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Fusaric Acid Production by Different Isolates of *Fusarium oxysporum* f. sp. *ciceris* and Effect of Selected Culture Filtrate Containing Fusaric Acid on Dynamics of Plant Pigments in Chickpea

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ABSTRACT

Background: Biochemical variation with respect to fusaric acid (FA) production by various isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc) was not known and the effect of FA on the pigment dynamics of chickpea was not studied earlier.

Methods: The quantity of FA production by 14 isolates of Foc was estimated through HPLC. Further, the effect of culture filtrate (CF) containing FA of ITCC 7682 and ITCC 7681 on the dynamics of plant pigments was studied on resistant cv. WR315 and susceptible cv. JG 62 under Phytotron growing conditions during 2019-2021.

Result: Isolate ITCC 7681 was recorded to produce the lowest quantity of FA (126.88±5.56 mg L^{-1}); whereas isolate ITCC 7682 produced the highest quantity of FA (820.51±13.37 mgL^{-1}). Isolates ITCC 7682 and ITCC 7680 were recorded to be highly virulent initiating wilt symptoms within 19-24 hours of inoculation in chickpea seedlings of susceptible cv. JG 62. Culture filtrates of ITCC 7682 and ITCC 7681 caused a reduction of chlorophyll a (15.13-72.38% and 9.24-72.38%), chlorophyll b (16.28-89.83% and 13.95-97.74%), total chlorophyll (48.68-73.17% and 38.87-70.33%) and carotenoid (58.33-88.23% and 48.14-82.56%) contents 3 and 6 days after inoculation respectively. Reduction of all these pigments was significantly higher in susceptible cv. JG 62 than resistant cv. WR 315.

Key words: Chickpea wilt, Fusaric acid, *Fusarium oxysporum* f. sp. *ciceris*, Pigments, Toxin.

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc) is one of the most important biotic stresses after *Ascochyta* blight for low yield of chickpea in India. Chickpea wilt has been documented in all chickpea-growing states of India, with an incidence ranging from 14 to 32 percent and a total yearly loss of up to 10 percent (Dubey *et al.,* 2010). Various species of *Fusarium* have been known to produce non-host specific toxin, fusaric acid (Selim and El-Gammal, 2015; Shinde and Deshmukh, 2014; Bani *et al.,* 2014). FA, a phytotoxin, has been reported to involve in wilt disease development. Chemically FA, a picolinic acid derivative, was the first fungal phytotoxin identified from diseased host plants (Gäumann, 1957). Toxins have been recognized as key components in the progression of the illness. Toxin insensitivity can be used as a criterion for screening pathogen-resistant germplasm (Gengenbach *et al.,* 1977).

Worldwide, several investigators have tried to differentiate various isolates of *Fusarium oxysporum* f. sp. *ciceris* into pathogenic races based on morphological and molecular characteristics along with their pathogenic reactions in chickpea differential hosts. But none of them truly studied the quantitative variation in the production of FA by various isolates/races of chickpea wilt pathogen either *in vivo* or *in vitro*, which play a significant role in the wilt pathogenesis. Further, there is a lack of information on the effect of crude extract of toxin produced by the chickpea wilt pathogen on the plant pigments of chickpea in susceptible and resistant cultivars. Based on the above research gap, the present investigation is formulated with the objective of quantitative estimation of FA produced by different isolates of Foc *in vitro* and the effect of FA contained in the CF on the plant pigments with special reference to chlorophyll and carotenoid contents in chickpea seedlings.

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MATERIALS AND METHODS

Estimation of FA production by different isolates of Foc in culture filtrate

A total of fourteen isolates of *Fusarium oxysporum* f. sp. *ciceris* (Table 1) were used in this study. The entire study was conducted in the Division of Plant Pathology and partly in the Division of Agriculture Chemicals, Indian Agricultural Research Institute, Pusa, New Delhi 110 012 during 2019-2021.

