



PHYTOTOXINS AND THEIR ROLE IN DEVELOPMENT OF *Fusarium* WILT IN CHICKPEA

SUBRAMANIAM GOPALAKRISHNAN* AND VADLAMUDI SRINIVAS

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Telangana, India.

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ABSTRACT

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Phytotoxins are low molecular weight metabolites produced by plant pathogens that cause obvious damage to plant tissues and are known to be involved in plant disease. Phytotoxins from various formae speciales of *Fusarium oxysporum* such as fusaric acid from the banana pathogen *F. oxysporum* f. sp. *cubense*, beauvericin from the muskmelon pathogen *F. oxysporum* f. sp. *melonis*, and bikaverin and norbikaverin from the cotton pathogen, *F. oxysporum* f. sp. *vasinfectum* have been reported to cause wilt symptoms in their host plants. *Fusarium* wilt, caused by *Fusarium* sp. one of the serious disease of chickpea, is responsible for losses up to 100 per cent when conditions favour the disease. Chlorosis and wilting are common symptoms on the chickpea plants infected with *Fusarium* sp. These symptoms suggest that phytotoxins are involved in the *Fusarium* wilt disease of chickpea. Filtrates from cultures of *Fusarium acutatum*, caused permanent wilting of chickpea cuttings and killed cells in a bioassay. The phytotoxin from the culture filtrate was identified as 8-O-methyl-fusarubin. Knowledge of such phytotoxic metabolites provides insights into disease syndromes and may be exploited by conventional and molecular breeding to obtain crops resistant to plant disease.

KEYWORDS: Phytotoxins, *Fusarium* wilt, chickpea, disease development

INTRODUCTION

Chickpea (*Cicer arietinum* L) is the third most important pulse crop after bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) on a world basis but of first importance in the Mediterranean basin and South Asia. It is grown in 33 countries on an area of about 11.5 million hectares (Bidyarani *et al.*, 2016) and India accounts for about 65 per cent of the world's chickpea production (FAOSTAT, 2014). Cultivated chickpea are divided into two major groups "desi" and "kabuli". Chickpea seed is mainly used as food because of its high protein (12-31 %) and carbohydrate (52-71 %) contents (Awasthi *et al.*, 1991). Global yields of chickpea (968 kg ha⁻¹) have been relatively stagnant (FAOSTAT, 2013) for the last five decades in spite of using various advanced and molecular breeding approaches, extensive use of synthetic fertilizers and pesticides that in addition created environmental and health concerns. Productivity may be considerably improved if the adverse effects of abiotic and biotic stresses are reduced. The major abiotic stresses are cold, heat and drought. Of these, drought is the major limiting factor as chickpea is grown on residual soil moisture as a post-rainy season crop. Advancing sowing dates in certain regions can alleviate the effect of moisture stress and thereby increasing the yield. Chickpea is also sensitive

to salinity and this is an important problem in India and Pakistan. Numerous studies have shown that soil salinity inhibits legume growth and development and decreases nodulation and nitrogen fixation (Mensah and Ihenyen, 2009; Egamberdieva *et al.*, 2013). Salt tolerant genotypes are available but these lines do not generally yield well. Lowering the water table through improved drainage can be effective in areas where it is high (ICRISAT, 1997).

Chickpea suffers from about 172 pathogens consisting of fungi, bacteria, viruses and nematodes of which 38 are soil-borne. *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *Ciceri* (FOC) are the most serious and are responsible for wet root rot, collar rot and wilt, respectively, and cause losses as high as 60 to 70 per cent when conditions favour disease (Anjaiah *et al.*, 2003). The foliar diseases which may damage chickpea are blight caused by *Ascochyta rabiei* and grey mould caused by *Botrytis cinerea*. Bacterial blight caused by *Xanthomonas cassiae* was also found damaging in India (Nene, 1980). Important viral diseases include stunt, chlorosis and dwarfing, mosaic, proliferation and necrosis caused by Pea Leaf Roll Virus, Chickpea Chlorotic Dwarf Virus, Alfalfa Mosaic Virus, Cucumber Mosaic Virus and Lettuce Necrotic Yellow Virus, respectively (Horn and Reddy, 1996). Among the nematodes, *Meloidogyne* spp.,

*Corresponding author, E-mail: s.gopalakrishnan@cgiar.org

Heterodera spp. and *Pratylenchulus* spp. cause heavy losses of the crop in several countries (Ansari *et al.*, 2002). *Fusarium* wilt and *Ascochyta* blight are considered to be the two most devastating diseases of chickpea (Hamid *et al.*, 2001).

