccctttg tacacaccgc ccgtcgctac taccgattga atggcttagt gagacctccg
gattgacatt cagaagttgg aaacaacatc tgtctgccga gaatttgttc aaacttggtc
atttagagga agtaaaagtc gtaacaaggt ttccgtaggt gaacctgcgg aaggatcatt
aatgattttt taagcaaacc aaagcttttt tttataaaaa gtgaggtttt gcgaatgtat
ttaaaacccc cactcttaaa aaaaatatat tttaattcat ataaaatgaa taaaaaaaaa
gatcactttc aacaacggat ctcttggctc tcgcatcgat gaagaacgca gcgaaatgcg
ataggtagtg tgaattgcag atgtaacgtg aatcatcgaa tctttgaacg caaattgcac
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ttgcttttgt aaatgtttat cggatttgag ccgtcttcta ttttttttta attaaagtgg
cttaaaatgt atttttggt atcgaaaaac gttattttac gatcaaattg attaaaaaaa
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ttcattcgta tacgattttt taatttttga cctcaaatca ggtaagaaca cccgctgaac
ttaagcatat caataagcgg aggaaaagaa aataacaatg attcccctag taactgcgag
tgaagaggga taagctcaaa ttttaaatct gttcggttct acctgacaga gttgtaattt
aaagaaacgt tttctgcgtc ttgagttaat caaaatcctt tggaatgagg tatcatggag
ggtgacaatc ccgttcatgg ttaattctaa gatgctttat gatacgtttt cgaagagtcg
agttgtttgg gattgcagct caaaatggga ggtaaatttc tcctaaggct aaatattggc
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agcttcgatc ttgtattgaa gtgttatagc ctttggtaaa tgtgatgatt aggatcgagg
attgcaacga ataccctttt gggctatccg cctgatctct gatacgtggc cttggttaag
aaagcgtgct tatggtattg aggtattacg gtcaaaaggt tagaacggat tacattcgtt
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Based on the comparison of Glomeromycetes (26S) with LSU 0061 Primer. Sequence obtained from sample with sequences available at NCBI database, samples are identified to be a species of the genus *Glomeromycetes* The most probable species is *Glomus aggregatum.*


KF836967.1(glomeromycetes)
JX276907.1(fungi)
JX276910.1(fungi)
KF849663.1(glomeromycetes)
KJ701496.1(glomeromycetes)
KJ701461.1(glomeromycetes)
KF849667.1(glomeromycetes)
KF849615.1(glomeromycetes)
KF849668.1(glomeromycetes)
KJ701484.1(glomeromycetes)
KJ701483.1(glomeromycetes)
KJ701497.1(glomeromycetes)
HF970212.1(glomeromycetes)
JX096617.1(fungi)
JF439176.1(glomeromycetes)
JF439167.1(glomeromycetes)
MH590070.1(glomeromycetes)
HF674440.1(glomeromycetes)
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MG183939.1(glomeromycetes)
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JX999967.1(fungi)
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KF060319.1(glomeromycetes)
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KF060320.1(glomeromycetes)
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HF548863.1(glomeromycetes)
HF548858.1(glomeromycetes)
Query_141413(Glomusaggregatum)
KF836938.1(glomeromycetes)
0.02