Culturing of the isolates

Each of the 14 isolates of *F. oxysporum* f. sp. *ciceris* was grown in 250 ml of sterile Czapek-Dox broth liquid medium separately in 500 ml conical flasks which were inoculated with 5 mm disc (1 disc per flask) of 7 days old culture of Foc. Three replications were maintained for each of the 14 isolates and incubated for 21 days at 25±2°C.

Estimation of fusaric acid

Extraction of FA from Foc culture filtrate was carried out following Pritesh *et al.* (2010). Fusaric acid from different isolates of Foc was estimated using an HPLC (Alliance, Waters Crop., Milford, Mass., USA), equipped with an e2695 quaternary pump, 20 µL loop auto-injector, 2998 photodiode array detector (PDA). The retrieved data were analyzed using the software program “Empower 2”. The stationary phase, C18 column (Hypersil ODS; 250 mm × 4.6 mm × 5µ, Thermo Fischer Scientific, USA) was used for the separation of FA using a gradient solvent system consisting of, solvent A: H_2O acidified with 0.1% FA and solvent B: CAN with 0.1% FA, v/v. Initially, the gradient system started with 90% A for 2 min, then decreased to 20% in 18 min, followed by an increase to 90% in the next 2 min. The flow rate of the gradient phase was maintained at 0.5 mL/min. The total run time for the analysis was 20 min. The detector response was recorded at 270 nm. Three replications were maintained for each isolate. Spectral analysis was conducted to compare the detected peaks within similar retention times in all samples with a spectral pattern of pure FA standard (Sigma Aldrich, USA). FA accumulation was determined by measuring the area under the curve at 270 nm (optimal wavelength) with the diode array detector (DAD).

Inoculation of chickpea seedlings with CF

The seeds of two chickpea cvs. WR 315 and JG 62 were procured from the Division of Genetics and Plant Breeding, IARI, New Delhi, surface sterilized with 1% sodium hypochlorite for 2 min, rinsed in sterile water and then soaked in distilled water for 24 hrs. Later, the seeds were allowed to germinate for two weeks following the paper roll method.

Each CF of the 14 isolates was taken into three different concentrations such as 100%, 50% and 25% separately. 15 days old seedlings of JG 62 were transferred to the test tubes containing 15 ml of CFs of 100, 50 and 25% concentrations of each isolate. One chickpea seedling was put in each test tube. Five replications were maintained for each treatment. Time (hours) taken by CF of different isolates at different concentrations to initiate/produce wilting/drooping symptoms were observed and recorded for virulence study.

Effect of FA contained in culture filtrate of Foc on pigment dynamics of chickpea seedlings

The CF of isolates ITCC 7682 and ITCC 7681 which were estimated to produce the highest and lowest amount of FA respectively were taken to further study their effects on pigment dynamics in chickpea seedlings. The 25% concentration of crude CF of isolates ITCC 7682 and ITCC 7681 was prepared by adding sterile distilled water in the ratio of 1:3. A commercially available FA standard (Sigma Aldrich, USA) was procured and 25 ppm concentration was used as a positive control. Therefore, T_1 = 25% concentration of CF of the highest FA producing and highly aggressive Foc isolate ITCC 7682; T_2 = 25% concentration of CF of the least FA producing and least aggressive Foc isolate ITCC 7681; T_3 = Positive control with 25 ppm concentration of commercial FA standard; T_4 = Negative control with sterilized distilled water.

Seven days old seedlings were carefully transferred into 60ml sterilized tubes (two seedlings/tube) filled with 40 ml Hoagland’s solution (Garland, 1992) and were allowed to grow for a week under proper growth conditions (18-20°C, 70-80% RH) at National Phytotron Facility, IARI, New Delhi. Then, Hoagland’s solution in the tube was replaced with different treatments as mentioned above. So, 15 days old chickpea seedlings were given treatments with 25% CF of Foc isolates ITCC 7682, ITCC 7681 and 25 ppm conc. of commercial FA. Treatments were given to both WR 315 and JG 62 seedlings with three replications.

