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Interacting climate change environmental factors effects on *Fusarium langsethiae* growth, expression of *Tri* genes and T-2/HT-2 mycotoxin production on oat-based media and in stored oats.

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ABSTRACT

The objectives of this study were to investigate the impact that interactions between key climate change (CC) related environmental factors of temperature (20, 25, 30°C), water activity (a_w; 0.995, 0.98) and CO_2 exposure (400, 1000 ppm) may have on (a) growth, (b) gene expression of biosynthetic toxin genes (*Tri5, Tri6, Tri16*), and (c) phenotypic T-2/HT-2 production by *Fusarium langsethiae* on oat-based agar medium and in stored oats. Fungal growth was optimum at 25°C and 0.995 a_w and reduced significantly at 30°C and intermediate stress (0.98 a_w, elevated CO_2 (1000 ppm) exposure by approx. 4-fold. Lag phases prior to growth paralleled these results with the longest lag phase in this treatment (24 hrs). On oat-based medium, the relative *Tri5* gene expression was increased in elevated CO_2 conditions. The expression of both the *Tri6* and *Tri16* genes was reduced when compared to control (20°C, 0.995 a_w, 400 ppm), especially in elevated CO_2 conditions. In stored oats, the *Tri5* gene expression was reduced in all conditions except at 30°C, 0.98 a_w, elevated CO_2 where there was a significant (5.3-fold) increase. The expression of the *Tri6* was slightly over-expressed in elevated CO_2 and the *Tri16* gene was upregulated, especially in elevated CO_2 conditions. For mycotoxin production, both on oat-based medium and in stored oats the production was higher at 25°C when compared to 30°C. In stored oats, at 0.98 a_w, elevated CO_2 led to higher T2/HT-2 toxin production at both 25 and 30°C with a significant increase (73-fold higher) at 30°C. In elevated CO_2 conditions, *Tri16* (Spearman test; 0.68; *p-value*=0.0019) and *Tri5* gene expression (Spearman test; 0.56; *p-value*=0.0151) were correlated with T-2+HT-2 production. Nine T-2 and HT-2 metabolites were detected by LC-MS/MS including a new dehydro T-2 toxin and the conjugate, HT-2 toxin glucuronide (in plantae). The new dehydro T-2 toxin was the most abundant metabolites and showed correlation (R2=0.8176) with T-2 production.

This is the first study to examine the impact of CC factors on growth and mycotoxin production by a strain of *F. langsethiae*. The influence of such scenarios on relative risk of oats contamination with these toxins in relation to the food security agenda is discussed.

KEYWORDS: climate change, temperature, carbon dioxide, water stress, growth, biosynthetic genes, Type A trichothecenes, mycotoxins, oats

Introduction

Oats production has increased in northern Europe, including the UK and Ireland over the last few years because of its health benefits (Marshall et al., 2015; Thies et al., 2014). Ripening oats can become infected by *Fusarium* species such as *Fusarium langsethiae* and *F. sporotrichioides* although they do not show any visible symptoms (Imathiu et al., 2017). These fungi also contaminate the ripening grain with type A trichothecenes (T-2/HT-2 toxins). There are EU-wide recommendations on maximum contamination limits for T-2+HT-2 toxins (Commission Recommendation 2013/165/EU). Currently, the indicative levels are 1000 µg/kg for T-2+HT-2 in unprocessed oats and 200 µg/kg in oats for direct human consumption (European Commission 2013/165/EU, 2013). However, because of the lack of symptoms, it is difficult to evaluate the relative toxin contamination levels present in a crop at harvest without chemical analyses of representative samples.

Climate change (CC) and food security has attracted significant attention in the last decade. This has also resulted in a focus on the impacts that interacting abiotic factors of elevated temperature (+3-5^{o}C), fluxes in wet and dry conditions (drought stress) and elevated CO_2 (400 ppm vs 800-1200 ppm) might have on fungal pathogenicity and mycotoxin contamination of staple food crops (Magan et al., 2011; Medina et al., 2017). Interacting environmental factors, especially of temperature and water availability, are critical in determining colonisation and mycotoxin contamination of cereals including oats (Magan et al., 2010; Medina and Magan, 2011, 2010; Mylona and Magan, 2011). Recently, there has been interest in the resilience of mycotoxigenic fungi, including *Fusaria*, under such climate-related environmental parameters. While limited data is available on effects of CC scenarios on mycotoxigenic fungi, some studies have focused on cereal:pathogen systems. These include maize and both *F. verticillioides*

