Online Available at: https://sciplatform.com/index.php/wjb/editor/submission/730

ISSN (Online) = 2522-6754 ISSN (Print) = 2522-6746

WORLD JOURNAL OF BIOLOGY AND BIOTECHNOLOGY

Research Manuscript www.sciplatform.com

Morpho-molecular identification of *Fusarium oxysporum* as the causal agent of dry rot of carrot in Pakistan

[a] Salman Ghuffar, [b] Muhammad Sajjad Saeed, [b] Waseem Abbas, [b] Muhammad Iqbal, [c] Nasir Mehmood, [a] Umar Sabtain, [d] Muhammad Ussama Yasin, [a] Abdul Qayyum, [e] Muhammad Rauf

[a] Vegetable Research Station Sahiwal, Punjab, Pakistan,
[b] Vegetable Research Institute, Ayub Agriculture Research Institute , Faislabad, Punjab, Pakistan,
[c] Rawalpindi Women University, Pakistan,
[d] Plant Pathology Research Institute, Ayub Agriculture Research Institute Faislabad, Punjab, Pakistan,
[e] Vegetable Research Station Karor, Lal Essan, Punajb.

Authors' Contribution Ghuffar S., M. S Saeed, U. Sabtain, M. Iqbal, M. Rouf, A. Qayyum conducted the survey and symptom based identification, W. Abbas, M. U. Yasin performed the morphological characterization, N. Mehmood, indetified tha pathogen at molecular level.

*Corresponding Author's Email Address: nasirm20@gmail.com

Review Proccess: Peer review

ABSTRACT

Fusarium rot or dry rot is an emerging threat to carrot field, which is responsible for significant economic losses worldwide. The objective of this study was to identify *Fusarium* species associated with dry rot of carrot (*Daucus carota)*. For this porpuse, ten isolates of *Fusarium* sp. were collected from symptomatic carrot roots at Vegetable Research Station Sahiwal (VRSS). During morphological studies, fungal colonies were observed whitish, cottony with abundunt arial mycelium. In microscopic observations, macroconidia was falciform and measured 12.1 to 30.7 (L) × 3.6 to 5.8 (W) µm with 2 to 3 septations, while elliptic microconidia of 5.8 to 8.6 (L) × 2.9 to 3.6 (W) µm with none or one septate. For molecular characterization, three isolates (ON955520, ON955053 & ON955054) were amplified using ITS1 & ITS4 primers. Sequence comparison revealed 99-100% genetic homology with previously reported isolates of *F.*oxysporum. To our knowledge, the occurrence of fusarium rot caused by *F.*oxysporum on carrot field is the first time reported in Pakistan which can cause reduction in crop yield.

Keywords: Morphological characterization, molecular identification, ITS primer, sequencing, phylogenetic analysis.

