Interacting Temperature and Water Activity Modulate Production of Destruxin A by *Metarhizium anisopliae* on *Galleria* Larvae-modified Agar Based Media *Invitro*

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Abstract

Destruxin A produced by some strains of entomopathogenic *Metarhizium anisopliae* is an important secondary metabolite which has been reported as a virulence factor. This study evaluated the effect of temperature and water activity (a_w) on destruxin A (DA) production by two strains of *M. anisopliae* (V275 and Ma 275.86) cultured on Oxoid Technical Agar modified with homogenized insect larvae *invitro*. Only one of the strains (V275) produced DA on the substrate and there were significant variabilities in the amount of DA at different a_w and temperature regimes. Concentrations of DA were further modulated by interactions of temperature and a_w. The retention time for DA in the HPLC profile was 15.5 minutes and the highest peak was at 0.98 a_w and 35°C while the lowest was at 0.96 a_w and 35°C. The abiotic interactions that supported biomass production appeared different from what was required for production of DA. The optimal temperatures for biomass formation varied significantly with a_w. At 0.995 a_w and 25°C, highest biomass was produced while at relatively lower a_w levels (0.98 and 0.96) the optimum temperature was 30 °C. There was a strong positive correlation (r = 0.455, n = 25, p = 0.023) between temperature and DA concentration.

Introduction

M.anisopliae is an important entomopathogenic fungus widely reported as a biocontrol agent (BCA) against many insect pests (Narladkar *et al.*, 2015; Borisade, 2015). The cyclic hexadepsipeptides, destruxins produced by some strains of this fungus have been described as a virulence factor ((Kao *et al.*, 2015) and many structurally related destruxins have been isolated from *Metarhizium* strains, out of which the most predominant are destruxins A, B and E (Wang *et al.*, 2003), and they are insecticidal (Amiri-Besheli *et al.*, 2010).

Having recognized the importance of destruxins and their roles in pathogenicity, it is important that studies are conducted to understand the factors which affect production and develop a simple growth medium for screening destruxin producing strains of *M. anisopliae* which can be potentially formulated into biopesticides.

Currently, there is no information on the influence that interacting temperature and a_w) may exert on this important fungal group in relation to production of secondary metabolites. In this study, the impact of interacting temperature (25, 30 and 35 °C) and a (0.995, 0.98, 0.96) on the amount of destruxin A (DA) produced was evaluated for two strains of *M. anisopliae* (V275; Ma 725.05) on a growth medium containing homogenized *Galleria* larvae. The possible implications of temperature and

water stress on destruxin production in relation to pest management and climate change was discussed.

Materials and methods

Source of fungal strains and Galleria mellonella.

The two strains of *M. anisopliae* (V275 and Ma 275.86) were respectively supplied by Prof. T. Butt (Swansea University, UK) and Prof. Dave Chandler (Warwick University, UK). Third instar *G. mellonella* larvae reared on cereal and honey-based meal were purchased from a commercial insectary in the UK.

P*roduction of seed culture*

Agar plugs of the two strains of *M.anisopliae* were sub-cultured on Sabouraud Dextrose Agar (SDA, Oxoid Ltd, a_w = 0.995) in 9 cm Petri dishes and incubated at 25 °C inside a sealed polythene bag. Conidia from 3 weeks old culture were harvested with 10 ml reverse osmosis water (Thermo-Scientific, Barnstead ™ Lab Tower ™ RO) containing 0.05% Tween 80 liter^{-1} (Borisade & Magan, 2014). The conidia suspension was poured into 25 ml Universal bottles and conidia concentration was determined using a haemocytometer and a microscope (Olympus ABHZ, Olympus, UK) and standardized to 1×10^6 conidia ml^{-1} by serial dilution.

Preparation of Galleria larvae-modified Agar based media

Oxoid Agar Technical (Agar No. 3) media was modified with *Galleria mellonella larvae* homogenate to encourage production of destruxins by the fungi. The larvae were killed by freezing at -20 °C for 30 minutes. These were rinsed thereafter in de-ionized water and divided into approximately 20 g sub-samples. The larvae sub-samples were frozen in liquid Nitrogen and transferred into a freeze dryer (MechaTech Systems LSBC50) for 5–6 days. The freeze-dried larvae (Moisture content = 7%) were crushed using ceramic mortar and pestle and added into either unmodified- (a_w = 0.995) or glycerol-modified (a_w = 0.98 and 0.96) Technical Agar at the rate of 100 g freeze- dried insect homogenate litre^{-1} of media. The media was autoclaved and poured into 9 cm Petridishes. The a_w of the media were checked using water activity meter (Novasina Humidat-IC-II, Switzerland) and they were found to be within 0.005 accuracy to the target levels.