(fumonisins) and *Aspergillus flavus* (aflatoxins), wheat and *F. graminearum* (deoxynivalenol) and coffee and *A. westerdijkiae* and *A. carbonarius* (ochratoxin A). Studies with *A. flavus* colonising maize grain showed that while growth was relatively unaffected, structural and regulatory biosynthetic genes involved in mycotoxin production (*aflD*, *aflR respectively*) and phenotypic aflatoxin B_1 production were stimulated under three-way interacting CC conditions of temperature (+4°C), water stress (0.99/0.98 vs 0.90 water activity, a_w) and elevated CO_2 (existing values of 350 ppm vs 650 and 1000 ppm CO_2) (Medina et al., 2017, 2015a). Indeed, transcriptomic analyses has suggested significant effects on secondary metabolite clusters, sugar transporters and stress related genes (Gilbert et al., 2017). Studies by Vaughan et al. (2014) showed that while infection of ripening maize cobs was increased under elevated temperature and CO_2 scenarios, fumonisin contamination was not increased. Subsequent studies included drought stress, which showed a stimulation of fumonisins (Vaughan et al., 2016). Studies of colonisation of stored coffee by *A. westerdijkiae* and *A. carbonarius* and ochratoxin A contamination suggested that there may be variability in the effects of these interacting climate change related environmental factors on different mycotoxigenic fungi. Thus, for *A. westerdijkiae* there was a stimulation of toxin production, while for *A. carbonarius* there was no effect on toxin contamination of this commodity (Akbar et al., 2016).

Studies on the ecology of *F. langsethiae* have compared different strains from northern European countries and identified optimum conditions for growth as being between 0.98-0.995 a_w and 25ºC (Medina and Magan, 2010). Production of T-2+HT-2 was highest at 20-25°C with freely available water (0.995 a_w; Medina and Magan, 2011). However, less is known about the effect of three-way interacting climate change factors in relation to the resilience of *F. langsethiae* and impacts on growth, gene expression and T-2/HT-2 toxin contamination.

The biosynthetic pathway for T-2+HT-2 production by *F. langsethiae* includes 15 genes located in 1 major *TRI* cluster and two independent sub-clusters. The *Tri5* cluster groups 12 genes including the *Tri5* encoding for trichodiene synthase (the first step in the pathway for trichothecene production) and *Tri6* encoding for a *Tri* positive transcription factor. The *Tri1-Tri16* sub-cluster includes 2 genes, among these *Tri16* is encodes a acyltransferase specific to the T-2+HT-2 production pathway (Lysoe et al, 2016).

The objectives of this study were to examine the effect of three-way interacting CC environmental factors of temperature (20, 25 and 30°C), water availability (0.995 vs 0.98 a_w) and elevated CO_2 (400 ppm vs 1000 ppm CO_2) on: (a) growth, (b) biosynthetic genes involved in trichothecene production (*Tri5, Tri6* and *Tri16*) and (c) phenotypic T-2 and HT-2 contamination on oat-based agar media and in stored oats.

Materials and methods

Fungal strains

Fusarium langsethiae (strain FE2391) isolated from U.K. grown oats (Worcestershire) was grown for 7 days on potato dextrose agar (PDA) at 25°C. This isolate is a known type A trichothecene producer and was kindly supplied by Dr P. Jennings, FERA, York, U.K.

Medium preparation and inoculation

Whole oats harvested in UK were milled in a Waring Laboratory & Science homogeniser model 7009G (Waring Laboratory Science, CT, USA) during 5 min at maximum speed. Two % (w/v)

oat flour and 2 % (w/v) agar (Technical agar No. 2, Oxoid) were added to the water. The water activity (a_w) of the unmodified medium was 0.995. This was modified by the addition of glycerol to obtain conditions representing intermediate water stress (0.98 a_w) based on ecological data from previous studies (Medina and Magan, 2010). The media were autoclaved for 15 min at 121ºC. Sterile cellophane layers (8.5 cm) were carefully placed on the surface of the media with a surface-sterilised forceps avoiding any air bubbles. The treatments and replicates were inoculated with 4 mm agar discs taken from the growing margins of the stock culture colony using a surface-sterilised cork borer, with the discs carefully placed centrally in each 9 cm Petri plate. In all cases 5 replicates were used per treatment. The diametric fungal growth was measured daily for 10 days.