INTRODUCTION: The carrot (*Daucus carota* L.) belongs to *Apiaceae* family is one of the popular fleshy edible storage root vegetable grown worldwide (Que *et al.*, 2019). In Pakistan, carrots are cultivated on an area of 13.95 thousand hectares, with 241.91 thousand tones production which occupies 3rd rank among winter vegetables (Noor *et al.*, 2020). In Punjab province, two major carrot growing districts are mainly Sheikhupura and Kasur contributes about 39% of the total production. Despite its significance as a rich in dietary carotenoids, source of vitamin A and contains appreciable quantities of thiamine and riboflavin. In Pakistan, several biotic and abiotic factors has been recorded for the low yield and among biotic factors, insects (Hussain *et al.*, 2017) virus (Urooj *et al.*, 2016), bacterial, nematode and fungal diseases has achived significant value. The carrot is known to suffer numerous carrot diseases responsible for significant yield losses (Khoo *et al.*, 2011). Among them, *Fusarium* rot or dry rot caused by *Fusarium oxysporum* considered the most destructive and economically meaningful disease (Zhang *et al.*, 2014). This disease is responsible to reduce carrots' nutritional value, shelf life, and destroy their aesthetic appearance. The harmfulness of *Fusariun genus* among vegetables has increased dramatically worldwide in past few decads based on environmental conditions, cultivation technology and variety resistance which leads to losses upto 60% (Gullino *et al.*, 2012). Moreover, Fusarium fungi can produce several toxins, which play an essential roles in disease developement, food safety concerns and carcinogernic to living organisms (Milićević *et al.*, 2010). *Fusarium* genus belongs to order hypocoreales of class Ascomycetes causing vascular wilt which could be observed in the field in patches on all growing stages with following symptoms such as drying of leaves along with un-developed roots showing reduced proliferation (Stoilova and Chavdarov, 2006). Favaourable enviornemntal conditions for fusarium rot to become in epidemic form is dry, hot spring season with temperature in between 22 to 25 °C respectively (Agrawal *et al.*, 1993). The pathogen is responsible for causing wilting are seed or soil borne in nature which can live in the soil for several years without having suitable host in the form of resting spores as a survival structure known as Chlamydospores (Chaudhary and Kaur, 2002). In addition, *Fusarium* species were traditionally identified on morphological means such as, size and shape of micro & macro-conidia, parameters related to vegetative compatibility groups and on the basis of host specificity (Abbas, 2019). But such of these aspects have some limitations for further identification of *Fusarium* among species and sub-generic groupings. Therefore, now-a-days, use of molecular techniques become more efficient and accurate for the determination of fusarium species (Visser, 2006). However, molecular approaches such as PCR techniques has been used as a tool for studying the identification of several fungal species and evolutionary genetics (Fravel *et al.*, 2003). For the phylogenetic study of Fusarium species, ITS gene have been introduced to study the taxonomy of this genus more precisely (Geiser, 2004).

OBJECTIVES: The present study was carried out to identify the *Fusarium* sp. collected for the first time in carrot fields in Vegetable research Station Sahiwal (VRSS) Punjab, Pakistan from diseased carrot samples based on morphological characters, molecular and pathological characterization via Koch's Postulate fulfillment.

MATERIALS AND METHODS: Cultural and morphological studies: A survey was conducted in March, 2022 from Vegetable Research Station, Sahiwal, Punjab, Pakistan where carrot crop (T-29) was cultivated for seed production. Infected samples of dry rot were collected based on the symptoms appeared as yellowing and wilting of leaves, root surface becomes dry which expressed light to brown irregular shaped cankers later on whitish mycelium developed on the lesions shown in (figure 1A) with the average disease incidence reported up to 70%. Infected samples were brought to Plant Pathology Research Institute, Faislabad for further cultural and morphological studies by using protocol given by (Ghuffar *et al.*, 2021). After 5th day of incubation, color of each colony was characterized on cultural and morphological means based on fungal taxonomic key (Leslie *et al.*, 2006).

Molecular analysis: For molecular characterization, The DNA was extracted from three isolates (VRSCfo01, VRSCfo02and VRSCfo03) by using Prem Man® Ultra sample preparation Reagent (Applied Biosystem, Foster City, CA), following manufacturer instructions. Protocol was followed by White (1990) for the prepration of 50 µL polymerase chain reaction (PCR) mixture and thermocycler PCR conditions for amplification of isolates with ITS1 & ITS4 primers respectively. DNA after amplification was verified by running 6µl of the PCR product on a 1% agarose gel (figure 2). Furthermore, after purification of PCR product with Gel band purification kit (GFX ™). The PCR product was subjected to Macrogen Korea Sequence Facility center for sequencing the purified DNA samples. Bio Edit v. 7.0.5.2 software was used for editing the Sequences manually (Hall, 1999). BLAST searches were performed to find out the genetic similarty between the ITS sequences through Gen Bank database BLAST program which was helpful in the construction of ITS phylogenetic tree. Alignment of Sequences were performed in the CLUSTAL-X v. 1.81 (Thompson *et al.*, 1997) and for the construction of phylogenetic tree Neighbor-Joining (NJ) analysis was performed in MEGA v. 7 (Kumar *et al.*, 2016).

