Research Journal of Agriculture and Forestry Sciences _____ISSN 2320 – 6063
Vol. **5(7),** 12-19, July **(2017)** *Res. J. Agriculture and Forestry Sci.*

Morphological and metabolic characterization of wilt disease (*fusarium oxysporum* f. sp. *ciceri*) in chickpea (*Cicer arietinum* L.)

Jasminkumar Kheni*, Vishal Kothari, Khyati Rathod, Visha Rathod, Shital Padhiyar, Rohit Bhalara, R.S. Tomar, M.V. Parakhiya and B.A. Golakiya
Department of Biotechnology, Junagadh agricultural University, Junagadh, Gujarat-362001, India
jash_9999@yahoo.com

Available online at: www.isca.in
Received 30th April 2017, revised 27th June 2017, accepted 5th July 2017

Abstract

Wilt disease of chickpea caused by the diminish pathogen Fusarium oxysporum f. sp. ciceri (foc) is a serious disease of chickpea (Cicer arietinum L.) and it cause approximately 10-15% loss in annual yield. This phytopathogenic fungus invades the plant via roots, colonizes the xylem vessels and prevents the nutrient flow, finally resulting in wilting of the whole plant thus it is also known as vascular wilt. For the experiment the wilt causing fungi has been isolated from recently wilted wilt susceptible chickpea (JG-62). From the damaged root part, 4 isolates of F. oxysporum were purified. The purified colony of F. oxysporum ranged diameter of 3.0 - 4.0 cm PDA medium at fourth day of inoculation. The mycelia characters had white to creamy and tinge pink, margins slightly lobed or smooth on PDA medium. This identification was further confirmed by Microcopy, Scanning electron microscopy (SEM) and 18s rRNA Sequencing of ITS region of Fungi. Dendogram created from sequences represents the strain isolated from wilt sick plot is highly similar to Fusarium oxysporum. Further, we carried out metabolic profiling of foc in GC-MS. The polar and non polar metabolic phase was separated. We identified total 49 metabolites in foc among them, the higher intensity of sugar, sugar alcohols, amino acids and fatty acid esters was found. This study can provide metabolomic and morphological study to understand pathogenesity of chickpea wilt.

Keywords: Wilt Disease, plant pathogen interaction, GC-MS, SEM.

Introduction

Chickpea (*Cicer arietinum* L.) is the one of the most widely cultivated cool season legume, globally. India is the leading country in chickpea production[1]. Chickpea is prior source of protein for humans. Therefore the chickpea is of significance to food and nutritional security in the developing world. However, due to some of the pathogens like *Fusarium wilt* and *ascochyta blight*, cause the yield loss of chickpea in national and international breeding practices. *Fusarium oxysporum* have different races and it cause the wilt disease in chickpea and affects the all major chickpea cultivars[2].

Wilt disease penetrate through the soil to roots and blocks the xylem plant cell, resulting in the obstruction of nutrient supply as wilting progresses, ultimately leading to plant death. This wilt fungus has tendency to survive for many years in soil even without any nutrient source or its host, poses a serious challenge for disease management[3].

The identification of *Fusarium* species is mainly based on its different morphological characters *viz.*, shapes and sizes of macro- and microconidia, presence and absence of chlamydo spores, colony appearances, pigmentations and growth rates on PDA medium[4]. Therefore, this technique is highly dependable for the strains differentiation at the intra specific level in the study of Fusarium taxonomy[5,6].

Metabolism is the complex process due to highly diversify nature of metabolites, and it is also associated with metaboloic pathways associated with different biological process. In organism to obtain as much information about the metabolome requires clear attention on sample preparation, selective extraction protocols, and analytical techniques combined with statistical analysis[7,8]. Furthermore, the metabolomic complexity is varying with organism so method should be optimized for each type of organisms of interest. Therefore, the optimized extraction protocol is considered as one of the most important quality parameter in microbial metabolome.

In this study, we examined metabolomics and morphological characters in *Fusarium oxysporum* f.sp *ciceri*. The extraction protocol for Fusarium was developed with optimized number of different metabolites, whereas minimizing the analytical errors. In this present work the GC-MS was utilized. The GC-MS provides quick detection of metabolites based on retention times (RT) and fragmentation patterns of different components.

Materials and methods

Isolation and 18s rRNA identification of fungus: The wilt causing fungus was isolated from recently wilted plants of JG-62 from wilt sick plot of Pulse research station, JAU, Junagadh. This fungus was utilized for identification. The fungal genomic DNA was extracted from mycelia grown in 250 ml of PDB at

28°C for 5 days. The mycelia were harvested from broth and lyophilised and stored at -20°C for further process. The genomic DNA for PCR was extracted by using HiMedia fungi DNA isolation kit. The ITS region of fungi, including ITS2, ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTTATTGATATG3') were amplified. The amplification was performed in 30 µl reaction volume with 0.1 mM of each dNTP and 100pmol of both forward and reverse primer. Veriti PCR (Thermo fisher) was programmed for initial denaturation at 94°C for 4 min, and 35 cycles at 94°C for 1 min, 55 °C for 1 min, and 72°C for 1 min. The amplification was completed with a final extension at 72°C for 5 min. Further it was sequenced by ABI 3130 capillary sequencing.

Microscopic observation: Wilt infected chickpea roots and isolated fungi both were subjected to microscopic examination. The light electron microscopy and SEM both were performed for morphological characterization. For the observations, wilt damaged roots were cut in to transverse and longitudinal sections. The fungal biomass and root sections were mounted with double-adhesive tape on aluminum stubs and vacuumed in concentrator plus (Eppendrof, Germany), further it was coated with gold (7 nm) in sputter coaters. The prepared specimens were examined with high vacuum at an accelerating voltage of 10– 15 kV under SEM (EVO 18 Carl Zeiss, Germany).