Effect of interacting temperature and a_w on DA production

Cooled agar media were overlayed with sterile sheets of cellophane overlay-disk (8.5 cm). The centre of each cellophane lined plate was inoculated with 1µl of the prepared standard conidia suspension (1×10^6) using Eppendorff Micropipette (1–20 µl capacity). Three replicate plates for each of the strains were prepared and sealed with parafilm, placed in sealable nylon and incubated at 25, 30 and 35 °C for 14 days.

Experimental design

Completely randomized design (CRD) which was run in triplicates was used to describe the variabilities in the rate of DA production in relation to the two strains, three temperatures and three a_w levels.

Extraction of destruxins from agar

Fungal mycelia were separated from the agar media by removing the colonies

growing on the cellophane surface and the weight of the agar and fungal biomass were determined. The agar from each plate was sliced using a surgical blade and kept in 25 ml standard glass bottles. Toxins were extracted by pouring 25 ml 1:1, v/v (dichloromethane: ethyl acetate) into the sliced agar inside the bottles followed by agitation on a rotary shaker at 50 rpm for 48 hours at 25 °C. Thereafter, the solvent was decanted into 15 ml standard glass bottles and evaporated inside a fume cupboard to dry. After drying, 1.5 ml HPLC grade methanol (Aldrich, Purity $\geq$ 99.9%) was poured into each bottle and turned gently to rinse the extracts. The methanol extract was withdrawn into HPLC vials (Agilent) with a micropipette and kept at -40 ^{o}C until required for HPLC analysis to quantify the amounts of DA produced.

HPLC procedures and quantification of DA

The HPLC system used for DA analysis was an Agilent 1200 series system (Agilent, Berks., UK) with a diode array detector and auto sampler (ALS, G1329, Agilent), auto-sampler thermostat (G1330B, Agilent), thermostarted column compartment (TCC, G1316A, Agilent), on-line degasser (G1379B, Agilent), and binary pump (G1312A, Agilent). The column was an Agilent Zorbax Eclipse plus C_{18} 4.6 × 150 mm, 3.5 µm; preceded by an Agilent Zorbax Eclipse plus C_{18} guard cartridge. Signals were processed by Agilent Chem Station software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA).

Analyses were performed in the gradient mode; solvent A was water and solvent B, acetonitrile. Gradient conditions were initiated by holding for the first minute with 10% B, after this the conditions were changed linearly to 60% B for 15 min (minute 16). The composition was then changed linearly to 100% B in 10 min (minute 26) and maintained for 5 min as a cleaning step in order to improve the results. After cleaning, conditions were returned to the initial 10% B. The flow rate of the mobile phase was 1 ml min^{-1} and injection volume was 25 µl.

Standard DA was obtained from Sigma-Aldrich. The initial stock solution prepared from the standard was made into 1000 ppm by diluting with 1 ml methanol (HPLC grade). Other concentrations, (50, 20 and 10 ppm) were derived from the diluted stock and used as standards for the HPLC. Lower dilutions (1 and 5 ppm) were prepared from subsequent diluents. A multi-level calibration was done and the graph of the peak heights against the concentrations of the standards was plotted (Fig. 1). The equation of the curve ($y = 32.824x + 14.954$, $R^2 = 0.9998$) was used to calculate concentrations of DA in the treatment samples. The crude extracts were loaded into the HPLC device and DA quantification was performed.

Statistical analysis

Data on DA concentration was subjected to Analysis of Variance (ANOVA) procedure and significant difference in concentration was separated using Tukey's Honestly Significant Difference (HSD).

Pearson partial correlation was used to explore the relationship between temperatures, a_w and the amount of DA in the substrate. Analysis was done using the IBM SPSS Statistical software Version 21, Armonk, NY: IBM Corp.