Pathogenicity test: For the confirmation of virulence nature of fungal pathogen, pathogenicity test was carried out. For this purpose, carrot seeds (cv. T-29) were grown in pots having sterilized soil . The soil was infested with *Fusarium* isolates by adding spore suspension with concentration of 1 × 10^4 CFU/g soil. Plants grown in non-infested soil served as a controls. There were three replicates per treatment (Zhang *et al.*, 2014).

RESULTS AND DISCUSSION: Cultural and morphological identification : A total of 10 isolates of *Fusarium* sp. causing dry rot of carrot were subjected to morphological charaterization. During

observation, isolates were found cottony, white with arial mycelium shown in (table 1 & figure 1 B). Maximum (L ×W) of macro-conidia was 30.7μm ± 0.6× 5.8±0.3 while in case of micro-conidia maximum (L ×W) was 8.6 μm ± 0.3 × 3.6± 1.4 μm was recorded for isolate VRSCfo02, respectively.

Figure 1: Symptomology(A) and cultural (B) and morphological (C) characteristics of *Fusarium* sp. causing dry rot of carrot.

The shape of macro-conidia was found falciform with 2 to 3 septations while, elliptic shape micro-conidia was observed with 0 to 1 septation respectively (table 1 & figure 1C). Majority of isolates having 2 septations within macro-conidia while maximum none sepations were observed in micro-conidia. Seifert (2001) proposed that colony color of *F. oxysporum* is whitish, cottony with aboundant arial mycelium which is a distinct feature of this species within the genus Fusarium. Similar features were also found in the study. Nelson *et al.* (1983) illustrated that species of *Fusarium* can also be differentiated mainly on the basis of shape, length and width of macro-conidia. In the present study, the shapes of macro-conidia were recorded falciform which is an agreement with the previous study proposed by Leslie *et al.* (2006). In addition, the number of septation in macro-conidia is an important feature for fusarium characterization and helpful for species identification reported by Leslie *et al.* (2006). On the basis of their observations, the number of macro-conidial septations were considered for species indentification. Moreover, another significant criteria for species identification is studied by Burgess *et al.* (1989) which is size and presence or absence of micro-conidia in fusarium species showing a significant variation among the isolates. Mandhare *et al.* (2011) studies the conidial size of *F. oxysporum* from chickpea showing similar variations. During pathogenicity test, the same symptoms on seedling were observed after two weeks on all infested plants as previously described while no symptoms were observed on the control plants.

Table 1: Fusarium culture identification and morphological characterization.

Sr.	Isolation ID	Colony	Macro-conidia				Micro-conidia			
		Colony Color	Shape	Length (μm)	Width (μm)	Septation	Shape	Length (μm)	Width (μm)	Septation
1	VRSCfo01	whitish, cottony with abundant arial mycelium	Falciform	30.6±0.4	5.6±1.1	2	Elliptic	5.2±0.2	3.0±0	0
2	VRSCfo02	-	-	30.7±0.6	5.8±0.3	2	-	8.6±0.3	3.6±1.4	0
3	VRSCfo03	-	-	12.9±0.2	5.2±1.5	3	-	7.6±1.1	3.1±0.5	1
4	VRSCfo04	-	-	22.5±1.2	4.1±0.6	2	-	6.9±0.19	3.5±0.4	0
5	VRSCfo05	-	-	12.6±0.4	3.9±0	2	-	7.1±0.04	3.3±0.3	0
6	VRSCfo06	-	-	18.9±1.7	5.1±0.8	2	-	7.4±0.2	3.0±0	0
7	VRSCfo07	-	-	12.1±0.2	3.6±1.7	3	-	5.8±1.1	2.9	1
8	VRSCfo08	Whitsh and cottony	-	27.3±1.5	4.7±0.4	3	-	6.7±0.2	3.4±0.8	1
9	VRSCfo09	-	-	25.2±0	3.8.±1.7	3	-	5.1±1.1	3.2±0.4	1
10	VRSCfo10	-	-	29.2±1.5	4.2±0.46	2	-	6.9±0.27	3.1±0.5	0

Phylogenetic analysis: Morphologically and pathogenically characterized three fusarium isolates (VRSCfo01, VRSCfo02 and VRSCfo03) were further subjected to molecular characterization for the determination of genetic diversity of ITS gene region through phylogenetic analysis. The gel electrophoresis providing a single band length of 680 bp with amplified ITS product which was further used for sequencing and phylogenetic studies (figure 2).

