➔DOI : 10.15740/HAS/AJBS/9.2/251-254
e ISSN-0976-8343 | ■ Visit us : *www.researchjournal.co.in*

Asian Journal of Bio Science, Volume **9** | Issue 2 | October, 2014 | 251-254
Received : 07.08.2014; **Revised :** 03.09.2014; **Accepted :** 15.09.2014

RESEARCH PAPER

Estimation of fusaric acid from culture filtrate of *Fusarium udum* by thin layer chromatography

S.B. SHINDE AND H.V. DESHMUKH

Department of Plant Pathology and Agricultural Microbiology, Mahatama Phule Krishi Vidyapeeth, Rahuri, AHMEDNAGAR (M.S.) INDIA
Email : shindesb123@gmail.com

The investigation was undertaken to estimate fusaric acid from culture filtrate of *Fusarium udum* by using thin layer chromatography. Acidic pH (3.00), temperature 28 to 30°C and 20 days after incubation was found to be optimum for maximum fusaric acid production.

Key words : Thin layer chromatography, *Fusarium udum*, Fusaric acid

How to cite this paper : Shinde, S.B. and Deshmukh, H.V. (2014). Estimation of fusaric acid from culture filtrate of *Fusarium udum* by thin layer chromatography. *Asian J. Bio. Sci.*, **9** (2) : 251-254.

INTRODUCTION

Fusarium wilt is a soil borne disease of pigeonpea. Butler first reported it from Bihar in the year 1906. The disease appears in young seedling first in patches in the field and can extent to the entire field if the pigeonpea is repeatedly cultivated in the same field.

A large number of micro-organisms are known to produce toxic metabolites when cultivated on synthetic media. The pathogen produces fusaric acid toxin that is having phytotoxin properties and its high production has been correlated to virulence of pathogenic strains (Xu *et al.*, 1983 and Chakrabarti *et al.*, 1980).

Fusaric acid (FA) is one of the most important host non-specific toxins produced by several *Fusarium* species (Kern, 1972). Different parameters like pH, temperature and days of incubation also standardized and these parameters plays important role in fusaric acid production.

Fusaric acid has been administered to humans in clinical trials as an antihypertensive agent (Ibrahim *et al*., 2005) in the treatment of Parkinson's disease (Hidaka, 1971; Matta and Wooten 1973) and at dosage rates up to 1200 mg/day in the treatment of drug addiction (Pozuelo, 1976).

RESEARCH METHODOLOGY

Preparation of standard curve for fusaric acid :

An aliquot of 0.00, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg of commercial fusaric acid solution was pipetted out in a series of test tubes in triplicate set. The volume was made one ml with distilled water in each of these test tubes. Then the standard curve was prepared by plotting absorbance against the concentrations of commercial fusaric acid solution.

Estimation of fusaric acid :

Isolation and purification of fusaric acid from culture filtrate :

Grow the organism for 6-7 days in 100 ml of Czapeck's liquid medium in 500 ml conical flask. After seven days collect the culture filtrate and centrifuge at 2000 rpm for 20 min. Use the clear supernatant. Adjust the pH of the culture filtrate (10 L) with 5 N HCl to 4.0 and extract 3 times with ethyl acetate using 1.5 L of the solvent for every extraction. Allow at least 30 min. for each extraction. Pool the ethyl acetate extracts and reduce the volume to 100 ml on a rotary evaporator or hot water bath. Add 50 ml of water, adjust the pH to 8.0 with NaOH and extract with 150 ml of ethyl ether in a Soxhlet extractor for about 24 h. Dry the ether extract for about 2 hrs with 20 g of anhydrous sodium sulphate and distill off the ether. Extract the residue with 100 ml of hot petroleum ether (b.p. 60-90°) using a reflux condenser for five times changing the solvent at 30 min. interval. Combine the petroleum-ether extracts and evaporate on a hot water bath (Crystals of the toxin appear). Dissolve the residue in 1-2 ml

of ethanol.

Detection of fusaric acid :

Fusaric acid isolated from culture filtrates may be detected by paper or thin-layer chromatography Stefan (2005). Spot on Whatman no. 1 filter paper or Thin layer chromatography (TLC) sheet. Develop the chromatogram descendingly for 10-12 hrs in sec-butanol formic acid-water solvent system (75:15:10 v/v). Dry the paper for 14-16 hrs under a hood and spray Dragendorff's reagent or bromophenol blue (BPB). The sensitivity may be increased by adding a few drops of concentration H_2SO_4 to the Dragendor's reagent. Fusaric acid gives orange spot with the reagent while with bromophenol blue (BPB), a yellow colour, since fusaric acid forms a chelate with copper or iron, spraying the paper with 1 per cent copper sulphate (aqueous) followed by BPB.