Inoculation and storage of oats inoculated with* F. langsethiae *inoculum

Oats harvested in UK and 500 g sub-samples in resealable plastic bags were irradiated at 12-15 kGys gamma irradiation (SynergyHealth Ltd., Swindon, UK). This was done to remove surface contamination of the oats but retain germinative capacity. A moisture adsorption curve was developed by adding known amounts of water to 5 g sub-sample of oats which were stored for 48 hrs at 4°C. After equilibration at 25°C, the a_w of the different treatments was measured using an Aqualab 4TE (Labcell). The adsorption curve was used to calculate the amount of sterile water needed to modify the oats to the target a_w levels for storage at 0.995 and 0.98 a_w. The necessary sterile water was added to the two treatments (minus the inoculum volume) and stored at 4ºC for 48 hrs and shaken twice a day to allow adsorption and equilibration. A stock culture was flooded with 10 mL of sterile water + 0.005% tween 80 and the colony surface agitated with a surface sterilised loop. The spores were decanted and the concentration was determined using a

haemocytometer. This was diluted with sterile water and then 0.5 ml added to the oats to obtain a final concentration of 10^3 microconidia per gram of oats. The experiment was done with 5 replicates per treatment in all cases.

Effects of climate change factors on in vitro and in situ* F. langsethiae *colonisation and mycotoxin production

The treatments used in this study were 20, 25, 30ºC; a_w levels of 0.995, 0.98 and CO_2 concentrations of 400 ppm and 1000 ppm. The *in vitro* agar cultures, and stored oat grain treatments (5 grams, wet weight) in 40 mL jars with a perforated lid were placed in 13-L air-tight containers including two beakers of glycerol/water solutions (2 x 500 mL) to maintain the atmosphere at the same equilibrium relative humidity (ERH) as the a_w treatment. The treatments and replicates in different environmental chambers were flushed for 10 days, either with 400 ppm (air) or 1000 ppm CO_2 from a speciality gas cylinder (British Oxygen Company, Guildford, Surrey, U.K.). The containers were flushed daily at 3 $L.min^{-1}$ to replace 3x the volume of the incubation chamber.

After 10 days, the final colony size of treatments and replicates were measured in the *in vitro* studies, and the fungal biomass carefully removed from the cellophane surface, weighed, placed in Eppendorf tubes and frozen at -80^{o}C. The stored oat samples were immediately frozen in liquid nitrogen and stored at -80ºC until molecular and mycotoxin analyses.

Tri *gene expression analysis*

Fungal biomass from the oat-based medium were ground using the Precellys 24 following the method used by Leite et al. (2012). For oat samples, a pestle and mortar was used with liquid nitrogen to grind the samples for RNA extraction. Extraction was performed using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St Louis, Missouri, U.S.A.) according to the manufacturers' instruction. The RNA obtained was checked for quality RQI>7 by Experion (BioRad, Watford, UK) and purity ($ratio_{260/280}$=2-2.2, $ratio_{260/230}$>2) by Genova Nano (Jenway, Stone, UK), and stored at –80ºC. Reverse transcription was performed using the Omniscript® Reverse Transcription Kit (Qiagen, Hilden, Germany) using $Oligo(dT)_{18}$ following the manufacturers' instructions. Initially, a number of housekeeping genes were examined. Of these the Actin and Citrate synthase genes were chosen as a reference because of their stability in media and oats matrices (Table 1). For information on the effect of treatments on biosynthetic genes involved in trichothecene production three *Tri* genes were used. These were the *Tri5*, *Tri6* and *Tri16*. The reaction was performed using SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad, U.K.) with the primers (see Table 1) at a concentration of 100 nM in a reaction volume of 10µl. The qPCR program used was 95ºC for 30 s followed by 45 cycles of: 95ºC for 5 s, 59ºC for 30 s, 65ºC for 5 s.

The data obtained from the qPCR was treated with the software Bio-Rad CFX Manager™ and Excel tools to calculate the ΔΔCq of the *Tri5*, *Tri6* and *Tri16* genes. The ΔΔCq data are presented as relative gene expression in comparison to the control condition defined as 20°C, 0.995 a_w and 400 ppm CO_2.

Quantification of T2/HT-2 toxins from oat-based matrices

After 10 days, agar plugs (Ø 9 mm) were taken across the colony to obtain between 0.5 to 1.0 g of fungal biomass + agar in 2 mL Eppendorf tubes. These were stored at -20ºC until analyses. Samples were thawed and extracted by mixing the agar plugs with 1 mL of methanol:water (80:20, v:v). The tubes were shaken for 90 min at 400 rev. min^{-1} at 25ºC in the dark. They were then centrifuged at 13,000 g for 15 min and 750 µL of supernatant removed and re-extracted again in the same way. Extracts were dried in a miVac evaporator (Genevac, Ipswich, UK) for 7 h. The samples were resuspended in 300 µL of acetonitrile:water (50:50, v:v) and filtered through a 0.45 µm PVDF filter (type) into HPLC vials and stored at -20°C until analyses.