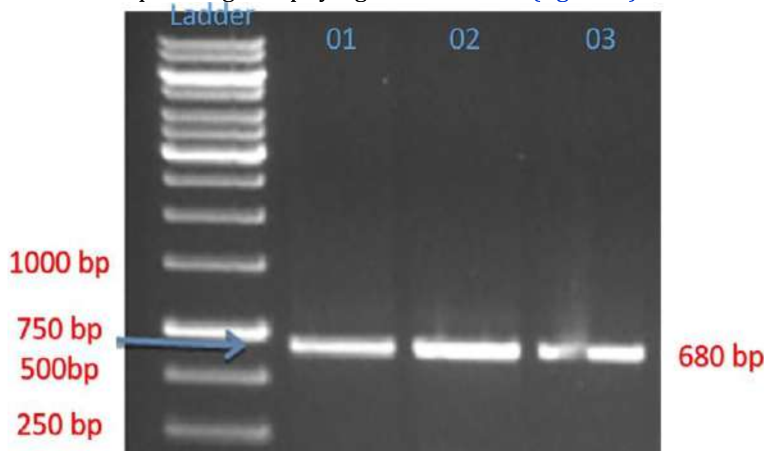

Figure 2: Molecular identification of ITS region from three representative isolates of *Fusarium* sp.

After submission of final sequences in the NCBI public data base under the accession numbers of ITS gene (ON955520, ON955053 & ON955054) exhibiting more than 99% genetic similarity with previously sequenced isolates of *F. oxysporum* available at public nucleotide data base system. Phylogenetic analysis of ITS of *F. oxysporum* revealed that isolates from different district of origin had varying levels of genetic diversity. In phylogenetic tree the reference sequences were used as *F. proliferatum*, *F. fujikuroi*, *F. annulatum*, *F. verticillioides* respectively. Furthermore, *Alternaria alternata* were used as outgroups shown in (figure 3).

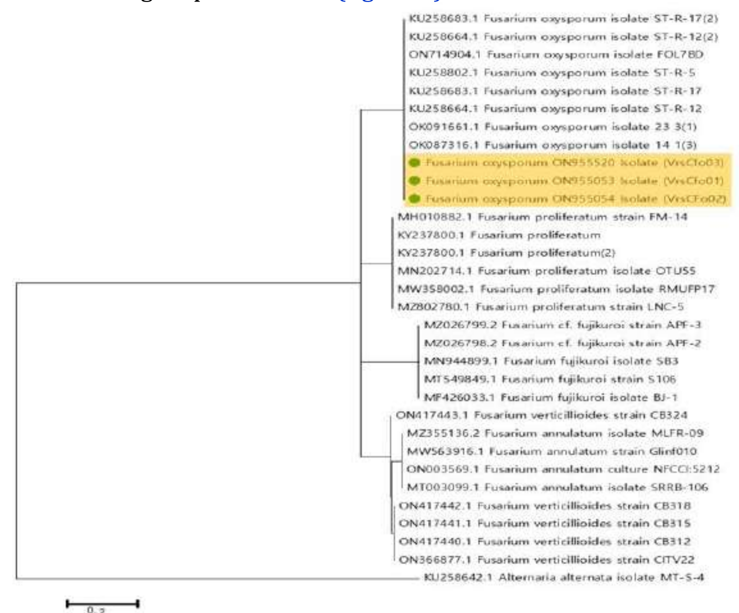

Figure 3: Phylogenetic Tree based upon MUSCLE alignment of the ITS region of rDNA nucleotide equences of *Fusarium oxysporum* isolates causing dry rots of carrot.