Metabolomic analysis: Metabolite extraction: Metabolite extraction and derivatisation steps prior to GC-MS analysis were performed using the two-phase methanol/ chloroform method with slight modifications[9]. The samples were crushed in liquid Nitrogen and then, Methanol (1.0 ml) was added to 50 mg of dried fungal biomass incubated for 15 min at 70°C. The extracts were then immediately mixed vigorously with 1 volume of distilled deionised water. Finally, 750 µl of chloroform was added to the mixture before phase separation by centrifugation at 10,000 rpm for 10 min. Aliquots of polar and non-polar supernatant (250 µl) were dried in turbo vacuum evaporator for 2 – 3 h.

Derivatisation: The dried extracts were re-dissolved in 50 µl of pyridine and sonicated for 10 min before methoximation step by adding 40 µl of 20 mg/ml methoxyamine HCL with pyridine[10]. The mixtures were then sonicated again for 5 min and incubated with constant agitation for another 90 min at 37°C. The trimethylsilylation (TMS) step was performed by adding 250 µl MSTFA to the extracts, followed by incubation for 1 h at 37°C. The extracts were then cooled down to room temperature for at least 1 h prior to GC-MS analysis. For the non polar extract, only the TMS step with MSTFA was performed.

Analysis of fungal metabolites by GC-MS: The polar and non polar samples were mixed in equal volume. Mixtures of polar and non polar samples were analyzed using GC-MS (Shimadzu 2010, Japan). A sample volume of 2 µl was injected with a splitless mode. The GC column used for the analysis was DB17MS, 30 m × 0.25 mm. The initial oven temperature was set at 50°C for 3 min, and then raised to a target temperature of 400°C in 9 min at a rate of 10°C/min. Helium was used as the carrier gas at a rate of 1ml/min. All the chromatogram peaks were compared with the NIST08 library database and the retention time index of common primary and secondary metabolites. The mass spectra were analyzed using the Scan, SIM and FASST software of Shimadzu. After GC-MS analysis, all the peak areas were integrated and then normalized to obtain the peak area ratios for all metabolites in each chromatogram. Three replicates were used in this study.

Results and discussion

Isolation of fungal cultures: The wilt causing pathogenic fungi has been isolated from recently wilt infected roots of chickpea (JG-62) (Figure-1a). The colony characteristics of isolated fungi were studied. The colony diameter of the purified pathogenic fungi ranged from 3.0 - 4.0 cm on the fourth day after inoculation on PDA medium (Table-1). The fungal mycelia of the isolates were smooth, tinge pink and white to creamy in colour and, sparse to abundant than PDA (Figure-1b). Further, the purified fungi were identified as *Fusarium oxysporum* f. sp. *ciceri.*

Table-1: Microscopic observations of *F. oxysporum.*

Sr. No	Morphological characters	Observations
1.	Colony colour	White to creamy and tinge pink
2.	Colony diameter	3.0 - 4.0 cm
3.	Pigmentation	Tinge pinkish to brown
4.	Mean length of macroconidia	21.25 ± 5.5 µm
5.	Mean width of macroconidia	2.75 ± 0.5 µm
6.	Chlamydospores	Round and thick-walled
7.	Chlamydospores diameter	12.0 µm

18s rRNA identification of fungus: The ITS region amplification of *Fusarium* was identified during this experiment. The ITS1 and ITS4 regions were amplified at ~600bp. Total 3 Isolates from chickpea wilt plot were identified based on the 18S rRNA amplicons. The sequences from all fungal isolates were further examined through BLAST searches and through direct comparisons with potentially related species. All the Amplified bands from the gels showed 96 to 100% identity with published *Fusarium oxysporum* sequences in NCBI database.

The highest matches found with *Fusarium oxysporum* f.sp *ciceri*. A phylogenetic tree recovered for fungal isolates (Figure-2). Isolate_jk1 is one of the closest to *Fusarium oxysporum* f.sp *ciceri* sequence.