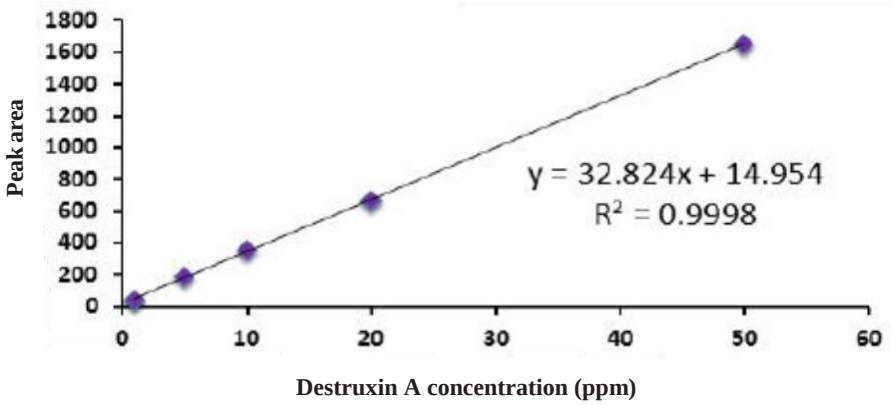

Fig. 1. Calibration curve of DA standards in relation to peak heights.

Results

The HPLC chromatograms and variabilities in DA production

DA was not detected in the crude extracts of Ma 725.05, therefore the data was not shown. The HPLC profiles for the second strain, V275 showed that the retention time for DA was approximately 15.5 minutes and there were variabilities in the peak heights at different levels of temperature and a_w interactions (Figs. 2, 3 and 4). The highest peak was recorded at 0.98 a_w and 35°C and the lowest was at 0.96 a_w and 35°C.

DA concentrations at different levels of interacting temperature and a_w

Fig. 5 shows the effect of interacting temperature and a_w on the concentrations of DA secreted into the agar. The effect of the interactions was significant on the concentration of DA produced (Table 1). At 0.995 a_w and 25 °C, the mean concentration of DA in the agar was significantly lower than the concentration at 30 and 35 °C.

At 0.98 a_w, the optimum temperature for DA production was 35 °C. Also at 0.98 a_w, DA production was favoured by increasing the temperature (25–35 °C). At this a_w level, the mean concentration of DA in the samples incubated at 30 °C were significantly higher than those which were incubated at 25 °C. In contrast, at 0.96 a_w, increase in temperature from 25–35 °C significantly reduced the amount of DA secreted into the agar.

The effects of each abiotic factor as well as their interactions had significant impact on biomass weight (Table 2).The optimum temperature for biomass production varied at different a_w levels (Fig. 6). At 0.995 a_w, highest biomass was produced at 25 °C while at relatively lower a_w levels (0.98 and 0.96) the optimum temperature was 30 °C.

Partial correlation analysis was used to explore the relationship between a_w and DA concentration (µg/g) of agar while controlling the effect of temperature (Table 3). There was a small and negative partial correlation between a_w of the media, if the temperature is controlled (r = -0.087, n = 25 and P = 0.713)

In contrast, there was a strong positive correlation between temperature and amount of DA secreted into the agar, controlling for

