Research Article

Neocosmospora rubicola: An Unrecorded Pathogen from Pakistan Causing Potato Stem Rot

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Abstract | Potato (*Solanum tuberosum*) is one of the important staple crops over the world. Potato crop is threatening by a number of biotic stresses which not only affect plant health and yield but also the quality of the produce. Among biotic diseases, fungal stem and tuber rot are the most common diseases that cause significant loss in potato production in Punjab province, Pakistan. *Neocosmospora rubicola* was found as a new pathogen causing stem rot of potato in the potato growing area of district Kasur. Symptoms of the *N. rubicola* infected potato plants were necrotic stem lesions near the collar region. Causal organism was isolated from the infected tissues, purified and identified on the basis of morphological characters, nucleotide sequences of internal transcribed spacer region (ITS) and partial beta tubulin gene. Phylogenetic analysis was also conducted to determine the phylogenetic relationship of this species with other reported species of this genus. Pathogenic potential of the isolate was verified by artificially inoculating the spores of pathogen in healthy plants. Appearance of same symptoms and re-isolation of *N. rubicola* from infected tissue confirmed Koch's pathogenicity postulates. Association of *N. rubicola* causing stem rot in potato is never reported before in Pakistan.

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Introduction

Potato (*Solanum tuberosum* L.) is a tuber crop which is ranked as the world's fourth largest food crop after wheat, rice and corn (Iqbal *et al*., 2019). Potato tubers are rich in carbohydrates, vitamins, minerals and other phytochemicals that contribute in human health and reduce health related risks of consumers (King and Slavin, 2013). Productivity of potato crop is affected by a large number of insects, viral, bacterial and fungal pathogens. Important potato fungal diseases in Pakistan are early and late blight, *Fusarium* rot and wilt, powdery mildew, black scurf, wart and *Rhizoctonia* canker (Majeed and Muhammad, 2018; Shahzadi *et al*., 2020). *N. rubicola* has been reported as a causative agent of stem rot of *Hylocereus costaricensis* in China (Zheng *et al*., 2018) and root rot of *Glycyrrhiza uralensis* in Korea (Kim *et al*., 2017). This fungus is also known to cause root rot in pear plant (Tang *et al*., 2017).

In the Genus *Neocosmospora,* approximately 900 species are including and nearly 100 of plant families act as spanning host of these strains. Previously, the species of *Neocosmospora* were included in the genus of *Fusarium.* However, Nalim *et al.* (2011) placed these species in the *Fusarium solani* group as members of *Neocosmospora*. But later, phylogenetic analyses of

DNA sequence data from six loci (act, ITS, LSU, rpb1, tef1 and tub), with integrated morphological characterizations of *Neocosmospora,* it is recognized as separate genus (Hirooka, *et al.*, 2012). Ubiquitously, the species of this genus survive extensively in living and dead plants, soil, air, and water (Lombard *et al.*, 2015).

Hence, the most popular theory for mode of action of Neocosmospora species reveals that they inhibit eukaryotic cells, by different ways including suppression of protein, DNA, and RNA biosynthesis, restraining mitochondrial function, cell division and membrane function. (Karlovsky *et al.*, 2016). The aim of this study was updating the statistics of disease distribution and varietal susceptibility trend in various potato growing regions of Punjab, Pakistan and Designing of comprehensive strategies for integrated disease management (IDM).

Materials and Methods

During a survey conducted in April 2017 and 2018, potato fields in Kot Radha Kishan (31˚10'21N 74˚5'59E), District Kasur, in Punjab province, Pakistan, to monitor potato rot diseases were found to be infected with unknown disease. A total of ten fields randomly selected from five villages of district Kasur. To ensure unbiased data, recording of the field scouting was made on cross zigzag or parallel line so that equal opportunity for each site could be ensured. During survey, a total of 365 plant tissue specimens were treated in lab for isolation of fungi. Among the test specimens, 25 fungi belonging to various species were isolated and five species of fungi were seen to be more frequent. Among the other frequent fungi, *Neocosmospora rubicola* was found to be a new pathogen associated with potato stem rot. Symptomatic infected plants initially showed water-soaked brown to black lesions on the lower stems, near the collar (Figure 1). With the spread of infection, potato plants gradually wilted and then died. To study the possible pathological causes of this disease, infected potato plants were brought to the Seed and Postharvest Pathology Laboratory, Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore. Stem sections of 2-3 mm^2 healthy control and symptomatic tissues were surface-disinfected via immersion in 2% (w/v) sodium hypochlorite solution for one min followed by three washings with sterile distilled water. Surface sterilized stem sections were dried on sterilized filter paper and then placed aseptically onto Potato Dextrose Agar (PDA) containing Petriplates. Inoculated Petriplates were incubated at 26 ± 2 ˚C and observed regularly for the growth of pathogen. Emerging fungal mycelia from the inoculated stem sections were transferred to fresh PDA Petriplates aseptically for purification.