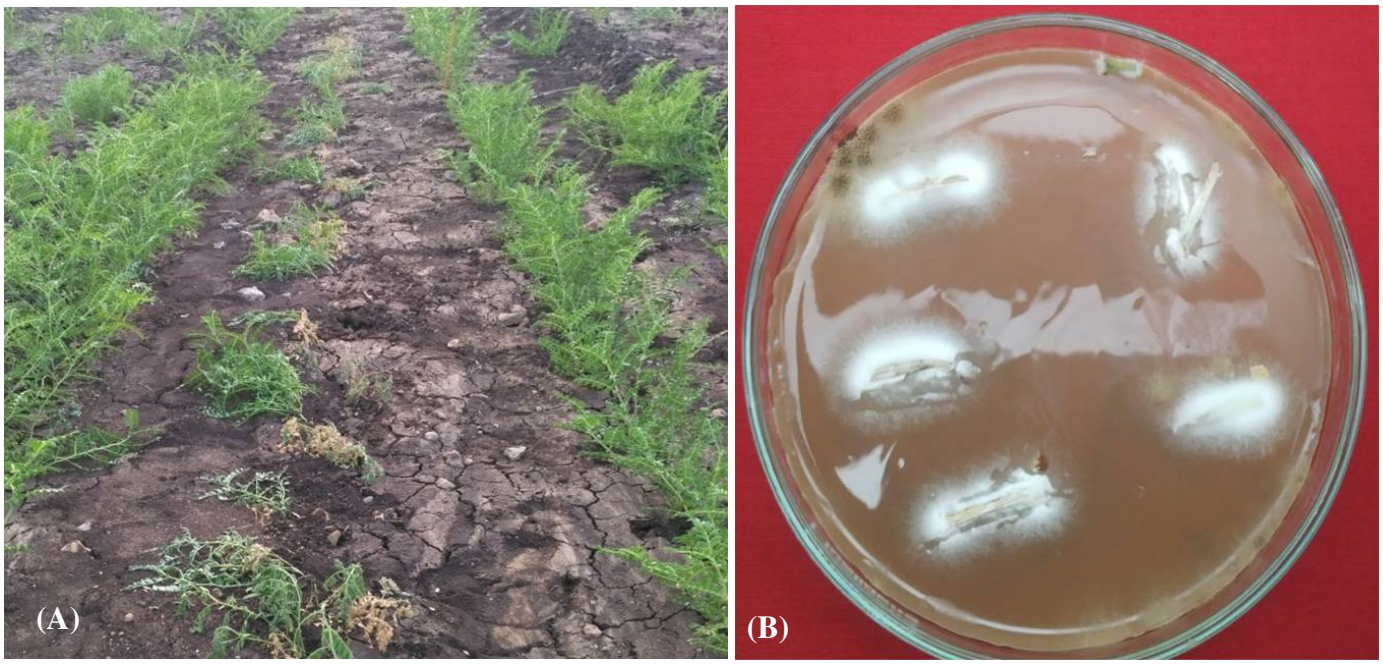

Figure-1: Recently wilted chickpea plants of JG-62 (A); Fungi isolated from roots of wilted chickpea plants (B).

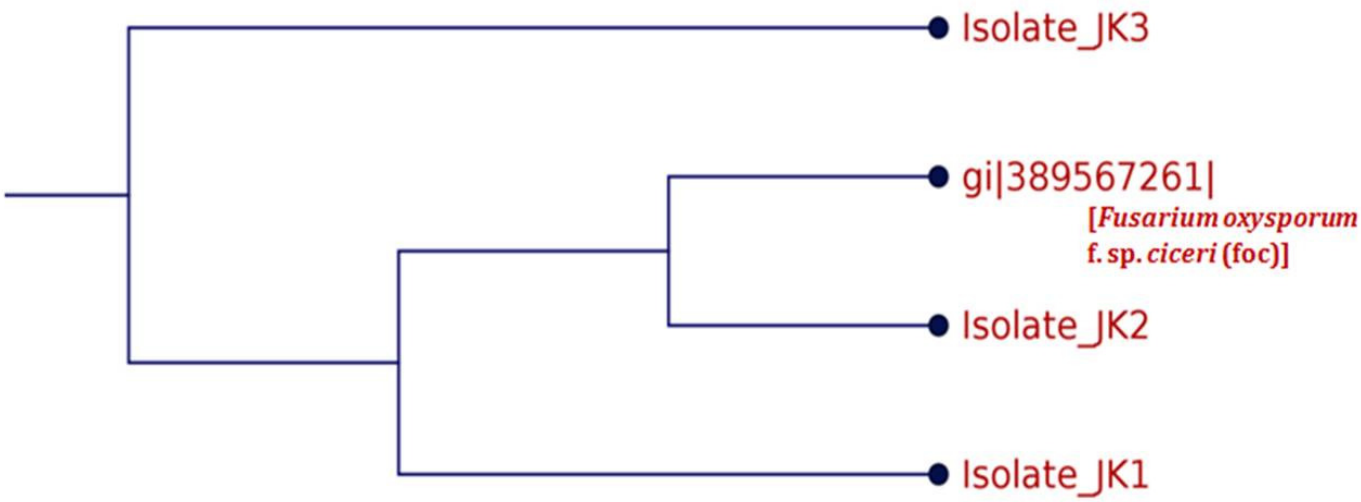

Figure-2: Phylogenetic tree from three isolates of Fusarium sp., Isolate JK2 were used for further study.

Microscopic observation: The microscopic observation has been taken by using light and scanning electron microscopy. The morphology of root infection, myselium, macro and micro conidia and chlamydospores has been observed. The chlamydospores formation of *F. oxysporum* was produced inside of the globose or subglobose false heads with a size of 12.0 µm in diameter (Figure-3). The arrangement of microconidia and macrocodidia was observed on the aerial surface of of the strains of *foc* mycelium. The size of macro conidia is almost doubled than microconidia. The macrocnidia of foc contains 5-6 septa inside the micro conidia. The sizes of chlamydospores and macroconidia are varies with different *fusarium* species and some *Fusarium* sp. cannot form micro conidia[11]. The microscopic observations are depicted in Table-1. Further, the scanning electron micrograph of *Fusarium* was studied (Figure-4). Growing of fungal mycelium on root surface of chickpea during infection stage was studied (Figure-4A). Mycelium growth increased rapidly and after 9 days from inoculation and penetrates in to the root and blocks the xylem of plants and it cause wilting of the chickpea plants (Figure-4B). It was observed that during compatible interactions, *Fusarium* mycelium penetrates in to the xylem through root apex and grow towards the stem[12]. It was also observed that in the plant, the fungus spreads to the tissue and vessels and invades them via growing mycelium. While in the vessels the fungus produces chlamydospores and micro-conidia. Here, in our study the growth and accumulation of microconidia was found inside the plant vessels (Figure-4C).