Samples were collected separately for each treatment from WR 315 and JG 62. The samples were collected at 1, 3 and 6 days after inoculation (Dai). Three replications were maintained for each treatment for both resistant and susceptible cultivars.

Estimation of chlorophyll contents

Chlorophyll extraction was carried out following the method described by Manolopoulou *et al.* (2016) using the modified dimethyl sulfoxide (DMSO) method. 0.025 g of leaf sample from all the treatments at respective intervals were collected and placed separately in test tubes containing 5 ml DMSO and allowed to incubate at 60-65°C for 1 hour. Absorbance was measured at 645 nm and 663 nm in Nanodrop Spectrophotometer. A clear DMSO without any leaf samples was used as blank. Determination of chlorophyll a, chlorophyll b and total chlorophyll were carried out following Manolopoulou *et al.* (2016) expressed as mg g^{-1} fresh weight.

Estimation of carotenoid contents

Leaf samples (~ 0.025 g) from all the treatments at respective intervals were collected and placed separately in test tubes containing 5ml DMSO and allowed to incubate at 60-65°C

for 1 hour. Absorbance was measured at 470 nm in Nanodrop Spectrophotometer. A clear DMSO without any leaf samples was used as blank.

Standard statistical calculations like Duncan multiple tests *etc.* were done wherever required following SPSS Software 16.0 version.

RESULTS AND DISCUSSION

Quantitative estimation of FA production by various isolates of Foc

Analysis of HPLC data indicated (Table 1) that *in vitro* productions of FA in Czapek Dox medium by different isolates of Foc were variable (126.88-820.51 mg L^{-1}). Among them, New Delhi isolate (ITCC 7681) was recorded to produce the lowest quantity of FA (126.88±5.56 mg L^{-1}); whereas Sri Ganga Nagar isolate (ITCC 7682) produced the highest quantity of FA (820.51±13.37 mgL^{-1}) followed by Udaipur isolate ITCC 7680 (750.05±2.24 mgL^{-1}), Satara isolate ITCC 7688 (663.31±14.58 mgL^{-1}) and Dholi isolate ITCC 7687 (616.75±17.55 mgL^{-1}). Production of FA by Alwar isolate ITCC 7675 (520.3±22.05 mg L^{-1}), Jaipur isolate ITCC 7674 (537.9±6.20 mg L^{-1}) and Jabalpur isolate ITCC 7692 (542.22±14.22 mg L^{-1}) were statistically at par but higher than Ludhiana isolate ITCC 7679 (296.45±7.18 mg L^{-1}), Hisar isolate ITCC 7678 (311.51±3.01 mg L^{-1}), Rewa isolate ITCC 7693 (321.48±4.15 mg L^{-1}) and Sikohpur isolate ITCC 7677 (323.72±11.91 mg L^{-1}) which were again at par among themselves. Guntur isolate ITCC 7689 (393.77±8.25 mg L^{-1}) and Raichur isolate ITCC 7690 (459.58±24.34 mg L^{-1}) were also recorded to produce a significant quantity of FA.