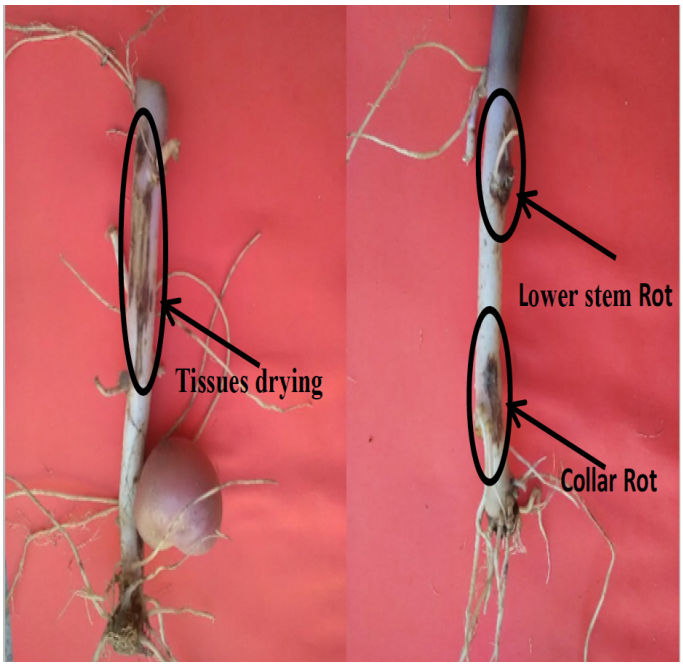

Figure 1: *Infection development of N. rubicola on potato stem.*

To verify the morphology-based identification, the fungi isolates were grown on PDA at 25˚C for 7 days. For morphology-based study, initially colony characteristics (culture color from front and reverse side, growth pattern, presence of growth zone, colony elevation, presence of submerged or aerial mycelium, types of conidia, presence of water drops or other exudates etc.) were recorded. Microscopic studies were carried out on 3 days to 3 weeks old cultures in water and Trypan blue mounts. Studies on cultural characteristics were initiated on 3rd day showing the appearance of the hyphae, to 21 days till the complete development of conidia. The optical microscope (BOECO; Model BM-120), was used to study of conidia and fungal mycelium. On the basis of morphological features, complete morphological description of fungal strain was prepared. Species were using the authentic taxonomic literature (Leslie and Summerell, 2008).

Genomic DNA of fungal pathogen was isolated using Akhtar *et al.* (2014) protocol. PCR was executed using subsequent primers; Internal Transcribed Spacer (ITS) of rDNA and partial beta tubulin gene

(Chowdhary *et al*., 2019). For the amplification of selected DNA fragments, 5-10 ng total genomic DNA was used as template. PCR amplification was performed in Thermal Cycler in a total volume of 30 µl using 2X AmpMasterTM Taq (Gene All Biotechnology Co., Ltd). PCR reactions were carried out following the steps; Initial denaturation at 94 ˚C for 5 min followed by 35 cycles of amplification each consisted of 30 sec denaturation at 94 ˚C, 30 sec primer annealing at 55 ˚C, 30 sec amplification at 72 ˚C and 7 min final extension at 72 ˚C. Resulting nucleotide sequences were visualized on a 1.5% agarose gel for 30 minutes and then PCR products were sequenced by SolGent (Daejeon, Korea). The acquired nucleotide sequences were deposited in GenBank (rDNA-ITS regions ID: MG976818 and partial beta tubulin gene ID: MH016281) and differentiate with the pool nucleotide sequences existing in GenBank by BLAST.