Time course of fusaric acid production :

100 ml of Czapeck's liquid medium in 250 ml conical flasks were inoculated with 5 mm disc (3 to 4 discs per flask) of 7 days old culture of *Fusarium udum* and incubated at 26°C. Culture filtrate was harvested at 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 days by filtration through Whatman no. 1 filter paper and used for fusaric acid (FA) quantification as previously outlined.

Effect of pH on fusaric acid production :

100 ml of Czapeck's liquid medium was poured into each of the 250 ml flasks and the pH was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 using 1 M NaOH or 1 M HCl. Each pH medium was dispensed into 250 ml conical flasks and all flasks were autoclaved at 121°C for 15 min. The flasks were then inoculated with 5 mm mycelial disc of *F. udum* and were incubated at 26 $\pm$ 2°C for 19 days in static condition. Clear filtrate was used for fusaric acid (FA) quantification as previously outlined.

Effect of temperature on fusaric acid production :

100 ml of Czapek's liquid medium was poured into each of the 250 ml conical flasks were incubated with 5 mm disc of 7 days old culture of *F. udum* and incubated at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50°C for 19 days. Culture filtrate was harvested after 19 days of incubation by filtration through Whatman no. 1 filter paper and used for fusaric acid (FA) quantification as previously outlined.

RESEARCH FINDINGS AND ANALYSIS

The findings of the present study as well as relevant discussion have been presented under the following heads:

Time course of fusaric acid production :

Data presented in Table 1 and revealed that extraction of fusaric acid from culture filtrates showed increase in fusaric acid gradually from 4th day (60 µg/lit) of incubation and reached the maximum at 20th day (606 µg/lit) and later on it remained constant. These findings were confirmed by qualitative and quantitative methods by using thin layer chromatography (TLC) and spectrophotometric analysis at 254 nm. As the incubation time increased, the fusaric acid level in the medium also increased . These results indicates that the fungal culture filtrate should be used after 20 days of incubation for *in vivo* and *in vitro* studies.

Table 1 : Fusaric acid production by *F. udum* at different days of incubation

Sr. No.	Days of incubation	Fusaric acid production (µg/lit.)			
		I	II	III	Mean
1.	4	60	60	60	60
2.	6	120	125	135	126.66
3.	8	310	260	305	291.66
4.	10	335	315	335	328.33
5.	12	325	395	395	371.66
6.	14	365	420	420	401.66
7.	16	455	485	465	468.33
8.	18	550	550	550	550.00
9.	20	560	615	645	606.66
10.	22	570	600	650	606.66
11.	24	600	685	635	606.66
12.	26	570	615	635	606.66
13.	28	570	615	635	606.66
14.	30	560	595	645	600.00
	S.E. ±				17.71
	C.D.(P=0.05)				51.32
	C.V. (%)				06.85

These findings are in agreement with Durate and Archer (2003) who reported that defined culture medium such as Czapeck-Dox induce greater production of toxic metabolite (fusaric acid) than the potato sucrose medium. Toxic metabolite (fusaric acid) on Czapeck-Dox reached a peak after 20 days of static incubation at 25°C and declined subsequently. The fusaric acid production was intentionally studied to establish a relation between the growth of fungus and toxin production and it found to be positively related with each other.

The present findings are in line with the work of Bacon *et al.* (1996) who surveyed 78 strains of *Fusarium moniliforme, F. crookwellense, F. subglutinans, F. sambucinum, F. napiforme, F. heterosporum, F. oxysporum, F. solani* and *F. proliferatum* for their ability to produce fusaric acid and reported that the fungi produces maximum fusaric acid 15 DAI.

Parmar *et al*. (2010) extracted fusaric acid from 100 ml of culture filtrate and observed increase in fusaric acid production gradually from 3 DAI (2.5 mg), reached the maximum at 10 DAI (6.5 mg) and declined later. These findings are not in agreement with the present findings.

Fusaric acid production by *F. udum* at different pH levels :

The effect of pH of medium on fusaric acid production are shown in Table 2. The pH level of the growth medium was found to influence the growth rate and fusaric acid production by *F. udum*.