The samples were injected into a HPLC-DAD Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a UV diode-array detector set at 200 nm with 600 nm as reference. The column used was a Poroshell® 120 EC C_{18} 100 mm x 4.6 mm (Agilent Technologies, Palo Alto, CA, USA). Separation and analyses were performed using the gradient mode with solvent A: water and solvent B: acetonitrile. Gradient conditions were 15 % of B for 3 min, then, switched to 30 % B after 1 minute, and then increased to 40 % B after 4 min, 50 % B after 2.5 min and finally 95 % B after a further 30 s. For 2.5 min, the conditions were kept at 95 % before going back to 15 % B in 30 s. The flow rate of the mobile phase was 1.2 $mL.min^{-1}$ and injection volume was 25 µL. Working standards were prepared from a T-2 and HT-2 stock solution (1 $mg.mL^{-1}$) supplied by Cambridge Bioscience (Cambridge Bioscience Ltd, UK). Signals were processed by an Agilent Chem-Station software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA).

LC-MS analysis of stored oat treatments

200 mg of ground oat samples were extracted according to the method described by (Meng-Reiterer et al., 2016). Briefly, 1 mL of acetonitrile:water:formic acid (79:20.9:0.1, v:v:v) was added to the 200 mg samples, vortexed for 30s, sonicated for 30 min and agitated on a ThermoMixer for 30 min (1400 rpm). Samples were spun at 4°C at 10,000 rpm for 6 min and 400 µL aliquots removed and diluted with 400 µL of LC-MS grade H_2O. The resulting mixtures were filtered into amber glass HPLC vials using a 0.45 µm PTFE syringe filter, (ChromeSpec). All the MS data were obtained using a Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific), coupled to an Agilent 1290 high-performance liquid chromatography (HPLC) system with a Zorbax Eclipse Plus RRHD C_{18} column (2.1 × 50 mm, 1.8 µm; Agilent) maintained at 35°C. The mobile phase was water + 0.1 % formic acid (A), and acetonitrile + 0.1 % formic acid (B) (Optima grade, Fisher Scientific, Lawn, NJ, U.S.A). Mobile phase B was held at 0 % for 0.5 min, before increasing to 100 % over 3 min. Mobile phase B was held at 100 % for 1.5 min, before returning to 0 % B over 0.5 min. Injections of 5 µL were used with a flow rate of 0.3 $mL.min^{-1}$. The following conditions were used for positive HESI: capillary voltage, 4.3 kV; capillary temperature, 400°C; sheath gas, 25 units; auxiliary gas, 15 units; probe heater temperature, 450°C; S-Lens RF level, 65. A top 5, targeted data dependent acquisition (tDDA) method was used that involved a full MS scan at 35,000 resolution over a 200-1100 *m/z*; automatic gain control (AGC) target and maximum injection time (max IT) was 3×10^6 and 128 ms respectively. An inclusion list of the previously identified T-2 and HT-2 metabolites (Meng-Reiterer et al., 2016) in the protonated, ammoniated or sodiated ionization forms was constructed (*m/z* of 467.2276, 484.2541, 489.2095 for T-2 and 425.2170, 442.2435 and 447.1989 for HT-2, respectively). When ion *m/z* signals above 5×10^5 were observed in a full MS scan that corresponded to the inclusion list, they were selected for MS/MS analyses. If no *m/z* signals from the inclusion list were present, the most intense ions were sequentially selected for MS/MS using

a 1.2 *m/z* isolation window and analysed at resolution of 17,500; AGC target, 3×10^6; max IT, 64 ms; stepped NCE 30 and dynamic exclusion of 5 s.

Statistical analysis

Statistical analyses were performed using the package JMP® 14 (SAS Institute Inc., 2016. Cary, NC, USA). Normality and homoscedasticity of each dataset (toxins and gene expression data) was checked using Shapiro-Wilk test and Welsh's test. Non-normality or variance homogeneity was confirmed even after multiple transformation trials performed. Thus, non-parametric test, Kruskal-Wallis, was performed. When significant differences were found (*p-value*<0.05), each pair were compared by a *post-hoc* Wilcoxon method. For gene expression and toxin production comparison, the Spearman test was used.