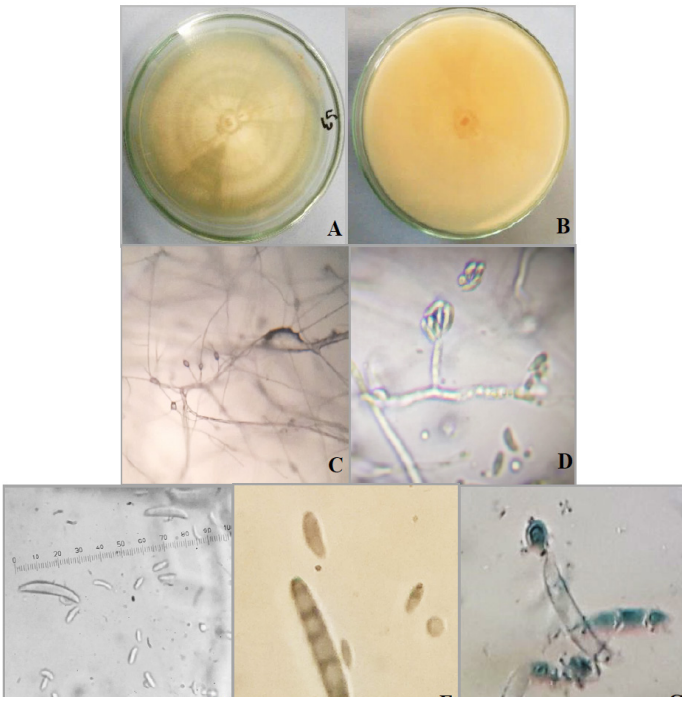

Figure 2: *Neocosmospora rubicola (FCBP 1565). A: Colony front; B: Colony reverse: C: Conidial attachment with conidiophores under stereoscope; D: Conidial attachment under 4X magnification of microscope; E, F and G: Conidia.*

Phylogenetic tree was aligned by MEGA6 Tamura *et al*. (2013) using maximum likelihood tree method (Tamura and Nei, 1993). The phylogenetic analysis involved nucleotide sequences of seven different species of *Neocosmospora* including *N. rubicola* (Lombard *et al*., 2015) retrieved from GenBank with the nucleotide sequences of respective genes of *N. rubicola* (FCBP1565).

Pathogenicity of isolated fungus was verified by inoculating three weeks old healthy plants with the spore suspension containing both micro and macro conidia of pathogen. Fresh fungus culture was obtained from single spore inoculum on PDA grown for 10 days at 25 ˚C. Conidial suspension; 3×10^4 conidia/ml was made in sterile distilled water and 30 µl from this suspension was injected into potato stems approximately 2 cm above the collar region. Injected areas were covered with sterilized moist cotton and sealed with cellophane tape. Same amount of sterilized water was injected in the stems of three healthy plants to serve as control. Plants were maintained in a green house at 26+2 ˚C.

Results and Discussion

Seven days old randomly selected cultures from three different isolates selected and characterized on the basis of cultural and morphological features. Fungal colonies grown on PDA were cream to pale yellow in color on both sides reaching 4-4.5 cm in diameter in seven days. Colonies were zonate with clear concentric rings, having regular margins without any kind of exudates. These morphological features are closely related with the finding of (Kim *et al*., 2017). Conidiophores were simple, unbranched, ranging 35-40 µm long. Abundant micro and macro-conidia were present. Macro conidia were cylindrical, curved, 3-4 septate, 8-12 x 1-2 µm in size and aggregated in slimy heads on conidiophores same as in case of *Fusarium* species. Lombard *et al*. (2015) reported that *Neocosmospora rubicola* macroconidia has 3–5-septate, cylindrical, straight or curving at both ends, beaked at both ends, his findings are in agreement with our study. Micro conidia were ellipsoidal to cylindrical in shape, 0-1 septate, 2-4 x 1-2 µm in size. On the basis of morphology features, causal fungus was identified as *Neocosmospora rubicola* (Lombard *et al*. (2015); Zheng at al., 2018). An agar slant of single spore culture of the test pathogen was deposited to First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab under the accession number FCBP1565.