Table 2 : Fusaric acid production by *F. udum* at different pH of growth medium

Sr. No.	pH	Fusaric acid production (µg/lit.)			
		I	II	III	Mean
1.	3.0	605	625	620	616.66
2.	3.5	545	565	555	555.00
3.	4.0	465	460	460	461.66
4.	4.5	420	405	420	415.00
5.	5.0	360	365	355	360.00
6.	5.5	355	345	345	348.33
7.	6.0	350	345	345	346.66
8.	6.5	355	345	350	350.00
9.	7.0	345	345	350	346.66
10.	7.5	255	260	250	255.00
11.	8.0	180	195	195	190.00
12.	8.5	160	205	180	181.66
	S.E. ±				05.27
	C.D. (P=0.05)				15.38
	C.V. (%)				02.47

The pH of the growth medium was found to influence the growth rate and fusaric acid production by *F. udum*. The acidic pH range was observed to be favourable or desirable for the growth of the fungi and also the fusaric acid production. The data revealed that maximum fusaric acid production (616.66 µg/li.) was observed around acidic pH at pH 3.00. With increase in the pH level towards the alkalinity, the fusaric acid production found to be decreased with least production of fusaric acid 181.66 µg/li. at pH 8.5. While at neutral pH level (6.0 to 7.0) the fusaric acid production was remained to be nearly constant.

The fungus could grow on medium having wide pH range than desirable one or neutral pH but the growth and fusaric acid production of the fungus was decreased with increase in pH of the medium. It was also observed that fungus could grow from pH 2.0 to 9.0 indicating survival of the fungus at all pH. It is noteworthy to mention that the fungal mycelium has got ability to grow at 2.0 to 9.0 pH level but could not produce fusaric acid at highly alkaline pH.

This results indicates that optimum pH of the medium should be 3.0 to have maximum fusaric acid production. These findings are in conformity with the work done by Nachmias *et al.* (1987) who reported that a change in pH before the media sterilization led to an increase in fusaric acid production, peaking at pH 3.00, suggesting that a higher initial pH of the culture medium may stimulate the production of toxic metabolites.

Nachmias *et al.* (1987) and Patel *et al.* (1987) adjusted culture media to pH 6.7 and 7.00 and used this culture media to promote production of *Verticillium dahlia* and *Curvularia lunata* toxins, respectively and reported that the optimum pH for toxin production was 5.00. These findings are in line with those obtained from present findings.

Onesirosan *et al*. (1975) also reported that the optimum pH for *Corynespora cassiicola* toxin production is 6.00 to 7.00 and toxin production was markedly reduced at 5.5 or 8.00 pH. These findings are in contrary to those obtained from present findings.

Fusaric acid production by *F. udum* at different temperature levels :

The data presented in Table 3 revealed that the temperature plays important role in fusaric acid production from the culture filtrate of *F. udum*. The most suitable temperature for maximum fusaric acid production was 28 to 30°C. The temperature below or above this range reduced the production of fusaric acid. The production of fusaric acid at 28 to 30°C was around 306.66 µg/li. Whereas, at 35°C temperature it was 246.66 µg/lit, at 40°C it was 108.33 µg/li and at 45°C temperature 55.00 µg/li. Temperature above 45°C and below 5°C did not allowed to fungus to produce fusaric acid at all.

Table 3 : Fusaric acid production by *F. udum* at different temperature levels

Sr. No.	Temperature(^{0}C)	Fusaric acid production (µg/lit.)			
		I	II	III	Mean
1.	0	0	0	0	0
2.	5	60	60	60	60.00
3.	10	155	155	150	155.33
4.	15	165	160	160	161.66
5.	20	200	205	215	206.66
6.	25	250	260	255	255.00
7.	30	305	315	300	306.66
8.	35	250	245	245	246.66
9.	40	105	115	105	108.33
10.	45	50	60	55	55.00
11.	50	0	0	0	0
	S.E.±				02.61
	C.D. (P=0.05)				07.65
	C.V. (%)				03.20

Raghuwanshi (1994) studied the high performance liquid chromatography (HPLC) of the root exudates and observed that temperature affect the flavonoid production by host, both qualitatively and quantitatively. In root exudates of the pigeonpea five peaks were detected at 30°C with retention times of 5.0, 6.0, 10.0, 11.0 and 12.5 min, whereas, at 37°C an additional peak corresponding to a retention time 9 min was also detected. Like the pigeonpea the relative proportion of the flavonoid decreased at higher temperature. Thus, the higher temperature changed the spectrum of flavonoids exuded by the pigeonpea but had no effect on the production of flavonoid in mungbean. These findings are in contrary to those obtained from present findings.