***Fusarium* wilt of chickpea**

Fusarium oxysporum is the causal agent of wilt of many plant species. All strains may exist saprophytically and some are considered to be non-pathogenic but many are well known for inducing wilt on a variety of plants (Fravel *et al.*, 2003). Often isolates are specific to particular hosts for example, *F. oxysporum* f. sp. *lycopersici* infects tomato and *F. oxysporum* f. sp. *cubense* infects banana (Fravel *et al.*, 2003). Wilt of chickpea is normally considered to be caused by *F. oxysporum* f. sp. *ciceri* (Padwick) Snyder and Hans, hereafter designated as FOC. Initially it was believed that formae speciales were specific to one host and hence the name was taken from the host. However, other species and formae speciales of *Fusarium* also cause wilt in chickpea (Di Pietro *et al.*, 2003; Gopalakrishnan and Strange, 2005). *Fusarium* wilt is prevalent in all chickpea-growing areas of the world, including India, Pakistan, Spain, Iran and Tunisia and is important where the chickpea-growing season is dry and warm (Dubey *et al.*, 2010). This disease causes yield losses up to 100 per cent under favorable conditions in chickpea (Anjaiah *et al.*, 2003; Landa *et al.*, 2004). Symptoms of *Fusarium* wilt in chickpea consist of epinasty, chlorosis of leaves, discoloration of vascular tissue and ultimately collapse of the whole plant (Hamid *et al.*, 2001). The disease may be diagnosed by sudden drooping of leaves and petioles, which may turn yellow and browning of vascular bundles and its colonisation by fungal hyphae, which are apparent when the stem is split open (ICRISAT, 1995). Seven races of FOC (0, 1, 2, 3, 4, 5 and 6) have been reported worldwide (Cachinero *et al.*, 2002).

Management of *Fusarium* wilt

FOC may be eliminated from the seed using the fungicide Benlate T (30% Benomyl + 30% Thiram) at 0.25% (Mandeel, 1996). FOC can survive in the soil for more than 6 years and also in symptomless carriers (Haware and Nene, 1982). Therefore it is not possible to control the disease by normal crop rotation. Soil solarisation reduced FOC population and incidence of wilt (Chauhan *et al.*, 1988) however; cost considerations

would limit the use of this technique in the commercial farming. Sterilisation of the soil by methyl bromide is not an option as it is both costly and environmentally damaging (Fravel *et al.*, 2003). Date of sowing seems to have an effect on the incidence of wilt by lowering the fungal attack but also yield.

Disease resistance is another way to control plant disease if satisfactory levels of long-lasting resistance can be incorporated into culturally desirable crop plants. Maintenance of high levels of resistance to disease is normally achieved by selection and hybridisation. Selection involves exposing plant populations to high disease pressure and selecting individuals that survive. Resistance can be also developed by mutagenesis using chemicals such as methyl or ethyl-methanesulphonate, diethyl sulphate or ionising radiation such as X or gamma rays. Generation of resistance by gamma radiation has been reported for diseases including wilt, blight, stunt and root rot of chickpea but none of these has yet reached commercial application. Although varieties of plants that are resistant to some fusarial diseases are known, e.g., tomato grown in greenhouses are resistant to common races of *F. oxysporum* f. sp. *lycopersici* (Fravel *et al.*, 2003), but there are several plants for which no dominant gene for disease resistance to *Fusarium* is known e.g. carnation, cyclamen and flax. Despite the presence of races of the fungus, chickpea in relation to FOC appears to fall into this category. Several workers have observed different patterns in the development of wilting symptoms when chickpea is infected with FOC. For e.g., Sharma *et al.* (2012) identified moderate level of resistance against *Fusarium* wilt on three breeding lines (ICCV 05527, ICCV 05528 and ICCV 96818) and one germplasm accession (ICC 11322).