Although important but phenotypic approach of fungal systematics is not sufficient for authentic identification of fungus (Wang *et al*., 2016). Therefore, molecular data in combination with the morphology are used for identification of fungi (Porras-Alfaro *et al*., 2014; Javaid *et al*., 2018). Primer pair ITS1/IT4 amplified approximately 650 bp while that of Bt_2a/

Bt_2b gave approximately 350 bp DNA fragment. PCR products were visualized on 1 % agarose gel along with DNA size marker and correct amplicons were sent for nucleotide sequencing. Resulting sequences were analyzed by nBLAST for similarity of amplified rDNA-ITS regions (GenBank ID: MG976818) and partial beta tubulin gene (GenBank ID: MH016281). BLAST results indicated that rDNA-ITS and partial beta tubulin gene of present fungal strain were 100% similar with that of their corresponding sequences of *N. rubicola* strain CBS 320.73 submitted under the accession numbers KM231799 and KM232061. It has been widely accepted that ITS nucleotide sequence in combination with any coding gene, for example, GAPDH, elongation factor or beta tubulin, is useful and accurate way of fungal species identification (Schoch *et al.*, 2012).

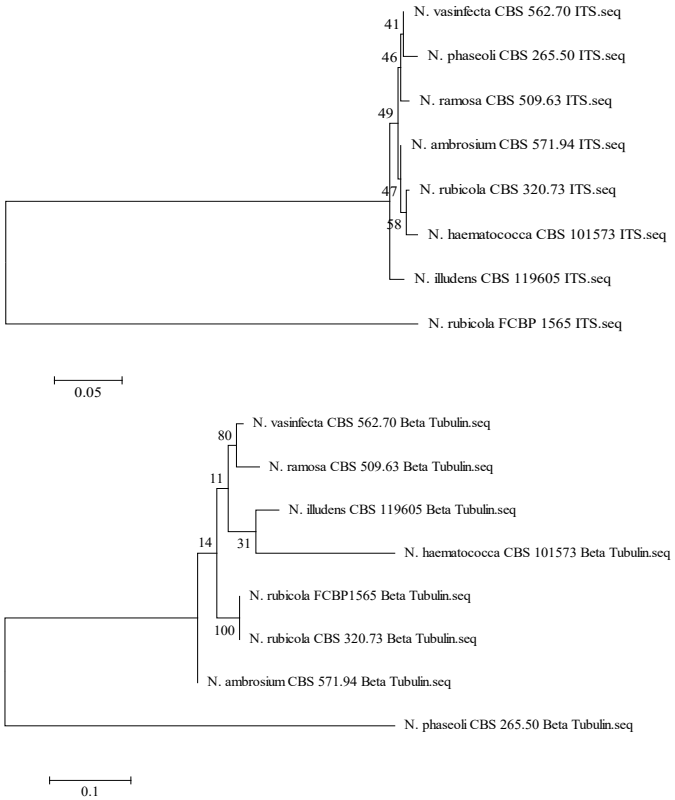

Figure 3: *Molecular phylogenetic analysis of ITS (a) and beta tubulin (b) nucleotide sequences.*

Phylogenetic tree, based on beta tubulin gene sequence (Figure 3B) depicted 100% similarity between *N. rubicola* strains CBS 320.73 and FCBP 1565. However, tree based on ITS nucleotide sequence (Figure 3A) exhibited higher similarity of both strains of *N. rubicola* and phylogenetic divergence from other species of the same genus. Hirooka *et al.* (2012) also apply both phylogenetic and morphological characterizations for the authorization of *Neocosmospora* species. The clustering percentage of taxa in tree is given next to the branches. The tree branch lengths are measured in term of substitutions per site. Evolutionary divergence between sequences of the strains used for phylogenetic tree analysis was also estimated by MEGA6 using the Maximum Composite Likelihood model (Tamura *et al.*, 2004) and shown in Table 1. The number of base substitutions per site between beta tubulin sequences of CBS 320.70 and FCBP1565 was zero that confirmed the identification of present study pathogen.