LITERATURE CITED

Bacon, C.W., Porter, J.K., Norred, W.P. and Leslie, J.F. (1996). Production of fusaric acid by *Fusarium* species. *Appl. & Environ. Microbiol.*, **62** (11) : 4039-4043.

Chakrabarti, O.K. and Basuchaudhary, K.C. (1980). Correlation between virulence and fusaric acid production in *Fusarium oxysporum f.sp. earthami Pnytopetbotoqie Z.*, **99**: 43-46.

Durate, M.L.R. and Archer, S.A. (2003). *In vitro* toxin production by *Fusarium solani* f. sp. *piperis*. *Phytopathologia brasileria*, **28** : 229-235.

Hidaka, H. (1971). Fusaric (5-butylpicolinic) acid, an inhibitor of dopamine beta-hydroxylase, affects serotonin and noradrenaline. *Nature*. **231** : 54-55.

Ibrahim, H.M., Yusoff, W.M.W., Hamid, A.A., Illias, R.M., Hassan, O. and Omar (2005). Optimization of medium for the production of β-cyclodextrin glucanotransferase using Central Composite Design (CCD). *Proc. Biochem.*, **40** : 753-758.

Kanniyan, J., Nene, Y.L., Reddy, M.V., Ryan, T.G. and Raju, T.N. (1984). Prevalence of pigeonpea diseases and associated crop losses in Asia, Africa and America. *Tropical Pest Mgmt.*, **30** (1) : 62-71.

Kern, H. (1972). Phytotoxine produced by fusaria. In : Wood, R.K.S., Balili, A. and Granidi, A. (Eds.). *Phytotoxins plant disease*. Academic Press, New York. pp. 35-48.

Matta, R.J. and Wooten, G.F. (1973). Pharmacology of fusaric acid in man. *Clin. Pharmacol. Ther.*, **14**(4) : 541–546.

Nachmias, S., Buchner, V., Tsror, L., Burstein, Y. and Keen, N. (1987). Differential phytotoxicity of peptides from culture fluids of *Verticillium dahlia* races 1 and 2 and their relationship to pathogenecity of the fungi on tomato. *Phytopathol.*, **77** : 506-510.

Nene, Y.L., Sheila, V.K. and Sharma, S.B. (1989). A world list of chickpea (*Cicer arientinum* L.) and pigeonpea [*Cajanus cajan* (L.) Millsp.] pathogens. Legumes pathology progress report-7 Patancheru, A.P. (INDIA), ICRISAT. 23 pp.

Onesirosan, P., Mabuni, C.T., Durbin, R.D., Morin, R.B., Rich, D.H. and Arny, D.C. (1975). Toxin production by *Corynespora cassiicola. Physiol. Plant Pathol.*, **5** : 295-298.

Patel, J.D., Patil, M.R. and Sapkal, P.N. (1987). Toxic effect of culture filtrate of *Curvularia lunata* (Wakker) Boedijin and *Alternaria tenuis* Auct. *PKV. Res. J.*, **11** : 189.

Parmar, Pritesh, Oza, V.P. and Subramanian, R.B. (2010). Optimization of fusaric acid production by *Fusarium oxysporum* f.sp. *lycopersici* using response surface methodology. *Indian J. Sci. & Tech.*, **3** (4) : 411-416.

Pozuelo, J. (1976). Suppression of craving and withdrawal in humans addicted to narcotics or amphetamines by administration of alpha-methylpara- tvrosine (AMPT) and 5-butylpicolinic acid (*fusaric acid*). *Cleve. Clin. Q.*, **43** : 89-94.

Raghuwanshi, A., Dudeja, S.S. and Khurana, A.L. (1994). Effect of temperature on flavonoid production in pigeonpea [*Cajanus cajan* (L.) Millsp] in relation to nodulation. *Biol. Fertil. Soils*, **17** : 314-316.

Stefan, E. (2005). Thin-layer chromatography-an appropriate method for fusaric acid estimation. *Biologia Bratislava*, **60**(1) : 104.

Xu, X.H., Gu, G.F. and Shen, F.D. (1983). Studies on the relationship between fusaric acid and cotton fusariam wilt disease. *Alta. Agric.* Univen Pekinensis, **9** : 29-35.