RESULTS

In vitro *studies on effect on three-way interacting climate change environmental factors on oat-based matrices*

Table 2 shows the effect of three-way interacting treatments on the lag phases prior to growth and the growth rates of *F. langsethiae* on the oat-based media. The lag phases prior to growth were generally <10 h with the exception of 30°C, 0.98 a_w, 1000 ppm CO_2 treatment where 24 hrs were required.

Growth of *F. langsethiae* was optimum at 25°C and 0.995 a_w and unchanged by exposure to elevated CO_2. For the 0.98 a_w treatment, growth was significantly (*p-value*<0.05) higher in elevated CO_2 conditions. At 30°C, the growth rate was lower when compared to 25°C regardless

of the a_w x CO_2 conditions. For both a_w levels tested, elevated CO_2 significantly (*p-value*<0.05) reduced the growth rate, particularly at 0.98 a_w with mycelial extension about 4x times less in the elevated CO_2 treatment.

Figure 1 shows the effect of three-way interacting treatments on the expression of the *Tri5, Tri16* structural and *Tri6* regulatory genes by *F. langsethiae* on oat-based media. For the *Tri5* gene, at 25°C, the relative expression was lower at 0.995 a_w and unchanged at 0.98 a_w (Figure 1a). In elevated CO_2 (1000 ppm), the *Tri5* gene expression was stimulated especially at 0.98 a_w. However, at 30°C, the gene expression remained unchanged, except for a stimulation of expression at elevated CO_2 with freely available water (0.995 a_w).

For the regulatory gene, *Tri6*, the expression was generally downregulated except for 25-30°C and 0.995 a_w (Figure 1b). At 25°C, the gene expression was lower under slight water stress of 0.98 a_w and existing CO_2 conditions (400 ppm) and downregulated at both a_w (0.995 and 0.98) levels when exposed to elevated CO_2 conditions. At 30°C, similar results were obtained. The highest downregulation was observed at 0.98 a_w independently of the CO_2 level.

For the structural gene *Tri16*, the expression was similar to the control in non-elevated CO_2 conditions. At both 25°C and 30°C in elevated CO_2 conditions, this gene was downregulated, especially at 0.995 a_w.

Figure 2 shows the impact of three-way interacting conditions of temperature, a_w and CO_2 levels on T-2+HT-2 production by *F. langsethiae* on oat-based media. At 25°C, there were similar amounts of T-2+HT-2 production under existing CO_2 conditions (400 ppm). In the elevated CO_2 treatment, the production of these two combined toxins was significantly higher at 0.995 a_w. At 30°C, the T-2+HT-2 production was significantly reduced regardless of the a_w and the CO_2 level used. The lowest T-2+HT-2 production by this strain of *F. langsethiae* was at 0.98 a_w under elevated CO_2 conditions.

Effect of the three-way interacting climate change environmental factors on Tri gene expression and T-2/HT-2 toxin production by F. langsethiae in stored oats

The effect of three-way interacting treatments on the expression of *Tri5, Tri16* structural and *Tri6* regulatory genes by *F. langsethiae* in colonised stored oats is shown in Figure 3. For the *Tri5 gene* at 25°C, the gene expression was downregulated independently of the a_w and CO_2 treatments used (Figure 3a). However, at 30°C, the gene expression was downregulated at 0.995 a_w and existing CO_2 concentration (400ppm) and stimulated at 0.98 a_w when exposed to elevated CO_2. For the *Tri6 gene*, the expression generally remained unchanged with the exception of 0.98 a_w and increased CO_2, where the expression was stimulated at both 25°C and 30°C (Figure 3b). The expression of the *Tri16* gene was stimulated under all conditions when compared to that at 25°C, 400 ppm CO_2 and freely available water (0.995 a_w). In addition, at 30°C, the gene expression of the *Tri16* was significantly stimulated at 0.98 a_w in elevated CO_2 conditions.

Figure 4 shows the impact of the three-way interacting climate change conditions on T-2+HT-2 contamination of stored oats. Under relatively conducive conditions of 25°C and 0.995 a_w and 400 ppm CO_2 similar T-2+HT-2 production occurred except when slight water stress was imposed (0.98 a_w). When temperature was increased to 30°C, the production of T-2+HT-2 toxins by *F. langsethiae* was significantly lower when compared to 20°C with freely available water (0.995 a_w). CO_2 concentration appeared to have no statistically relevant effect on toxin production at 0.995 a_w. However, at 0.98 a_w, there appeared to be some stimulation of T-2/HT-2 production, especially at elevated CO_2.