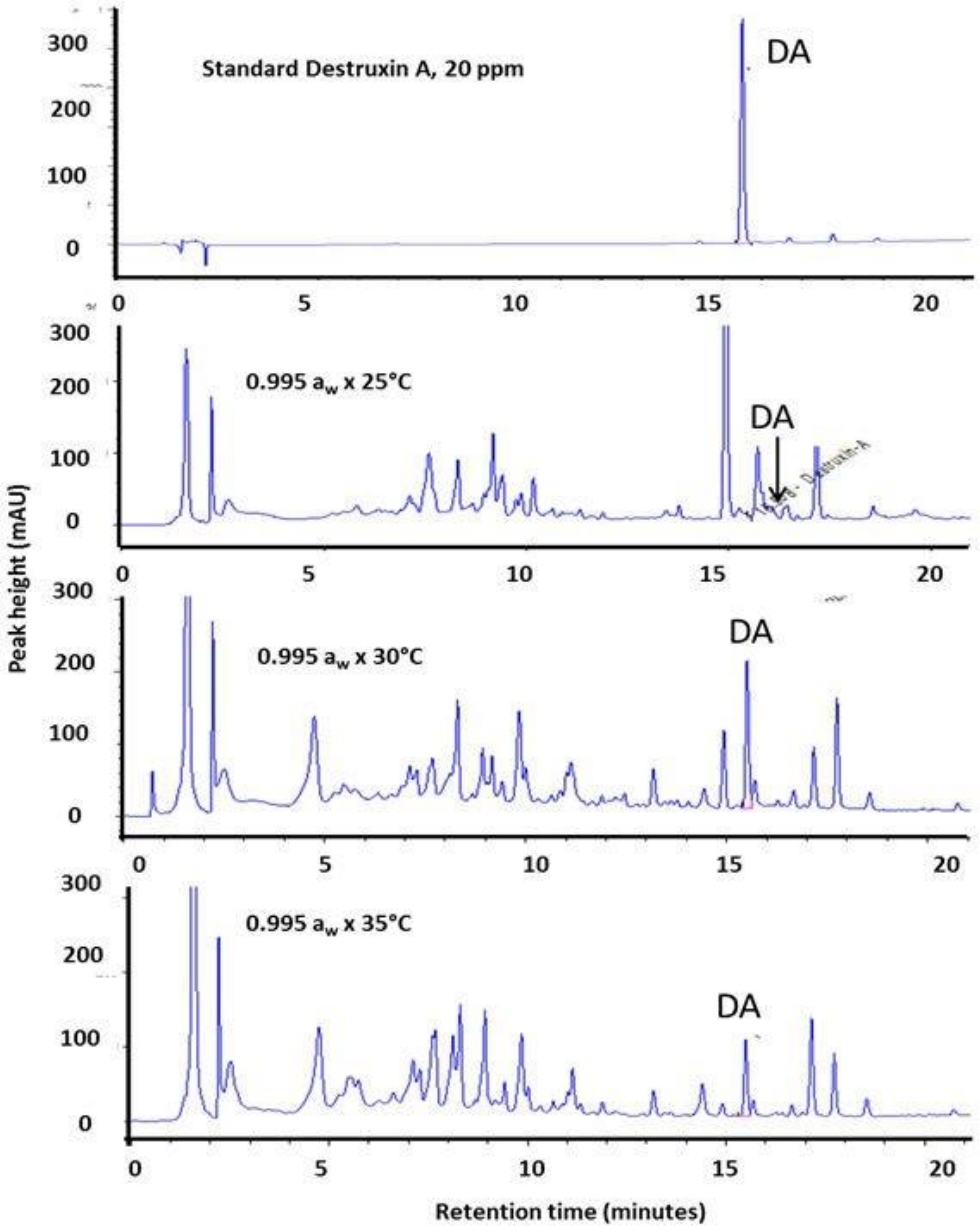

Fig. 2. HPLC profile showing signals of cyclic peptide products of crude extract from *M. anisopliae* at 0.995 a_w.