Biological control of plant diseases usually occurs by one or more of several distinct mechanisms. These include competition for nutrients, parasitism, antibiotics production and induced systemic resistance (Van Loon *et al.*, 1998). Biological control of the soil and seed-borne plant pathogenic fungi have been addressed using bacterial and fungal antagonists, to certain extent. Strains of *Bacillus* spp., *Pseudomonas* spp. *Trichoderma* spp. and non-pathogenic isolates of *F. oxysporum* were found not only to control FOC but also in helping the chickpea plants to mobilize and acquire nutrients (Postma *et al.*, 2003; Perner *et al.*, 2006; Gopalakrishnan *et al.*, 2015). Saprophytic *Fusarium* are able to suppress populations

of pathogenic *Fusarium* spp. by competing for nutrients in the soil, infection sites on the root and also in inducing systemic resistance (Fravel *et al.*, 2003). In wilt sick plot, Landa *et al.* (2004) reported that biocontrol agents, *Bacillus subtilis* GB02 and *Pseudomonas fluorescens* RG26, when applied alone and in combination with non-pathogenic *F. oxysporum* Fo 90105 delayed the disease onset and suppressed *Fusarium* wilt. *Trichoderma harzianum* and *Pochonia chlamydosporia* were found effectively controlled *Fusarium* wilt in chickpea (Khan *et al.*, 2011). *Streptomyces* spp. isolated from national parks in Kenya were shown to have antifungal activity against FOC (Nonoh *et al.*, 2010). Five strains of *Streptomyces* spp., isolated from herbal vermi-compost, were reported as having potential for biocontrol of *Fusarium* wilt in chickpea (Gopalakrishnan *et al.*, 2011). Although biological control often appears promising in specialised environments, disappointing results frequently are obtained in the field as several factors determine the survival and delivery of the antagonist. Therefore, the strategy to combat the disease should be to integrate different methods of control including cultural practices, use of resistant cultivars, biological control and chemical control. For instance, Singh *et al.* (2003) showed that two strains of *P. fluorescens* in combination with thiram @ 1.5 g kg⁻¹ effectively controlled collar rot of chickpea caused by *S. rolfsii* in both greenhouse and field experiments. There is a growing interest in the use of secondary metabolites, such as toxins, proteins, hormones, amino acids and antibiotics from microorganisms for the control of plant pathogens as these are readily degradable, highly specific and less toxic to nature (Doubou *et al.*, 2001). Hence, metabolites from microorganisms may be exploited for the control of *Fusarium* wilt.

Phytotoxins (metabolites) of microorganisms

Phytotoxins are low molecular weight compounds produced by microorganisms that cause obvious damage to plant tissues and are known with confidence to be involved in plant disease (Scheffer, 1983). Such damage may include wilting, water soaking, chlorosis and necrosis (Strange, 2003). For instance, the strawberry pathotype of *Alternaria alternata* produced AF toxin correlated with the pathogenicity of the isolates (Akamatsu *et al.*, 1997). Coriander seeds soaked in spore suspension of *F. oxysporum* f. sp. *corianderi* and partially purified toxins significantly lowered the seed germination and reduced the shoot and root lengths over the un-inoculated control (Gandhikumar and Raguchander, 2001).

Phytotoxins have been described in a number of well-documented reports as integral factors in disease development (Yoder, 1980; Scheffer, 1983) and have proved to be useful tools in the selection of resistant/tolerant plants (Daub, 1986). Phytotoxins may be classified as host-selective (host specific) or non-selective (non-specific). Host-selective phytotoxins are toxic to those plant species or cultivars that serve as hosts for the toxin-producing pathogen and lack toxicity towards non-hosts. A non-selective toxin may exhibit differential toxicity towards various plant species but toxicity is not highly correlated with the toxin-producer's host range (Knoche and Duvick, 1987). Host-selective toxins are found principally in species of *Alternaria* and *Cochliobolus* and non-selective toxins in species of *Fusarium*, *Ascochyta*, *Leptosphaeria* and also some species of *Pseudomonas* and *Xanthomonas* (Tables 1 and 2).