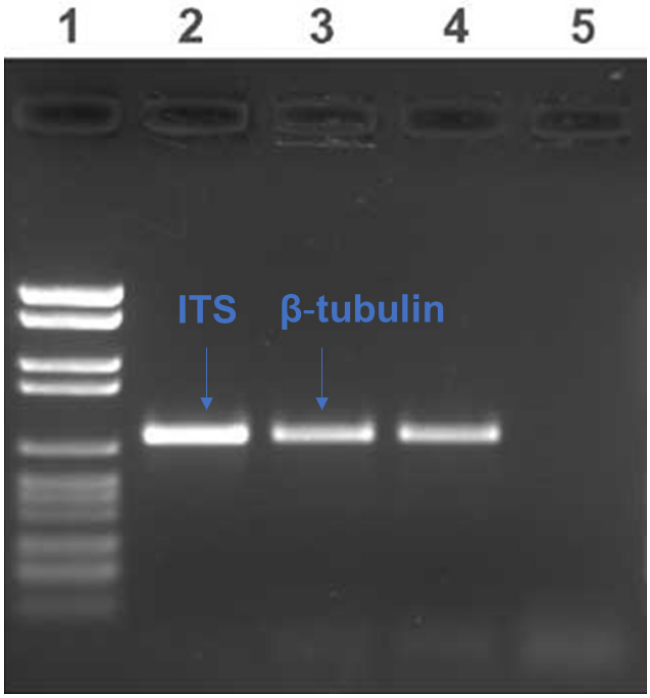

Figure 4: *Agarose gel electrophoresis of ITS and beta tubulin PCR products for the differentiation of N. rubicola.*

Artificially inoculated stems began to exhibit rotting symptoms after 10 days of injection while control stems remained healthy. Potato plant initially showed browning lesions near the soil line of stem and roots which developed into black resulting in cracking of stem. However, potato seeds in early stage may also be affected and lead to potato plants wilt or stunt growth. The vascular bundles of stem internally changed into light brown to blackish in colour. Therefore, potato plants are defoliated or exhibited chlorotic in leave tissues and in the case of severe infections cause great losses in potato production. *N. rubicola* was re-isolated from lesions of artificially inoculated plants thus satisfying Koch's pathogenicity postulates. Same protocol was followed by He *et al.* (2016) for confirmation stalk rot of maize. Based on the symptoms, morphological characteristics, molecular and phylogenetic data, *N. rubicola* was reported for the first time from Pakistan causing stem rot in potato plants.

Table 1: *Estimates of evolutionary divergence between nucleotide sequences.*

	N. vasinfecta CBS 562.70	*N. rubicola* FCBP 1565	*N. rubicola* CBS 320.73	*N. ramosa* CBS 509.63	*N. phaseoli* CBS 265.50	*N. illudens* CBS 119605	*N. haematococca* CBS 101573	*N. ambrosium* CBS 571.94	
N. vasinfecta CBS 562.70									ITS
N. rubicola FCBP 1565	0.592								
N. rubicola CBS 320.73	0.012	0.601							
N. ramosa CBS 509.63	0.008	0.606	0.017						
N. phaseoli CBS 265.50	0.010	0.608	0.023	0.015					
N. illudens CBS 119605	0.021	0.602	0.025	0.025	0.025				
N. haematococca CBS	0.014	0.596	0.010	0.019	0.023	0.027			
N. ambrosium CBS 571.94	0.006	0.596	0.006	0.010	0.017	0.019	0.012		
N. vasinfecta CBS 562.70									Beta tubulin
N. rubicola FCBP 1565	0.058								
N. rubicola CBS 320.73	0.058	0.000							
N. ramosa CBS 509.63	0.037	0.080	0.080						
N. phaseoli CBS 265.50	0.759	0.754	0.754	0.757					
N. illudens CBS 119605	0.080	0.097	0.097	0.094	0.834				
N. haematococca CBS	0.208	0.204	0.204	0.208	0.991	0.193			
N. ambrosium CBS 571.94	0.054	0.050	0.050	0.076	0.719	0.094	0.215		

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Novelty Statement

Neocosmospora rubicola has already been identified as a pathogen on several plants and crops around the world. This disease reports for the first time in Pakistan associated with the potato stem rot. So, our study regarding potato stem rot is unique and first.

Author's Contribution

MR conceptualized the study, formal analysis, methodology and writing of original draft/ manuscript. NA review and editing, supervision and technical input. SN supervised, reviewed and edited the manuscript. Provided technical guidelines during the study. MS designing of survey format and compilation and interpretation of data. AT field visits for collection of data and Figures and Graphs in Microsoft excel.

Conflict of interest

The authors have declared no conflict of interest.

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