The present work is supported by Aoki *et al.* (2003) which suggested that molecular study is helpful to identify the fungi on species level, which is difficult on the basis of morphological characterization. Likewise, Waalwijk *et al.* (2004) recommended that amplification of ITS grene with polymerase chain reaction (PCR) provides accurate identification of fungal pathogens as compared to conventional techniques.. Similarly, Mule et al. (2005)

illustrated that DNA sequencing is helpful for accurate identification of biotic diseases in species level. On the basis of molecular studies recommended that ITS gene is an efficient tool which could be used as a phylogenetic analysis to distinguish the Fusarium species as studied by Chandra *et al.* (2011) & Achari *et al.* (2020), As per our knowledge concluded that, this is the first report of *F. oxysporum* causing dry rot of carrot in Pakistan.

CONCLUSION: Identification through morpho-molecular means and pathogenicity tests are reliable tools for the confirmation of *F. oxysporum* causing dry rot of carrot in Pakistan. In summary, this research work provided the comprehensive factual picture of dry rot caused by *F. oxysporum* from Pakistan through morph-molecular identification.

CONFLICT OF INTEREST: Authors have no conflict of interest.

ACKNOWLEDGMENTS: It was highly aknowledge to the Governemnt of Punjab (GOP), Agriculture Department for providing funding facilities under the development project titled " Development of hybrid and OPVs in vegetables resilient to climate change" to complete this research work.

REFERENCES: Abbas, M. F., 2019. Prevalence and characterization of fungal pathogens associated with loquat (*Eriobotrya japonica*) in Punjab Pakistan. PMAS-Arid Agriculture University, Rawalpindi.

Achari, S. R., J. Kaur, Q. Dinh, R. Mann, T. Sawbridge, B. A. Summerell and J. Edwards, 2020. Phylogenetic relationship between australian *Fusarium oxysporum* isolates and resolving the species complex using the multispecies coalescent model. BMC genomics, 21(1): 1-20.

Agrawal, S., K. Singh and S. J. A. Lal, 1993. Plant protection of lentil in India. pp: 147-165.

Aoki, T., K. O'Donnell, Y. Homma and A. R. Lattanzi, 2003. Sudden-death syndrome of soybean is caused by two morphologically and phylogenetically distinct species within the *Fusarium solani* species complex—f. Virguliforme in North America and F. Tucumaniae in South America. Mycologia, 95(4): 660-684.

Burgess, L., P. E. Nelson and B. J. J. F. L. Summerell, 1989. Variability and stability of morphological characters of *Fusarium oxysporum* isolated from soils in Australia. Mycologia, 81(5): 818-822.

Chandra, N. S., E. Wulff, A. Udayashankar, B. Nandini, S. Niranjana, C. Mortensen and H. Prakash, 2011. Prospects of molecular markers in *Fusarium* species diversity. Applied microbiology biotechnology, 90: 1625-1639.

Chaudhary, R. and A. Kaur, 2002. Wilt disease as a cause of shift from lentil cultivation in Sangod Tehsil of Kota (Rajasthan). Indian journal of pulses research, 15(2): 193-194.

Fravel, D., C. Olivain and C. Alabouvette, 2003. *Fusarium oxysporum* and its biocontrol. New phytologist, 157(3): 493-502.

Geiser, D. M., 2004. Practical molecular taxonomy of fungi. Advances in fungal biotechnology for industry, agriculture, medicine: 3-14.

Ghuffar, S., M. Z. Ahmad, G. Irshad, M. A. Zeshan, A. Qadir, H. A. Anwaar, M. Z. Mansha, H. M. Asadullah, A. Abdullah and U. Farooq, 2021. First report of *Aspergillus niger* causing black rot of grapes in Pakistan. Plant disease, 105(05): 1570.