It was observed (Table 1) that isolate ITCC 7681 which exhibited the lowest quantity of FA (126.88±5.56 mgL^{-1}) took 110.4, 156 and 204 hrs time to exhibit wilt symptoms with 100, 50 and 25% conc. of CF respectively. Two isolates ITCC 7682 (820.51±13.37 mg L^{-1}) and ITCC 7680 (750.05±2.24 mg L^{-1}) were recorded to produce a significantly higher quantity of FA, took 19.2-24.0, 69.6-76.8 and 96.0-98.4 hrs to exhibit wilt syndromes with 100, 50 and 25% conc. of CF respectively. Isolates ITCC 7679, ITCC 7678, ITCC 7693 and ITCC 7677 which produced a moderate quantity of FA, showed wilting syndrome between 88.0-98.0, 15-141 and 141-180 hrs with 100, 50 and 25% conc. of CF respectively. Interestingly, isolates ITCC 7675, ITCC 7674, ITCC 7692, ITCC 7687 and ITCC 7688 which produced FA between 500-700 mgL^{-1} required 43.2-69.6, 74.4-98.4 and 93.6-105.6 hrs for wilting syndrome initiation with 100, 50 and 25% conc. of CF respectively. In general, it was found that isolates that secreted the maximum quantity of FA in CF took minimum time for wilt initiation and vice versa indicating the quantity of FA production was found to be negatively correlated with the time taken to initiate wilt syndrome. Here, Sri Ganga Nagar (ITCC 7682) and Udaipur (ITCC 7680) appeared to be highly virulent initiating wilt symptoms within 19-24 hours of inoculation in 100% conc. of FA. There is a definite correlation between the quantities of *in vitro* FA production and virulence or aggressiveness among the Foc isolates. In *Fusarium* wilt of tomato, Selim and El-Gammal (2015) demonstrated that all pathogenic isolates of *Fusarium oxysporum* f. sp. *lycopersici* varied in *in-vitro* production of FA. The isolates which secreted higher quantities of FA expressed severe disease in 4 weeks; conversely isolates producing lower quantities of FA took 6 weeks to express the severe disease. Bani *et al.* (2014) also observed variation in FA production by strains of *Fusarium oxysporum* f. sp. *pisi in vitro* and the pea leaf lesion size was found positively

Table 1: Estimation of FA production by different isolates of Foc in culture filtrate and their correlation with wilt syndrome initiation in chickpea cv. JG 62.

Isolate	Origin of isolate	#Estimated fusaric acid (mgL^{-1}) production	§Mean time (in hours) taken to show wilting symptoms		
			100% conc. of CF	50% conc. of CF	25% conc. of CF
ITCC 7674	Jaipur, Rajasthan	537.90±6.2^{e}	67.2^{cd}	88.8^{cdef}	103.2^{ef}
ITCC 7675	Alwar, Rajasthan	520.3±22.05^{e}	69.6^{cd}	98.4^{cde}	100.8^{ef}
ITCC 7677	Sikohpur, Haryana	323.72±11.91^{b}	88.8^{abc}	115.2^{bc}	141.6^{cd}
ITCC 7678	Hisar, Haryana	311.51±3.01^{b}	88.8^{abc}	141.6^{ab}	160.8^{bc}
ITCC 7679	Ludhiana, Punjab	296.45±7.18^{b}	98.4^{ab}	134.4^{ab}	180.0^{ab}
ITCC 7680	Udaipur, Rajasthan	750.05±2.24^{h}	24.0^{fg}	76.8^{ef}	96.0^{f}
ITCC 7681	IARI, New Delhi, Delhi	126.88±5.56^{a}	110.4^{a}	156.0^{a}	204.0^{a}
ITCC 7682	Sri Ganga Nagar, Rajasthan	820.51±13.37^{i}	19.2^{g}	69.6^{f}	98.4^{f}
ITCC 7687	Dholi, Bihar	616.75±17.55^{f}	57.6^{de}	84.0^{def}	93.6^{f}
ITCC 7688	Satara, Maharashtra	663.31±14.58^{g}	43.2^{ef}	74.4^{ef}	105.6^{ef}
ITCC 7689	Guntur, Andhra Pradesh	393.77±8.25^{c}	81.6^{bc}	100.8^{cde}	129.6^{de}
ITCC 7690	Raichur, Karnataka	459.58±24.34^{d}	81.6^{bc}	105.6^{cd}	122.4^{def}
ITCC 7692	Jabalpur, Madhya Pradesh	542.22±14.22^{e}	55.2^{de}	74.4^{ef}	110.4^{ef}
ITCC 7693	Rewa, Madhya Pradesh	321.48±4.15^{b}	96.0^{ab}	146.4^{a}	146.4^{cd}

#Mean±SE values for FA accumulation by different Foc isolates; §Mean time (hrs) taken to initiate wilt syndrome in different concentration. Different letters (a-i) denote significant differences ($p<0.05$).

correlated with the concentration of FA. The development of typical wilt symptoms was confirmed in cotyledons and lower leaves of tomato seedlings when roots are treated with FA (Lopez-Diaz *et al.,* 2018). A similar result was also reported by Wu *et al.* (2008) in watermelon seedlings which corroborates the results of our present study.