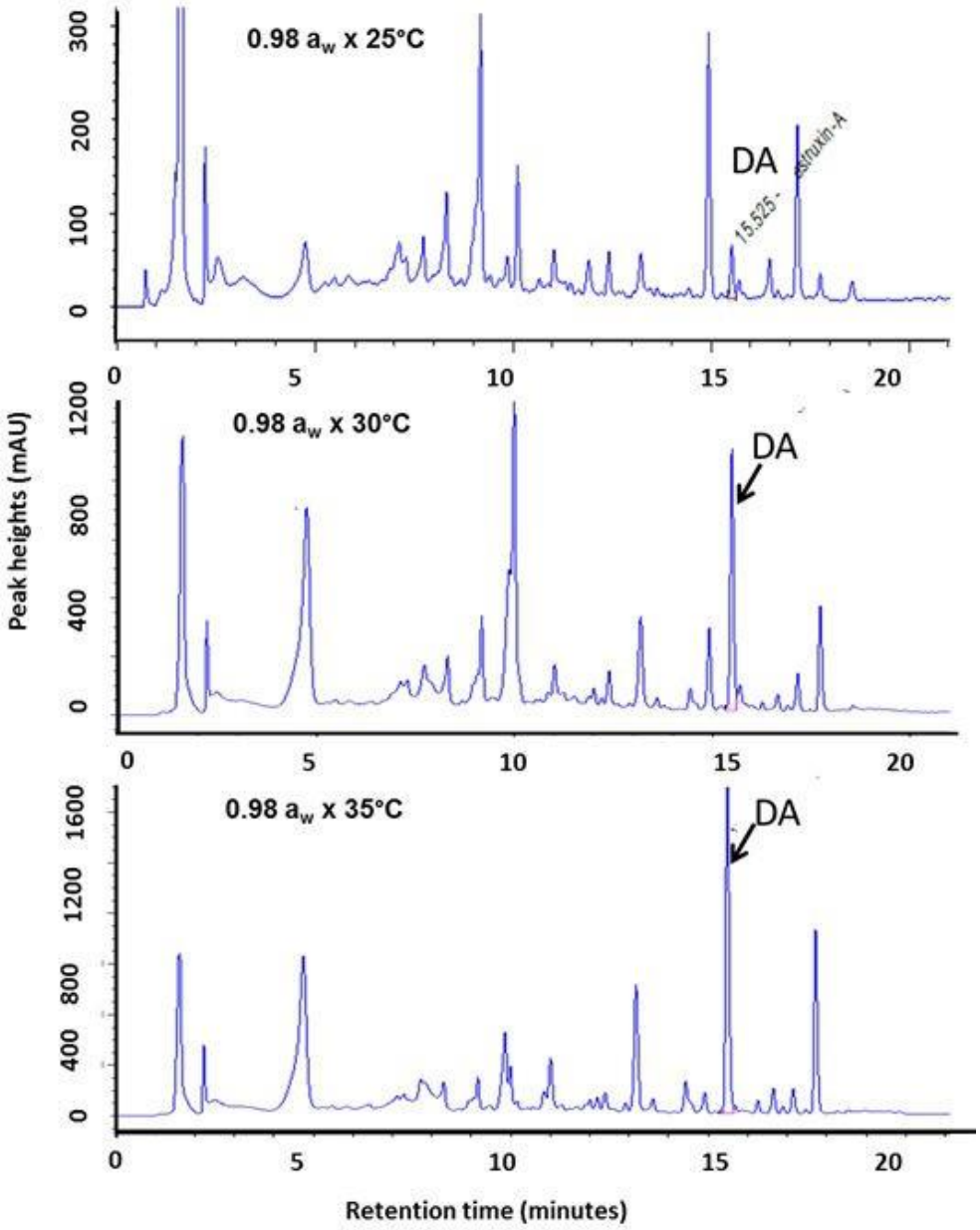

Fig. 3. HPLC profile showing signals of cyclic peptide products of crude extract from *M. anisopliae* at 0.98 a_w