Gullino, M. L., J. Katan, A. J. F. w. o. g. v. Garibaldi and o. crops, 2012. The genus fusarium and the species that affect greenhouse vegetables and ornamentals. American phytopathical society: pp: 5-9.

Hall, T. A., 1999. Bioedit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. In: Nucleic acids symposium series. [London]: Information retrieval Ltd., c1979-c2000.: pp: 95-98.

Hussain, A., M. Arif and A. Abbas, 2017. Management of aphid borne potato virus y (PVY) through chemical and bio-chemical methods. World journal of biology and biotechnology, 2(2): 159-163.

Khoo, H.-E., K. N. Prasad, K.-W. Kong, Y. Jiang and A. Ismail, 2011. Carotenoids and their isomers: Color pigments in fruits and vegetables. Molecules, 16(2): 1710-1738.

Kumar, S., G. Stecher and K. Tamura, 2016. Mega7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology evolution, 33(7): 1870-1874.

Leslie, J., B. J. J. F. L. Summerell and E. Brett A. Summerell., 2006. The *Fusarium* laboratory manual blackwell. 1-388.

Mandhare, V., G. Deshmukh, J. Patil, A. Kale and R. MPKV, 2011. Morphological, pathogenic and molecular characterization of *Fusarium oxysporum* f. Sp. Ciceri isolates from maharashtra, India.

Milićević, D. R., M. Škrinjar and T. Baltić, 2010. Real and perceived risks for mycotoxin contamination in foods and feeds: Challenges for food safety control. Toxins, 2(4): 572-592.

Mule, G., M. Gonzalez-Jaen, L. Hornok, P. Nicholson and C. Waalwijk, 2005. Advances in molecular diagnosis of toxigenic *Fusarium* species: A review. Food additives contaminants, 22(4): 316-323.

Nelson, P. E., T. A. Toussoun and W. Marasas, 1983. Fusarium species: An illustrated manual for identification.

Noor, A., K. Ziaf, M. A. Ghani, C. M. Ayub, I. Ahmad and M. J. P. Amjad, 2020. 35. Plant spacing effects on seed yield and quality of carrot cultivar t-29. Pure and applied biology, 9(4): 2563-2570.

Que, F., X.-L. Hou, G.-L. Wang, Z.-S. Xu, G.-F. Tan, T. Li, Y.-H. Wang, A. Khadr and A.-S. Xiong, 2019. Advances in research on the carrot, an important root vegetable in the apiaceae family. Horticulture research, 6.

Seifert, K. A., 2001. Fusarium and anamorph generic concepts. In: Fusarium: Paul E. Nelson memorial symposium amerocam phytopathological society, St. Paul, MN.

Stoilova, T. and P. Chavdarov, 2006. Evaluation of lentil germplasm for disease resistance to *Fusarium* wilt (*Fusarium oxysporum* f. Sp. Lentis). Journal of central european agriculture, 7(1): 121-126.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins, 1997. The clustal_x windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic acids research, 25(24): 4876-4882.

Urooj, M., U. Arif and A. Intikhab, 2016. A brief review for identification and detection of potato viruses. World journal of biology and biotechnology, 1(1): 33-37.

Visser, M., 2006. Molecular biological studies of the fusarium wilt pathogen of banana in south africa. University of Pretoria.

Waalwijk, C., R. van der Heide, I. de Vries, T. van der Lee, C. Schoen, G. Costrel-de Corainville, I. Häuser-Hahn, P. Kastelein, J. Köhl and P. Lonnet, 2004. Quantitative detection of *Fusarium* species in wheat using taqman. European journal of plant pathology, 110: 481-494.

Zhang, X., J. Hu, H. Zhou, J. Hao, Y. Xue, H. Chen and B. Wang, 2014. First report of *Fusarium oxysporum* and *F. Solani* causing *Fusarium* dry rot of carrot in China. Plant disease, 98(9): 1273-1273.

Except where otherwise noted, this item's licence is described as **© The Author(s) 2023**. Open Access. This item is licensed under a **Creative Commons Attribution 4.0 International License**, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this it are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.