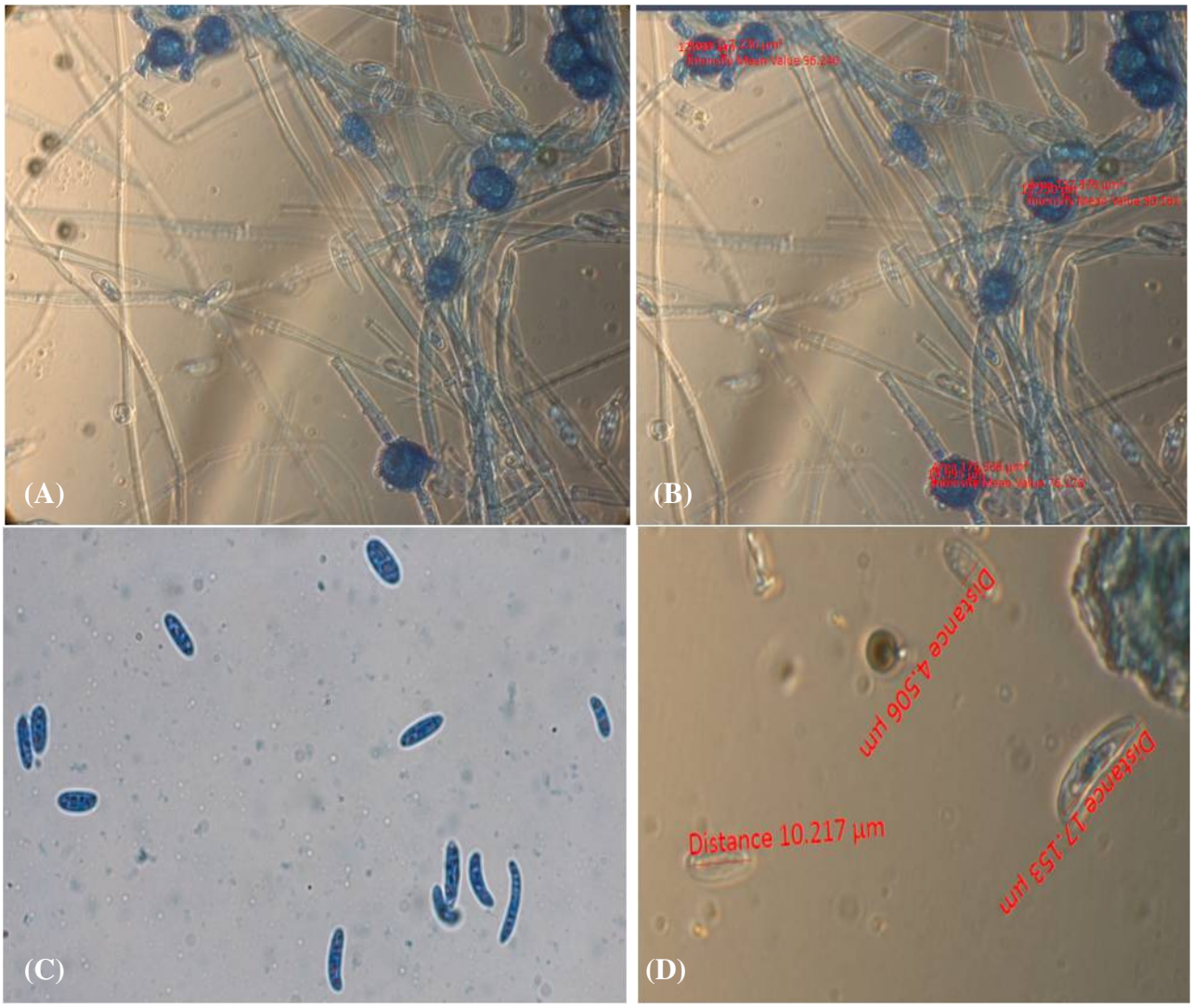

Figure-3: Light micrographs showing the chlamydospores ´s formation *of Fusarium oxysporum* f.sp *ciceri*. Indicated with arrow (A, B); the size of macro conidia and micro conidia was measured, conidia indicated with arrow (C, D).

Metabolomic analysis: The results pertaining to GC-MS analysis of the derivatised extract of *Fusarium oxysporum* f. sp. *ciceri* (foc) lead to the identification of a number of compounds. These compounds were identified through mass spectrometry combined with GC. The methanolic extract is the best solvent for fungal metabolites extraction[13]. Here, total of 49 compounds were identified, during pick integration duplicates were removed. The preliminary metabolomis study revealed that methanolic extract of foc contains reducing sugars, sugar alcohols, fatty acids, amino acids and other important metabolites. The chromatograph of GC-MS is shown (Figure-5). The graphs of all metabolites with average area percentage were drawn (Figure-6). In metabolic extract total 15 sugars and 6 sugar alcohols were identified (Figure-6A). Among them the highest level of fructose and xylulose were found. This indicates that the carbohydrates are the major sourse of fungal cell wall. The sucrose were also found as it polymer of glucans. The level of glucan is higher in fungal cell wall[14]. Total 8 organic acids were found in methanolic extract (Figure-6B). The Propanetricarboxylic acid (citric acid) is the major constituent among them. It was identified that citric acid is important metabolites of fungi and produce during fermentation process[15]. However, we found total 6 fatty acids among them palmitic acid and Octadecanoic acid founds higher in amount (Figure-6C). Furthermore, in fungi, plants, and animal tissues, the monounsaturated and Polyunsaturated fatty acids are predominant[16]. Here, some amino acids like alanine, glutamine, glycine and L-proline were identified from extract of *Fusarium* biomass (Figure-6D). In which the glyice and proline were in higher concentration comparatively. We also studied some organic acids founds during metabolic study. The concentration of inositol was detected next to citric acid. In that relation some fungal pathogens must be able to acquire inositol in order to proliferate and cause infection in their hosts (Figure-6F). The GC-MS study of *Fusarium oxysporum* f. sp. *ciceri* (foc) focused on its primery metabolomic constituents.