Table 3 summaries the relative impact of the three-way interacting climate change parameters on the *Tri5, Tri6* and *Tri16* gene expression and T-2+HT-2 production in both oat-based media and stored oats. This shows that elevated CO_2 stimulated *Tri5* gene expression, while there was no impact on *Tri6* and *Tri16* gene expression, with both being downregulated. Exposure to elevated CO_2 conditions resulted in a <2-fold change in T-2+HT-2 production. In elevated CO_2 conditions, *Tri6* gene expression (Spearman test; -0.70; *p-value*=0.0027) was inversely correlated with T-2+HT-2 production.

In the stored oat treatments, elevated CO_2 stimulated *Tri5* gene expression at increased temperature (30°C) regardless of the a_w level tested. Under slight water stress, the *Tri6* gene expression was higher regardless of the temperature tested. For *Tri16*, gene expression was stimulated only at 0.98 a_w and 30°C. The combined toxin production was higher at 0.98 a_w independent of the temperature tested. In elevated CO_2 conditions, *Tri16* (Spearman test; 0.68; *p-value*=0.0019) and *Tri5* gene expression (Spearman test; 0.56; *p-value*=0.0151) were correlated with T-2+HT-2 production.

Effect of the three-way interacting climate change environmental factors on non-targeted detection of T-2/HT-2 related metabolites production by F. langsethiae in stored oats

Samples were screened for previously reported T-2 and HT-2 related compounds (Meng-Reiterer et al, 2016) by accurate mass (< 3ppm) and when possible, their identity was confirmed by MS/MS. In addition to T-2 and HT-2 toxins, another 9 related metabolites were detected (Table S1).There were no analytical standards available for these additional metabolites and their relative abundances are represented by the peak area response of the precursor ion detected in full MS mode (< 3 ppm) (Table S2a and S2b).

Among the T-2 and HT-2 metabolites detected, The presence of T-2 toxin glucoside could not be confirmed; the intensity of putative *m/z* signals in the full MS spectra was insufficient to trigger an MS/MS scan. In total, three T-2 toxin related metabolites were detected and confirmed by MS/MS. Six HT-2 related metabolites were also detected (Table S2a). As with the measured concentrations of HT-2 toxin, no related metabolites were found in the control samples, and only hydroxy-HT-2 glucoside, out of the six, was found at 30°C, for an a_w of 0.98 (400ppm) (Table S2b). Two HT-2 toxin-diglucosides were detected and distinguished by different retention times. It is the first time that a new, dehydro T-2 toxin was detected and the conjugate, HT-2 toxin glucuronide was reported *in plantae*. The maximum abundant metabolite was the dehydro T-2 toxin at 25°C and an 0.995 a_w independently of the CO_2 conditions. The peak intensity of this newly identified dehydro T-2 toxin was strongly correlated with the measured concentration of T-2 toxin (R^2=0.8176).

DISCUSSION

This study has compared the effect of three-way interacting CC environmental factors on *F. langsethiae* , relative biosynthetic gene expression of three genes and phenotypic type A trichothecene production in oat-based medium and in stored oats. This has shown that the overall effects *in vitro* were less marked than that observed in stored oats. *In vitro,* only the *Tri5* gene expression was upregulated, especially at 30°C, in intermediate water stress and elevate CO_2 (1000 ppm), when compared to the control. Indeed, elevated temperature (30°C) was the only major factor impacting on T-2/HT-2 toxin production, regardless of the other imposed interacting environmental conditions. *F. langsethiae* usually prefers relatively cool and damp conditions for colonisation and indeed mycotoxin production (Medina and Magan, 2010, 2011). This species may well be less resilient under CC conditions, especially in elevated temperatures and water stress where growth was significantly reduced to about 35-40% compared to that under optimum conditions. This would of course influence the secondary metabolite production patterns. This was supported by the relative increase in production of the *Tri5* gene but not the *Tri6* or *Tri16* genes on oat-based media. Previous studies with more resilient *Fusaria* such as *F. graminearum* and *F. culmorum*, examined the effect of two-way interacting conditions of temperature and water stress on relative gene expression (6 different biosynthetic genes (*Tri4, Tri5, Tri6, Tri10, Tri12* and *Tri13*) and type B trichothecenes (deoxynivalenol, DON) production. This showed that at 0.995-0.95 a_w the expression of most of these genes was optimum at 25-30°C with a good correlation between *Tri* gene expression and phenotypic DON production (Schmidt-Heydt et al., 2010). More recently, Medina et al. (2015b) examined the effect of these two abiotic factors with exposure of CO_2 on growth of *F. graminearum* and also *F. verticillioides*. This showed that the pattern of growth was modified by the three-way interacting factors. However, effects on mycotoxins were not determined.