Plant pathogens produce a variety of secondary metabolites in culture that show phytotoxic activity but only a small proportion of these have a demonstrated role in plant disease. This is because of their low water solubility and the extreme sensitivity of the plants to solvents used to dissolve these compounds. Many of these phytotoxic compounds dissolve in solvents such as methanol, ethanol, dimethyl sulfoxide and acetone at a concentration of two to five per cent, which are extremely damaging to crop seedlings. These solvents upon further dilution usually causes the compound to precipitate, leaving a negligible concentration of the solution. However, determination of the role of phytotoxic compounds in pathogenesis (ability to cause disease) or virulence (severity of disease) is critical and hence pathogenicity studies should precede any effort to correlate toxin production with pathogenicity and virulence (Strange, 2007).

Purification of phytotoxins

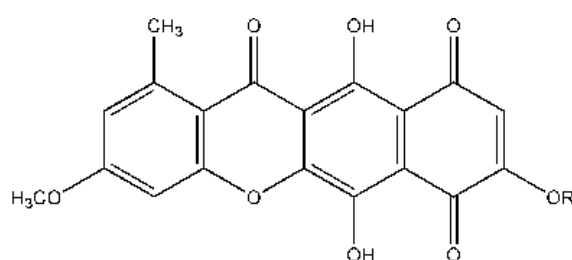
Although phytotoxins are thought to play a role in plant disease syndrome, particularly if the symptoms are expressed at the site of infection, they are usually difficult to extract from the infected plant. Phytotoxins which are of importance in plant disease syndromes are usually isolated from axenic cultures of pathogens. For examples, isolates of *Ascochyta rabiei*, the causal agent of blight in chickpea, produced the toxins, solanopyrones A and C when grown in Czapek Dox liquid medium (CDLM) supplemented with chickpea seed extract (Alam *et al.*, 1989) and also solanapyrone B when grown on CDLM

Table 1. Examples of host-selective toxins

Pathogen	Host	Toxin	Chemical class
<i>Alternaria alternata</i>	Japanese pear	AK-toxin	Epoxy-decatrienoic esters
	Strawberry	AF-toxin	Epoxy-decatrienoic esters
	Tangerine	ACT-toxin	Epoxy-decatrienoic esters
	Apple	AM-toxin	Cyclic tetrapeptide
	Tomato	AAL-toxin	Aminopentol esters
	Rough Lemon	ACR(L)-toxin	Terpenoid
<i>Bipolaris sacchari</i>	Sugarcane	HS-toxin	Glycosylated sesquiterpene
<i>Cochliobolus carbonum</i>	Corn	HC-toxin	Cyclic tetrapeptide
<i>Cochliobolus heterostrophus</i>	Corn	T-toxin	Linear polyketols
<i>Cochliobolus victoriae</i>	Oats	Victorin	Cyclized chlorinated peptide
<i>Mycosphaerella zeae-maydis</i>	Corn	PM-toxin	Linear polyketols
<i>Periconia circinata</i>	Sorghum	Peritoxin	Peptidyl chlorinated polyketide
<i>Pyrenophora tritici-repentis</i>	Wheat	P _{tr} ToxA	13.2-kDa protein
		P _{tr} ToxB	6.6-kDa protein

Table 2. Examples of non-host-selective toxins (Strange, 2003)

Pathogen	Host	Toxin	Chemical class
<i>Streptomyces scabies</i>	Potato	Thaxtomins	4-nitrotryptophan and phenylalanine groups linked in an L, L-configured cyclodipeptide
<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	Tobacco	Tabtoxin	Dipeptide of either threonine or serine linked to tabtoxinine- β -lactam
<i>Xanthomonas albilineans</i>	Sugarcane	Albicidin	Low molecular wt. compound with several aromatic rings
<i>Fusarium graminearum</i>	Wheat	Trichothecene	Trichothecenes are derived from farnesyl pyrophosphate, which is cyclized to form trichodiene and trichothecenes
<i>Fusarium moniliforme</i>	Maize	Fumonisin	Aminopentol esters
<i>Ascochyta rabiei</i>	Chickpea	Solanapyrones	Polyketide