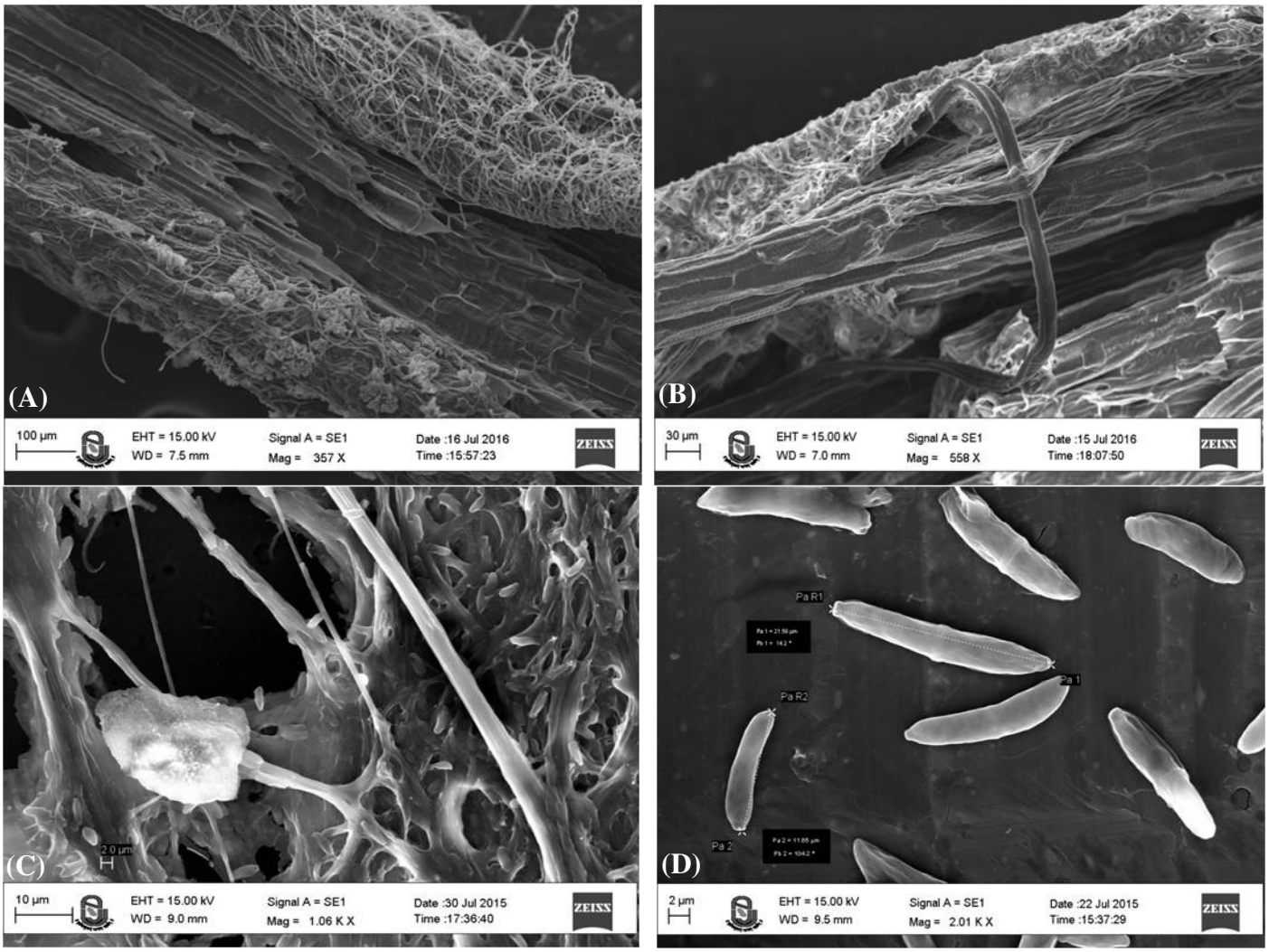

Figure-4: Electron micrograph *Fusarium* infection on chickpea root and penetration into the epidermis layer (A, B); The development and accumulation of microconidia inside the vessels (C); the difference between micro and macroconidia measured (D).

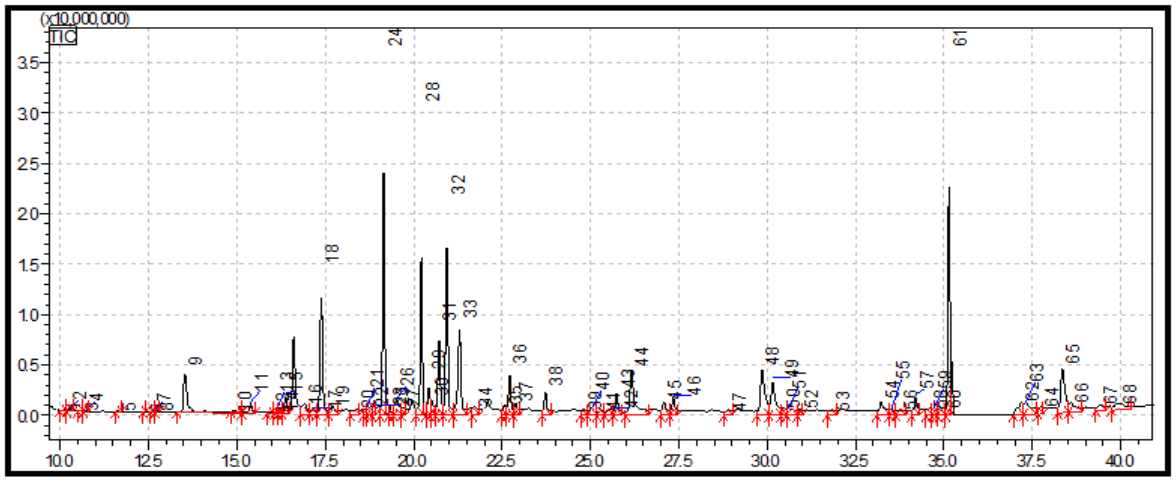

Figure-5: GC-MS Chromatograph of Methanolic extract of *Fusarium Oxysporum* F.sp.*ciceri*