Effect of FA contained in culture filtrate of Foc on pigment dynamics of chickpea seedlings

Chlorophyll content dynamics

Culture filtrates of ITCC 7682 and ITCC7681 and pure FA did not show a consistent or definite trend in effect on the content of chlorophyll at 1 Dai in both resistant and susceptible cultivars (Fig 1). A decrease in chlorophyll contents started at 3 Dai and continued till 6 Dai in all treatments in both WR 315 and JG 62. In general, the reduction of chlorophyll a, chlorophyll b and total chlorophyll were more in susceptible cv. JG 62 as compared to resistant cv. WR 315 in both 3 and 6 Dai. The highest reduction of chlorophyll contents (chlorophyll a, b and total) was recorded at only 6 Dai. Further, maximum reduction of chlorophyll a (72.38%), chlorophyll b (89.83%) and total chlorophyll (73.17%) were recorded in susceptible cv. JG 62 at only 6 Dai with the CF of ITCC 7682. However, reductions were 68.85, 38.18 and 64.49% in chlorophyll a, chlorophyll b and total chlorophyll respectively in resistant cv. WR 315 for the corresponding period with CF of ITCC 7682. There were also considerable reductions of chlorophyll contents (chlorophyll a, b and total) in both WR 315 and JG 62 at 3 Dai with CF of ITCC 7681 which contained approximately ~31.72 mgL^{-1} fusaric acid in 25% diluted filtrate (Fig1). Pure FA standard @ 25 mgL^{-1} also showed a substantial reduction in chlorophyll a (71.90%), chlorophyll b (94.92%) and total chlorophyll (67.0%) in JG 62 at 6 Dai, whereas, reductions were 0.55, 40.00 and 52.90% in chlorophyll a, chlorophyll b and total chlorophyll in WR 315 after 6 Dai. The highest reduction of chlorophyll contents was inflicted by CF of ITCC 7682 (6 Dai) which contained approximately 205.12 mgL^{-1} FA in 25% concentration as per the chromatogram data of HPLC analysis. Therefore, it is concluded that exposure of chickpea seedlings to FA which is phytotoxic to plants causes a reduction in chlorophyll contents in chickpea seedlings and the reduction in chlorophyll content is time and concentration-dependent. The findings of the current study are in conformity with watermelon wilt caused by *Fusarium oxysporum* f. sp. *niveum* (Wu *et al.,* 2008) and chickpea wilt (Khan *et al.,* 2004).

Carotenoid dynamics

It was observed that carotenoid content decreased in all treatments for all three intervals in both WR 315 and JG 62 with respect to the control. The reduction of carotenoid content (Fig 2) was found to slow down with the increased exposure time. The maximum reduction in carotenoids was 76.92% in WR 315 and 88.23% in JG 62 at 1 Dai with CF of ITCC 7682, but the highest reduction of carotenoid was 82.56% in JG 62 followed by a 50% reduction in WR 315 at 1 Dai with CF of ITCC 7681. The reduction of carotenoid content was 23.07, 18.51 and 20.68% in cv. WR 315 and 47.056, 42.85 and 25.00% in cv. JG 62 at 1, 3 and 6 Dai respectively with pure FA standard. The decrease in carotenoid content was more with the treatment of CF of ITCC 7682 and ITCC 7681 where FA concentrations were ~205.12 mgL^{-1} and ~31.72 mgL^{-1} respectively in 25% diluted filtrate. Overall reductions in carotenoid content were higher in cv. JG 62 than cv. WR 315 in all treatments. In the present study, the rate of reduction of carotenoids gradually slowed down with the increase of time of exposure of chickpea roots to CF with a higher concentration of FA. Exposure of chickpea root to the high concentration of FA for a long period of 3-6 days might have caused cell death and chlorophyll destruction, thereby synthesis of carotenoid content decreased with time. Similar results were reported in the *Fusarium* wilt of tomato (Singh *et al.,* 2017).