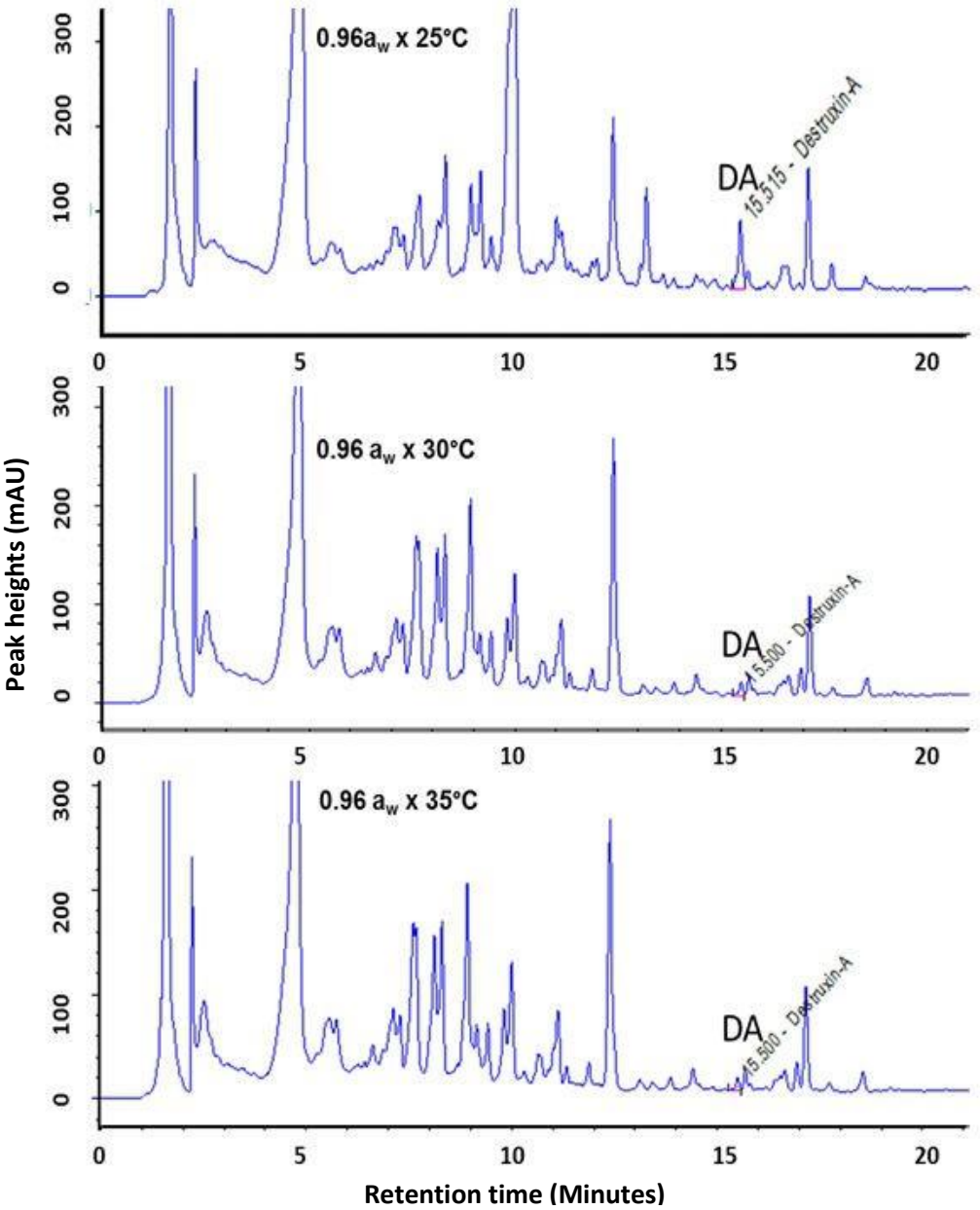

Fig. 4. HPLC profile showing signals of cyclic peptide products of crude extract from *M. anisopliae* at 0.96 a_w)

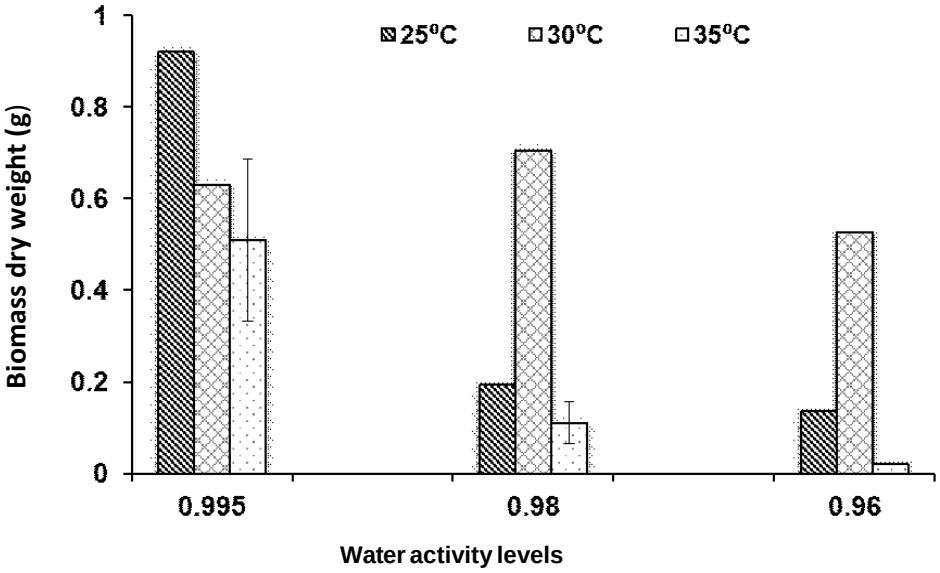

Figure 6. Dry weight of *M. anisopliae* biomass recovered from Technical agar based media at three a_w levels. Bars are standard error of the means where shown.