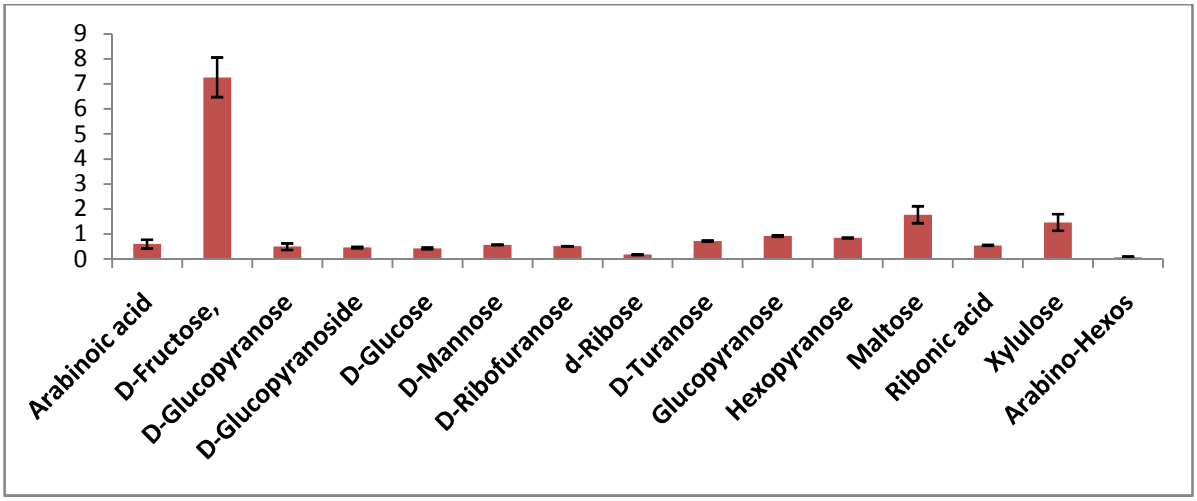

Figure-6A: Sugars

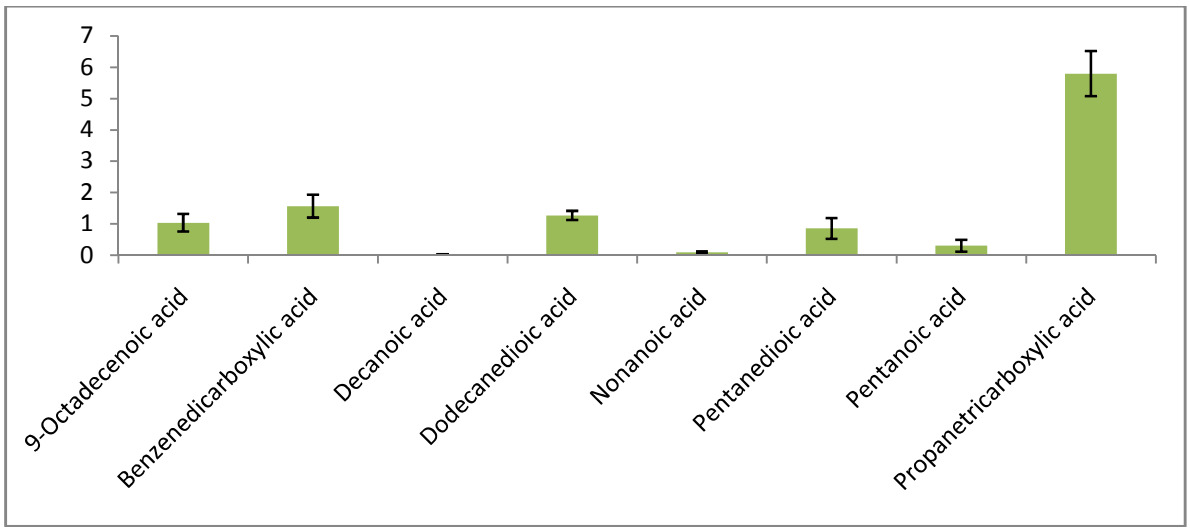

Figure-6B: Organic Acids

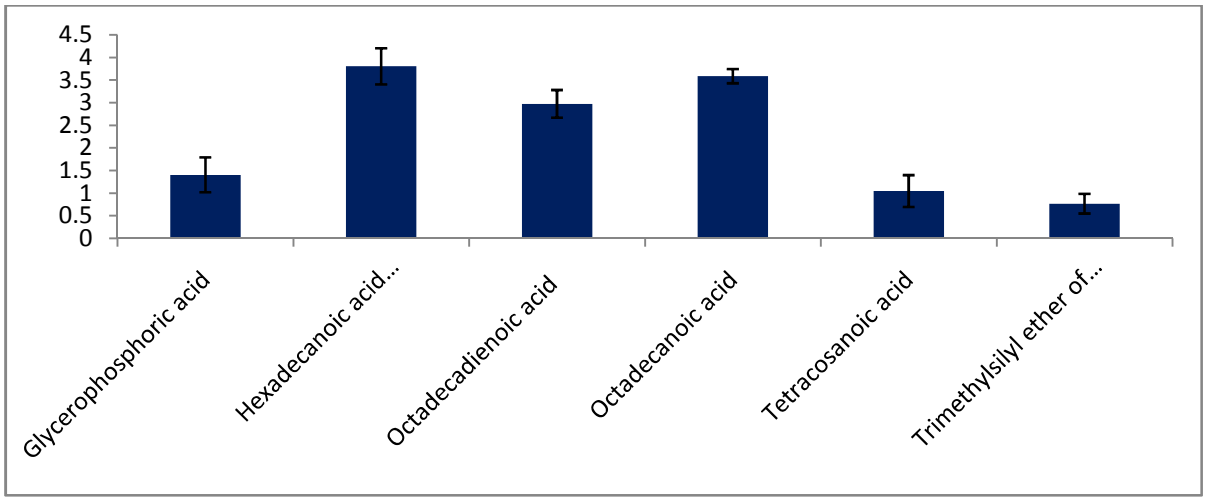

Figure-6C: Fatty Acids

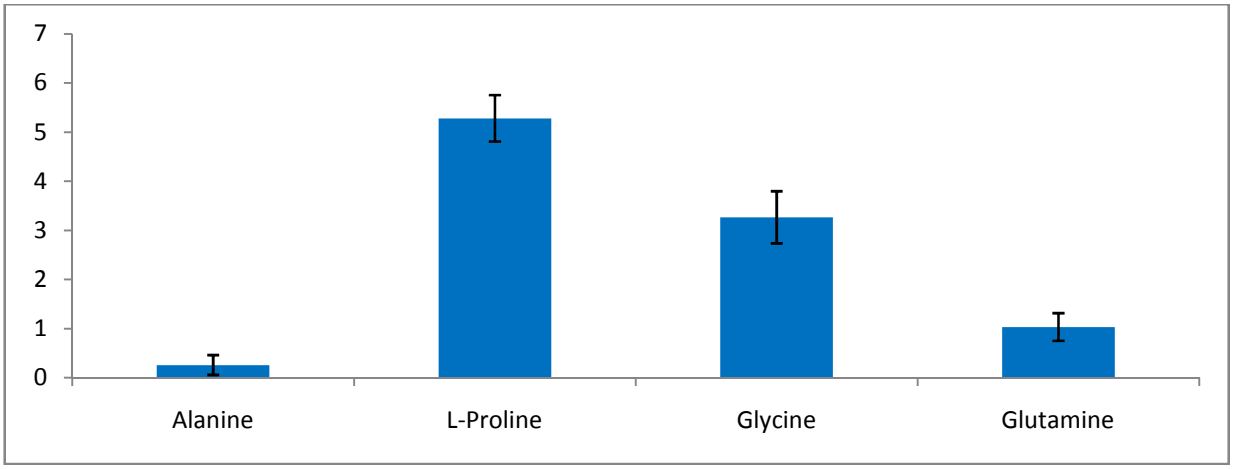

Figure-6D: Amino Acids

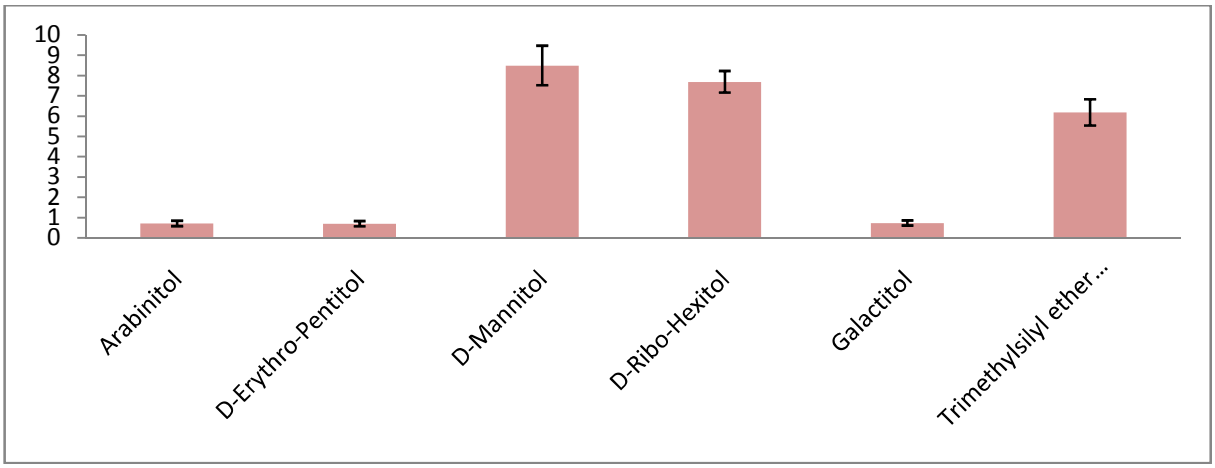

Figure-6E: Sugar Alcohols

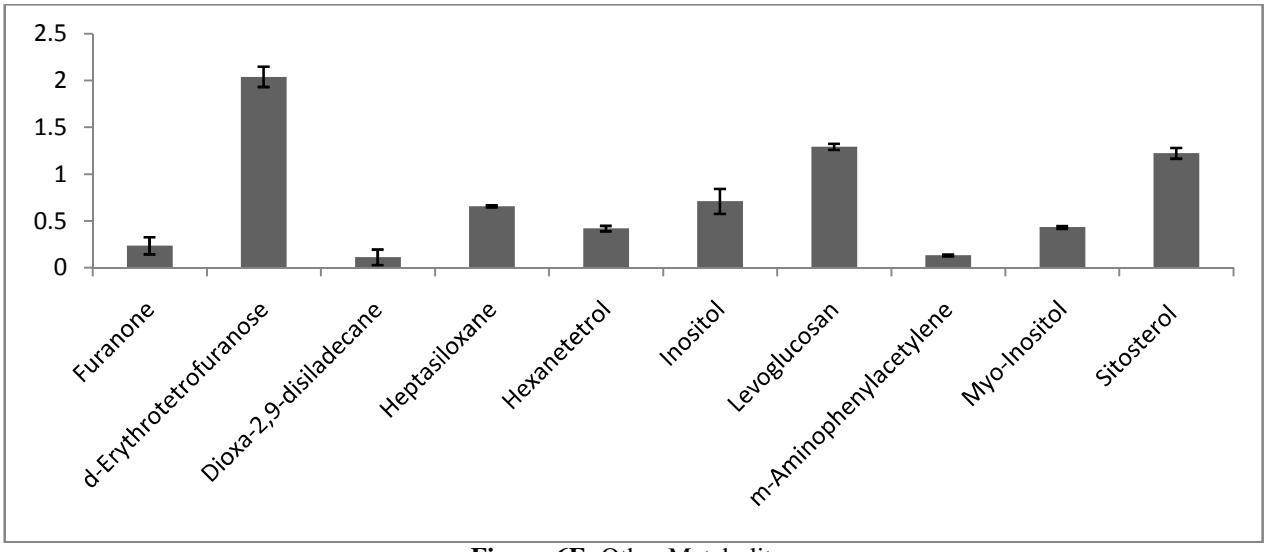

Figure-6F: Other Metabolites

Figure-6: Different metabolites identified in *Fusarium oxysporum* f.sp *ciceri*

Conclusion

From the above study it can be concluded that there are several morphological characters differs in the *Fusarium oxysporum* f.sp *ciceri*. The mode of penetrations of fungal myselium inside the plant vessel, and it blocks the xylem and stem which cause wilting in chickpea. The metabolomic study which provides the primary metabolite constituents of Fusarium and they are responsible for pathogenesity of fungi. This study can provide metabolomic and morphological study to understand pathogenesity of chickpea wilt.

Acknowledgement

We express our sincere gratitude to the pulse research Station, Junagadh Agricultural University for the provision of accession of the chickpea and utilization of chickpea wilt plot. This study was financially supported by the Junagadh agricultural university to carry out post graduate research work.

References