Bikaverin: R = -CH₃
Norbikaverin: R = -H

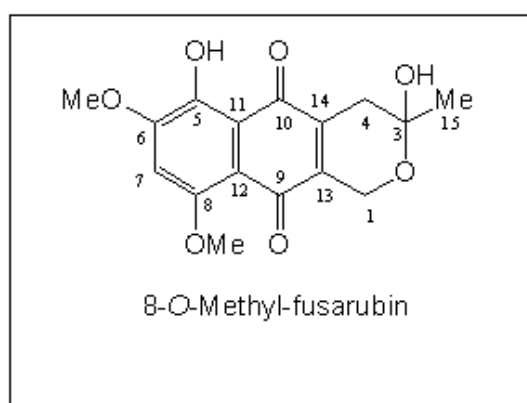


Fig. 1. Structures of bikaverin and norbikaverin (toxins from *F. oxysporum* f. sp. *vasinfectum*) and 8-O-methyl-fusarubin (Toxins from *F. acutatum*)

supplemented with metal cations, Zn, Ca, Cu, Co and Mn (Chen and Strange, 1994). These toxins were isolated by solvent partitioning with ethyl acetate and flash chromatography of the organic fraction on silica gel. The compounds were identified in the flash fractions by their characteristic UV spectra and those with similar spectra were combined. Purity of the compounds in the combined fractions was monitored by HPLC on an analytical C₁₈ column with aqueous mixtures of methanol, acetonitrile and tetrahydrofuran as mobile phases (Hamid and Strange, 1997).

Detection of an unknown toxin can be achieved by a suitable bioassay; preferably the assay should be rapid to perform, simple, sensitive and give quantitative results (Strange, 2003). Shohet and Strange (1989) suggested a bioassay technique in which cells were isolated from leaves of pigeonpea by a combination of enzyme digestion and mechanical agitation followed by incubation with culture filtrates of *Phytophthora drechsleri* f. sp. *cajani*. Phytotoxic compounds from *P. citrophthora* were assayed with tomato (non-host) and lemon (host) seedlings (Breiman and Barash, 1981) whereas tomato cuttings were used to assay toxins produced by *P. cactorum* in culture (Pligh and Rudnicki, 1979).

Phytotoxins from *Fusarium* species

Fusarium species produce complex mixtures of toxins that probably serve a variety of functions in allowing them to compete with other microorganisms and dominate their habitats. Species of *Fusarium* are known to produce mycotoxins, phytotoxins and some of the toxins, such as the trichothecenes, are toxic to both animals and plants (Desjardins *et al.*, 1992 and 1995). Bosch *et al.* (1989) reported that out of 62 isolates of *Fusarium*, obtained from pasture grass and soil from New Zealand, 82 per cent of the isolates were toxic to rats in feeding tests and of them 24 per cent were found severely toxic and caused haemorrhages of stomach, intestine, haematuria and finally death. Some of the compounds produced such as the trichotecene toxins, deoxynivalenol (DON), T-2 toxin and diacetoxyscirpenol (DAS) as well as zearalenone (ZEN) and the fumonisins have mammalian toxicity (Cawood *et al.*, 1991; Hussein and Brasel, 2001; L'vova *et al.*, 2003). Fumonisin mycotoxins (FB1 and FB2) produced by the fungus *Fusarium moniliforme* were extracted from the cultures of the fungus on maize meal with methanol/water (3:1) and further purified using Amberlite XAD-2, silica gel and reversed phase C₁₈ chromatography (Cawood *et al.*, 1991).