Studies on stored oats colonized by *F. langsethiae* showed different results when exposed to CC abiotic conditions. This was especially so when examining 30°C and intermediate water stress (0.98 a_w) and 1000 ppm CO_2 exposure. In this treatment there was a stimulation of all three *Tri* genes expression examined and a significant increase in T-2/HT-2 toxin contamination when compared to the controls (20°C, 0.995 a_w, 400 ppm CO_2). Indeed, there was a correlation between the relative expression of these genes and mycotoxin production. Although, *F. langsethiae* appears to be less resilient than other *Fusaria* such as *F. graminearum* and *F. verticillioides* based on data in relation to temperature x a_w stress and in some cases interactions with CO_2 exposure (Medina et al., 2015, 2013; Schmidt-Heydt et al., 2011, 2009). It was suggested that under temperature and intermediate water stress there may be two peaks of secondary metabolite production, the first under optimum and a second peak under abiotic stress conditions (Schmidt-Heydt et al., 2008, 2009).

Previous studies on three-way CC interacting abiotic conditions have predominantly been carried out with xerotolerant and xerophilic mycotoxigenic fungi (*A. flavus*, *A. carbonarious, A. westerdijkiae*) with more limited studies on *Fusaria*, predominantly on *F. graminearum* and *F. verticillioides* (Akbar et al., 2016; Medina et al., 2017; Váry et al., 2015; Vaughan et al., 2016). Of particular interest is the development of acclimatized strains of some of these species by culturing species for 5-10 generations in CC conditions and then comparing these with the original strains in terms of growth, mycotoxin production and indeed plant pathogenicity. Studies with *A. flavus* and *F. graminearum* suggest that the resilience of the strains of these species increased growth rate when grown under CC abiotic conditions and also produced significantly higher amount of mycotoxins and in some cases increased pathogenicity. Such studies have not yet been carried out with strains of *F. langsethiae* and this should provide data on whether

resilience of this species will be enhanced further when acclimatized over several generations of such three-way interacting conditions.

In this study, we have demonstrated for the first time that CC will have an impact on *F. langsethiae* growth, gene expression and T-2+HT-2 production (and related metabolites) under interacting CC conditions. The response to these three-way interacting abiotic factors was higher in stored oats in comparison with oat-based media. The highest impact of elevated CO_2 levels, was at high temperature (30°C) and slight water stress (0.98 a_w), where both gene expression and T-2+HT-2 and their metabolites including the new dehydroT-2 toxin were significantly increased. This type of molecular ecology study will be beneficial in understanding the resilience of such fungal pathogens under expected CC conditions and provide the type of information which can be effectively utilised for developing predictive models which can be utilised in evaluating the relative risks of mycotoxin contamination in the future.

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Table and Figure Legends:

Table 1:

Nucleotide sequences of primers for RT-qPCR assays designed for *Tri5, Tri6, Tri16*, actin and citrate synthase genes.

Table 2:

Effect of water activity (0.995, 0.98) × elevated CO_2 levels (400, 1000 ppm) at 25 and 30°C on the on *F. langsethiae* growth rate and lag time.

Table 3:

Impact of 1000 ppm CO_2 treatment on relative *Tri5, Tri6, Tri16* gene expression and T-2 and HT-2 production at the different temperatures, a_w and matrix tested. Control condition is 20°C, 0.995 a_w and 400 ppm.

Supplementary Table 1:

Detected T-2/HT-2 metabolites in oat samples

Supplementary Table 2:

Peak areas of detected T-2 (a) and HT-2 (b) related metabolites.

Figure 1:

Effect of water activity (0.995, 0.98) × elevated CO_2 levels (400, 1000 ppm) at 25 and 30°C on the relative expression of the *Tri5* (a), *Tri6* (b) and *Tri16* (c) on oat-based medium. Treatments with the same letter are not significantly different (Kruskal-Wallis; *p-value* <0.05) Control condition is 20°C, 0.995 a_w and 400 ppm.

Figure 2:

Effect of water activity (0.995, 0.98) × elevated CO_2 levels (400, 1000 ppm) at 25 and 30°C on T-2 and HT-2 production in oat-based medium. Treatments with the same letter are not

significantly different (Kruskal-Wallis; *p-value* <0.05) Control condition is 20°C, 0.995 a_w and 400 ppm.