TABLE 3

Partial correlation analysis showing the relationship between water activity, temperature and concentration of DA produced by M.anisopliae

Control Variables			*Water activity*	*Concentration*	*Temperature*
-none-[a]	Water activity	Correlation	1.000	–.077	0.000
		Significance (2-tailed)		.713	1.000
		Df	0	23	25
	Concentration	Correlation	–.077	1.000	.454
		Significance (2-tailed)	.713		.023
		Df	23	0	23
	Temperature	Correlation	0.000	.454	1.000
		Significance (2-tailed)	1.000	.023	
		Df	25	23	0
Temperature	Water activity	Correlation	1.000	-.087	
		Significance (2-tailed)		.687	
		Df	0	22	
	Concentration	Correlation	–.087	1.000	
		Significance (2-tailed)	.687		
		df	22	0	

[a]. Cells contain zero-order (Pearson) correlations.

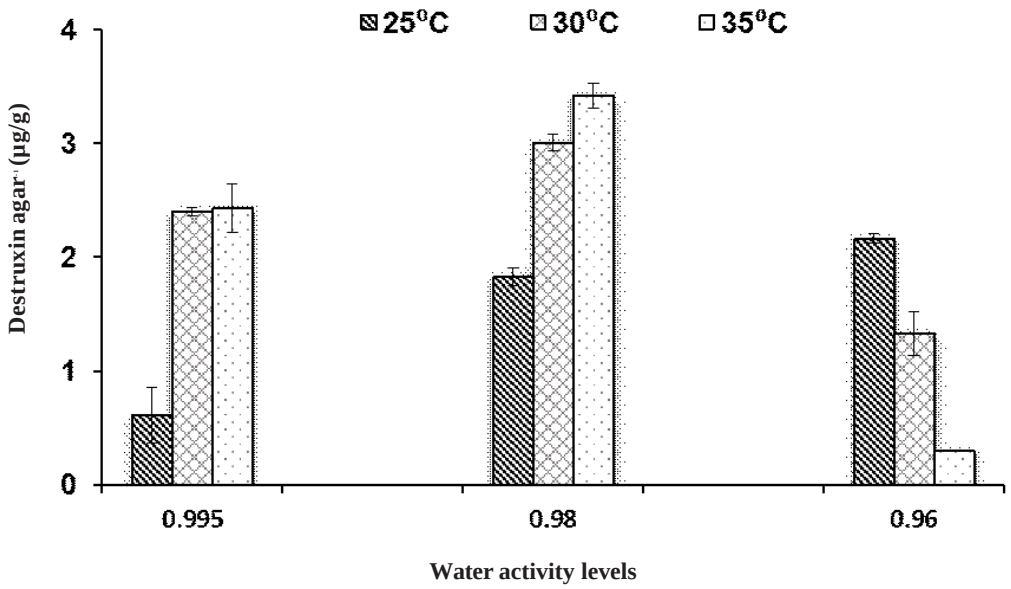

Fig. 5. Effect of a_w and temperature on destruxin A concentration in crude extract of *M. anisopliae*…

TABLE 1

Analysis of variance of the effect of a_w and temperature on concentration of DA produced by M.anisopliae (V275).

DF	*MS*	*F*	*Factors*
a_w	2	4.151	194.445*
temperature	2	1.186	55.543*
Factor interactions			
a_w x Temperature	4	2.451	114.828*
Total	25		

*Significant at the level $P < 0.05$

TABLE 2

Analysis of variance of the effect a_w and temperature on biomass weight of M.anisopliae (V275)

DF	*MS*	*F*	*Factors*
a_w	2	0.518	25.329*
temperature	2	0.372	18.187*
Factor interactions			
a_w x temperature	4	0.141	6.891*
Total	27		

*Significant at the level $P < 0.05$

a_w (r = 0.455, n = 25, P = 0.023), with increase in temperature (25–35 °C) associated with higher DA in the agar. An inspection of the zero order correlation (r = 0.454) suggested that a_w had a little influence on the relationship between temperature and DA concentration. The effect of temperature alone could help to explain 20.7% (r^2 = 0.2070) of shared variance of concentrations of the toxin measured in the substrate at different levels of interacting abiotic stress factors.