1. Food and Agriculture Organization of the United Nations (2017), http://faostat.fao.org/site/339/default.aspx/10/03/2017

2. Gurjar G., Barve M., Giri A. and Gupta V. (2009). Identification of Indian pathogenic races of Fusarium oxysporum f. sp ciceris with gene specific, ITS and random markers. *Mycologia*, 101(4), 484-495.

3. Haware M.P., Nene Y.L. and Natarajan M. (1996). Survival of *Fusarium oxysporum* f. sp. *ciceri* in the soil in the absence of chickpea. *Phytopathol,* 35(1), 9-12.

4. Leslie J.F. and Summerell B.A. (2006). The Fusarium laboratory manual. Blackwell Publishing Ltd; Oxford, London.

5. Hillis D.M. and Dixon M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic. *The Quarterly Rev. of Biol.,* 66(4), 411-453.

6. Mirete S., Patino B., Vázquez C., Jiménez M., Hinojo M.J., Sodevilla C. and Gonzá lez-Jaénm M.T. (2003). Fumonisin production by Gibberella fujikuroi strains from Pinus species. *Int. J Food Microbiol.,* 89(2), 213-221.

7. Hoque M.A., Ushiyama H., Tomita M. and Shimizu K. (2005). Dynamic responses of the intracellular metabolite concentrations of the wild type and pykA mutant Escherichia coli against pulse addition of glucose or NH3 under those limiting continuous cultures. *Biochem. Eng. J*., 26(1), 38-49.

8. Villas S.G., Roessner U., Hansen M.A.E., Smedsgaard J. and Nielsen J. (2006). Sampling and sample preparation. *Metabolome analysis: an introduction*, 39-82.