Sequence of *Glomus mosseae* : (26S) Sequencing data

caaaatggga ggtaaatttc tcctaaagct aaatattggc gagagaccga tagcaaaca
gtaccgcgag ggaaagatga aaagaacttt gaaaagagag ttaaatagta cgtgaaattg
ttggaaagga aacgattaaa gtcagtcata ccaacgggaa atcaaccttt tgagttcggt
cttgtgggtt tgaagagttt caaagccttc ggatttgtga gattgggatc tcttggtgca
ctttttcgta cggttagtca acatcggttt taatcattat aaaatggttg aaggaatgta
actttgattt cgatcgaggt attatagcct ttgacagatg taatgatcaa gaccgaggat
tgcaacggat acccttcagg gctattcgtc tgatctttgg tacttgtctt tagtatcgga
agcttgctaa cgatattagg gttcacgggt caaaggttgg aacggattaa attagcctac
taaaatggga ggtaaatttc tcctaaggct aaataacggc gagagaccga tagcgaacaa
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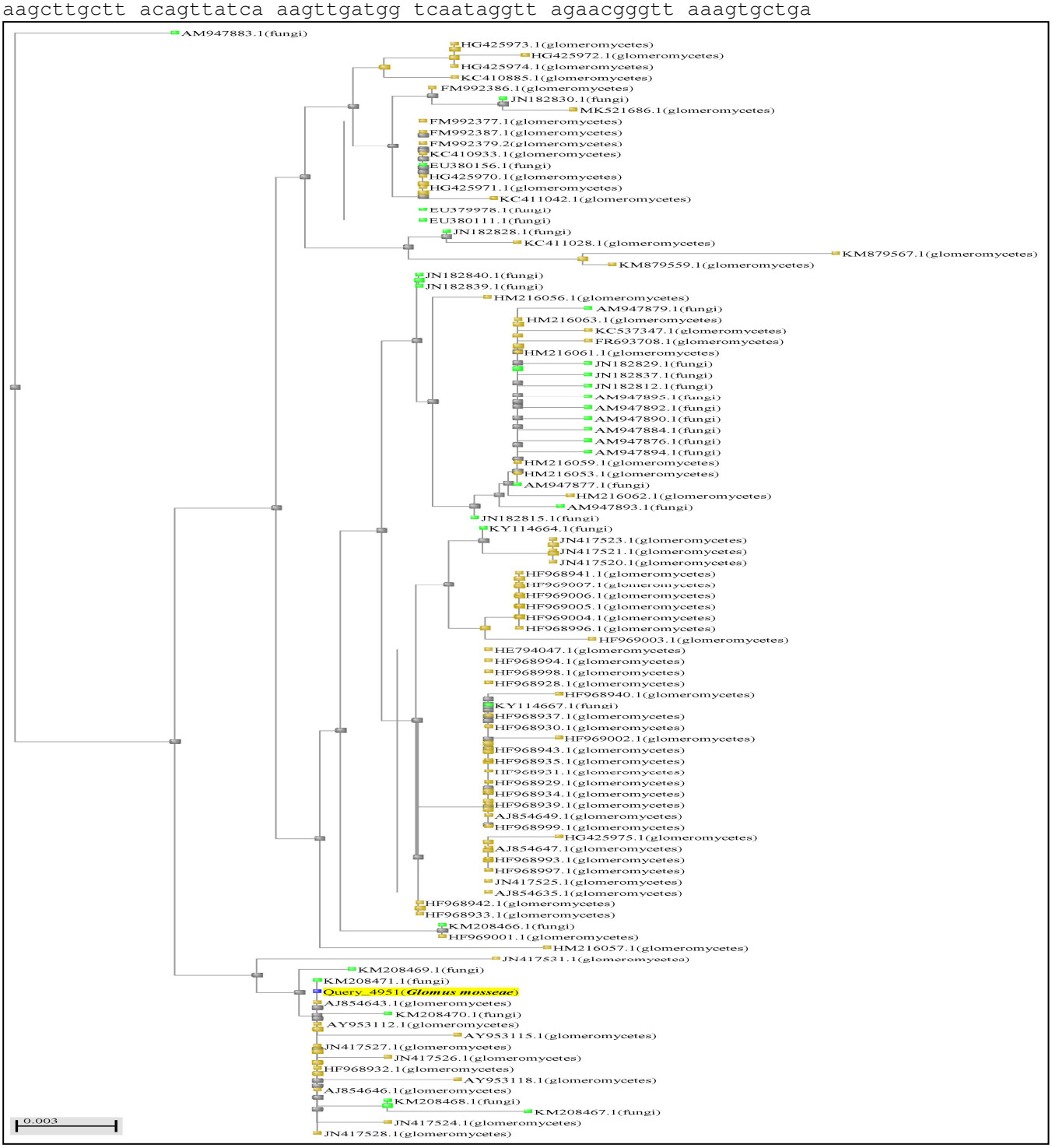

DISCUSSION

With two varieties of Quinoa (INIA - 431, INIA – 427) in this research successfully isolated good yield of DNA from the mycorhiza which was isolated from the rhizosphere soil of Quinoa. In present work by optimizing CTAB protocol which was given by Doyle and Doyle (1990) Isolated and identified two VAM fungi through CTAB technique those are *Glomus aggregatum* and *Glomus mosseae* The purity and concentration of DNA obtained, good purity range of DNA was seen Quinoa the identified organisms were *Glomus aggregatum* by using (LSU 0061 Primer) at (150bp) and one more organism was *Glomus mosseae* by using (LSU 0805 primer) at (120bp) by CTAB method.

Sequencing analysis: The PCR products of *G. aggregatum* and *G. mosseae* were purified by gel elution and the purified products were sequenced using Sanger dideoxy method. {ABI 3130 (48 capillary) or 3730Xl (96 capillary) electrophoresis instruments}.

Blast analysis of (26S) rRNA amplicons yielded most probable hits with the genus Gloeromycetes. The Blast sequence analysis, along with rRNA sequencing data indicates that the isolated species is *Glomus mosseae*. Based on the comparison of *G. mossae* (26S) with LSU 0805 primer. Sequence obtained from sample with sequences available at NCBI database, samples are identified to be a species of the genus *Glomeromycetes* The most probable species is *Glomus mossae.*

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