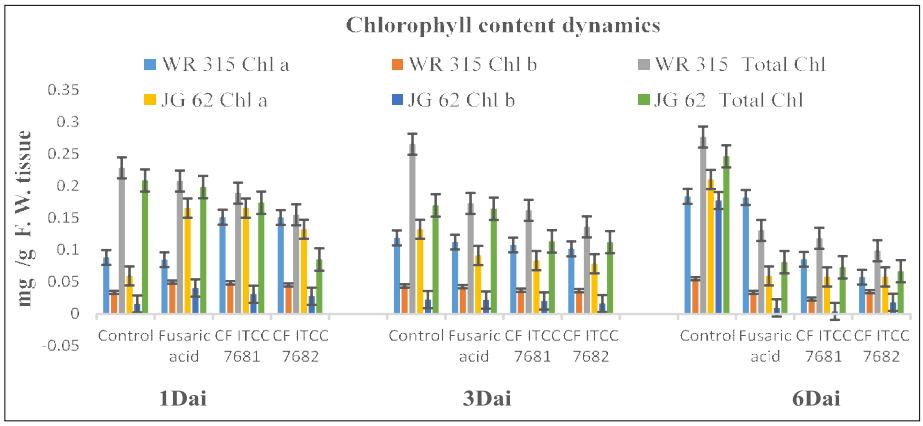

Fig 1: Effect of FA contained in culture filtrate on chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Total Chl) content in resistant cv. WR 315 and susceptible cv. JG 62.

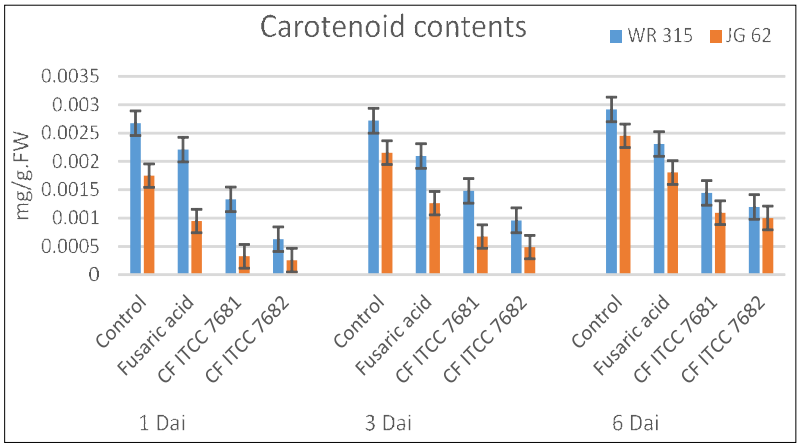

Fig 2: Effect of FA contained in culture filtrate on the carotenoid pigment in resistant cv. WR 315 and susceptible cv. JG 62.

CONCLUSION

Different isolates of Foc vary in their ability to synthesize FA in CF. The quantity of FA present in the CF has a direct correlation with the wilt syndrome initiation. The isolates which can produce a higher quantity of FA are more virulent and exhibit early wilting and vice versa. In the present study, Fusaric acid, a phytotoxin, has showed to reduce plant pigments such as chlorophyll a, chlorophyll b and total chlorophyll and carotenoid contents. This may be due to the degradation of chlorophyll or inhibition of chlorophyll synthesis leading to the development of yellowing or chlorosis which is the most prominent characteristic of wilting. Further, the reduction of plant pigment contents was higher in susceptible cv. JG 62 than resistant cv. WR 315 indicating the sensitivity of JG 62 to FA is more than that of WR 315. Chickpea cultivars less sensitive to the high concentration of fusaric acid could be used in the breeding programme for the development of resistant chickpea varieties for specific virulent race-dominated areas.

Conflict of interest: None.

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