Schaafsma *et al.* (1998) demonstrated a cheapest and reliable method for identifying and quantifying DON (produced by *Fusarium graminearum* and *F. culmorum* in maize) and zearalenone (produced by *F. graminearum* in stored grain) with thin layer chromatography. Several toxins from various formae speciales of *F. oxysporum* have been described as causing wilt symptoms in their host plants. These toxins include fusaric acid from the banana pathogen *F. oxysporum* f. sp. *cubense*, beauvericin from the pathogen of muskmelon *F. oxysporum* f. sp. *melonis*, and several polyketide toxins (including bikaverin and norbikaverin) from the cotton pathogen, *F. oxysporum* f. sp. *vasinfectum* (Thangavelu *et al.*, 2001; Moretti *et al.*, 2002; Bell *et al.*, 2003).

Phytotoxins from *Fusarium* species causing wilt in chickpea

Chlorosis and wilting are common symptoms of toxicosis and these symptoms are characteristic of the phenotypes of chickpea plants infected with FOC (Gopalakrishnan and Strange, 2005). Other symptoms of toxicosis on chickpea are epinasty of the leaves, discoloration of the vascular tissue and ultimately collapse of the plant (Hamid *et al.*, 2001). These symptoms suggest that phytotoxins are involved in the disease. Kaur *et al.* (1987) found that partially purified toxin from FOC inhibited callus growth in chickpea. Rao and Padmaja (2000) reported that crude culture filtrates of FOC, when diluted to 30 per cent with water, caused wilting of 1-week-old chickpea seedlings in 4-5 days. An isolate of FOC from Thal region of Pakistan, identified based on its morphology and pathogenicity, was further identified as *Fusarium acutatum* in the 16S rDNA analysis (Gopalakrishnan and Strange, 2005). Filtrates from cultures of *F. acutatum* grown on a defined liquid medium caused permanent wilting of chickpea cuttings and killed cells, isolated enzymatically from healthy plants, in a bioassay (Gopalakrishnan *et al.*, 2005).

Purification of the phytotoxins from the culture filtrates of *F. acutatum*

Toxic activity from the culture filtrates of *F. acutatum* was retained by a cyano solid phase extraction cartridge and the toxin was isolated by elution from the cartridge in acetonitrile and Si-gel thin layer chromatography of the eluate. Bioassay of the fractions were done as per the protocols of Hamid and Strange (2000). In brief, filtrates of cultures were tested for their toxicity to cells isolated

from chickpea leaflets using fluorescein diacetate to differentiate live and dead cells. This compound readily enters the live cells with intact plasma-membranes and, once inside the cell, is metabolised by esterases to give free fluorescein. As plasma-membranes are impermeable to fluorescein, the compound accumulates and imparts a yellow-green fluorescence to such cells, which may be viewed under fluorescent microscope.

Analytical HPLC of the compound on a cyano column with diode array detection gave a single peak with a homogenous spectrum and λ_{\max} 224 and 281 nm whereas NMR and mass spectrum studies showed that toxin was 8-*O*-methyl-fusarubin (Gopalakrishnan *et al.*, 2005). Naphthazarin toxin, produced by species of *Fusarium*, of which 8-*O*-methyl-fusarubin is one, have been implicated in disease syndromes in citrus (van Rensburg *et al.*, 2001) and cotton (Bell *et al.*, 2003). Such compounds attack membranes and could be responsible for the loss of the semi-permeability of the plasma-membrane as found in the cell assay reported by Hamid and Strange (2000). An attack on plasma-membranes could also explain wilting as chickpea is likely to depend in part of the turgor of parenchyma cells surrounding the stele for support.

CONCLUSION

There is a need to develop new control strategies for *Fusarium* wilt because of the increasing importance of *Fusarium* wilt in chickpea production. For instance, the Round Ready™ (RR) gene from *Agrobacterium* spp. strain CP4 is now used widely in soybean and cotton to prevent toxicity from the widely used herbicide glyphosate (Bell, 2003). Similar approaches could be used against toxins of FOC that are crucial for either pathogenesis or increased virulence. Foreign genes could also be introduced into biocontrol organisms to protect them from FOC toxins and further allow destruction of toxins in the rhizosphere. Hence, a holistic approach to FOC toxins should facilitate development of new control practices for *Fusarium* wilt of chickpea.

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