Discussion

We described the procedure of stimulating *M. anisopliae* destruxin production by modifying agar based substrate with homogenized *G. mellonella* larvae. Modification of substrate with homogenized insect larvae was done to mimic the conditions obtainable while the fungus is growing on dead insects. Previous studies on destruxin production employed liquid fermentation techniques and use heterogeneous substrates such as basmati rice (Wang *et al.*, 2004). Many strains of *M. anisopliae* which are capable of producing Destruxin on insect carcass fail to produce the secondary metabolite on these artificial substrates. Therefore, the results of screening for metabolite production on such media may be confusing. Thus, the method described in this study is reliable for screening Destruxin producing strains of *M. anisopliae*.

There is no previous report on the effect of interactions of temperature and a_w on destruxins production by entomopathogenic fungi. However, only one of the two tested strains, (V275) produced DA on the Modified Oxoid Technical Agar. Growth substrate have been demonstrated to profoundly influence destruxins production (Shah *et al.*, 2005). The study showed that further modulations of amount of destruxins occurred at different levels of interactions of temperature and a_w. Chen *et al.* (1999) reported that the optimum condition for production of destruxins A and B by a strain of *M. anisopliae* on rice, bran and husk medium was 71% moisture content and 0.921 a_w but the study took no account of the effect of temperature relations. Wang *et al.* (2004) previously investigated destruxins A and B production by *M. anisopliae* (V275) being one of the strains of *M. anisopliae* tested in the current study and another strain (V245) on Czapek Dox solid medium and found that none of them could produce cyclic peptide metabolites after incubation for 14 days. However, both produced DA and B in rice culture while none of them produced destruxin E. The study also showed that the effect of different carbon and nitrogen sources as well as their ratio (C:N), length of fermentation and aeration had significant impact on destruxins production.

Largely, investigations on biosynthesis of secondary metabolites by entomopathogenic fungi employ complex liquid media. The current study made use of a relatively simple solid substrate to stimulate destruxin production and the process of extraction and HPLC analysis were described explicitly. The media contained homogenized insect larvae and non-ionic solute, glycerol. This medium was capable of stimulating production of secondary metabolites by entomopathogenic fungi. Probably, one of the strains (Ma 725.05) is not a DA producing strain. Amiri-Besheli *et al.* (2000) investigated inter- and intra-

specific variation in destruxin production by eleven *Metarhizium* species and found that some strains could not produce destruxin. Similarly, Hu *et al.* (2006) investigated destruxin production among 80 strains of *M. anisopliae* and found that production levels vary among the strains and that absence of destruxin is probably characteristic to the different species.

It has been reported that temperature and water availability as well as their interactions cause significant modulation of growth with a more dramatic impact on sporulation (Borisade & Magan, 2014). In this study, there was no consistent relationship between amount of fungal biomas and concentrations of DA in the agar. For example, at 0.995 a_w and 25°C, biomass weight was significantly the highest and incidentally, destruxins concentration was at the lowest level. In contrast, the lowest biomass weight was recorded at 0.98 a_w and 35°C while the toxin concentration was at peak. At 0.96 a_w, generally low biomass was produced between 25–35 °C but the a_w level favoured higher toxin production at 25 °C.

It is interesting that increase in temperature from 30–35 °C combined with water stress (0.98 a_w) caused approximately 13% increase in the DA detected gram^{-1} agar and it was a significant increase. This suggests that temperature and water relations can alter the physiology of entomopathogenic fungi and their secondary metabolite production in an unpredictable way. This kind of information is essential and may give the picture of behaviour of entomopathogenic fungi in terms of toxigenesis and pest management under a climate change scenario, where higher temperature and drought are expected. For example, *M. Anisopliae* while growing as an endophyte in plants have been shown to utilize destruxins as a virulence factor against important agricultural pests. Destruxin production levels in such instances may therefore be affected by temperature and water relations.

Conclusion

Abiotic influences, temperature and water availability are capable of causing significant alterations in biosynthesis of important secondary metabolites by *M. anisopliae*. A screening criteria based on these abiotic parameters (temperature and a_w) can facilitate selection of strains which can be used for pest management, particularly under tropical climatic conditions.

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