9. Sanimah S. and Sarip J. (2015). Metabolomic analysis of *Carica papaya* variety Eksotika and Sekaki. *J. of Tropical Agril. and Food Sci.,* 43(2), 103-117.

10. Roessner U., Cornelia W., Kopka J., Trethewey R.N. and Willmitzer L. (2000). Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. *The Plant Journal,* 23**,** 131-142.

11. Sempere F. and Santamarina M.P. (2009). The conidia formation of several Fusarium species. *Annals of Microbiology*, 59(4), 663-674.

12. Fernández D.J., Landa B.B., Kang S., Jiménez-Díaz R.M. and Navas-Cortés J.A. (2013). Quantitative and microscopic assessment of compatible and incompatible interactions between chickpea cultivars and *Fusarium oxysporum* f. sp. *ciceri* Races. *Plos one,* 8(4). https://doi.org/10.1371/journal.pone.0061360

13. Madla S., Miura D. and Wariishi H. (2012). Optimization of Extraction Method for GC-MS based Metabolomics for filamentous fungi. *J Microbial Biochem Technol,* 4, 5-9. doi:10.4172/1948-5948.1000064

14. Levitz S.M. (2010). Innate recognition of fungal cell walls. *Plos Pathog,* 6(4), e1000758. https://doi.org/10.1371/journal.ppat.1000758

15. Ramesh T. and Kalaiselvam M. (2011). An experimental study on citric acid production by *Aspergillus Niger* using *Gelidiella Acerosa* as a substrate. *Indian J of Microbiol* 51(3), 289-293.

16. Scheuerbrandt G. and Bloch K. (1962). Unsaturated fatty acids in microorganisms. *The J of Biol Chem,* 237(7), 2064-2068.