Figure 3:

Effect of water activity (0.995, 0.98) × elevated CO_2 levels (400, 1000 ppm) at 25 and 30°C on the relative expression of the *Tri5* (a), *Tri6* (b) and *Tri16* (c) on irradiated oat grain. Treatments with the same letter are not significantly different (Kruskal-Wallis; *p-value* <0.05) Control condition is 20°C, 0.995 a_w and 400 ppm.

Figure 4:

Effect of water activity (0.995, 0.98) × elevated CO_2 levels (400, 1000 ppm) at 25 and 30°C on T-2 and HT-2 production on irradiated oat grain. Treatments with the same letter are not significantly different (Kruskal-Wallis; *p-value* <0.05) Control condition is 20°C, 0.995 a_w and 400 ppm.

Table 1.Nucleotide sequences of primers for RT-qPCR assays designed for *Tri5, Tri6, Tri16*, actin and citrate synthase genes.

Primers	Gene	Oligonucleotide sequence (5'-3')	Product length (pb)	Origin
actf	Actin	CCTATCTACGAGGGTTTCGCC	73	This article
acfr		AGTCGGTAAGATCACGACCAG		
citf	Citrate	CCCATGTTCAAGCTCGTCTC	88	This article
citr	synthase	CGTTAGGGTAGGGGTTCTTGG		
Tri5f	TRI5	GATCTGATGACTACCCTCAATTCCTT	71	(Marín et al., 2010)
Tri5r		GCCATAGAGAAGCCCCAACAC		
Tri6f	TRI6	CGCTTTCGAATATGGTGGTT	206	(Ferruz et al., 2016)
Tri6r		CCTACGGTGGAGCCTACAAA		
Tri16f	TRI16	GGTCTGGTCTAATCTTACA	147	(Ferruz et al., 2016)
Tri16r		CACGACATTACCCATATAAG		

Table 2.Effect of interacting abiotic factors of temperature, water activity and CO_2 on growth of *F. langsethiae in vitro* on oat-based matrices.

Temperature	**Water activity (a_w)**	**CO_2 treatment (ppm)**	**Lag phase (days)**	**Diametric growth rate ($mmday^{-1}$)**
20ºC	0.995	400	0.4 ± 0.2^{b}	13.2 ± 0.3^{b}
25ºC	0.995	400	0.3 ± 0.2^{b}	15.1 ± 0.6^{a}
		1000	<0.1	15.5 ± 0.5^{a}
	0.98	400	<0.1	11.6 ± 0.2^{c}
		1000	0.2 ± 0.1^{c}	$13.3\pm1.9^{a,b}$
30ºC	0.995	400	0.2 ± 0.3^{c}	8.6 ± 0.3^{e}
		1000	<0.1	7.9 ± 0.2^{f}
	0.98	400	<0.1	9.1 ± 0.1^{d}
		1000	1.0 ± 0.6^{a}	2.1 ± 0.6^{g}

<0.1 No Lag time calculated; S.D.: standard deviation. Treatments with the same letter are not significantly different (Kruskal-Wallis; *p-value* <0.05)

Table 3. Summary of the effect of climate change environmental factors on relative biosynthetic gene expression of *Tri5*, *Tri6* and *Tri16* and T-2+HT-2 toxins produced by *F. Langsethiae in vitro* on oat-based medium and in stored oats.

Temperature	**a_w**	**Matrix**	***Tri5***	***Tri6***	***Tri16***	**T-2+HT-2**
25ºC	0.995	Oat-based medium	↑ (x5.8)	↓ (x0.3)	↓ (x0.0)	=[1]
		Stored oats	=	=	=	=
	0.98	Oat-based medium	↑ (x2.4)	=	↓ (x0.0)	=
		Stored oats	=	↑ (x3.1)	=	↑ (x3.5)
30ºC	0.995	Oat-based medium	↑ (x4.1)	↓ (x0.2)	↓ (x0.0)	=
		Stored oats	↑ (x2.1)	=	=	=
	0.98	Oat-based medium	↑ (x2.4)	=	↓ (x0.1)	=
		Stored oats	↑ (x6.7)	↑ (x2.0)	↑ (x2.6)	↑ (x72.8)

[1]=variation lower than 2-fold. ↑ or ↓: variation higher/lower than 2-fold.Numbers between brackets refer to the fold-variation with respect to the control.

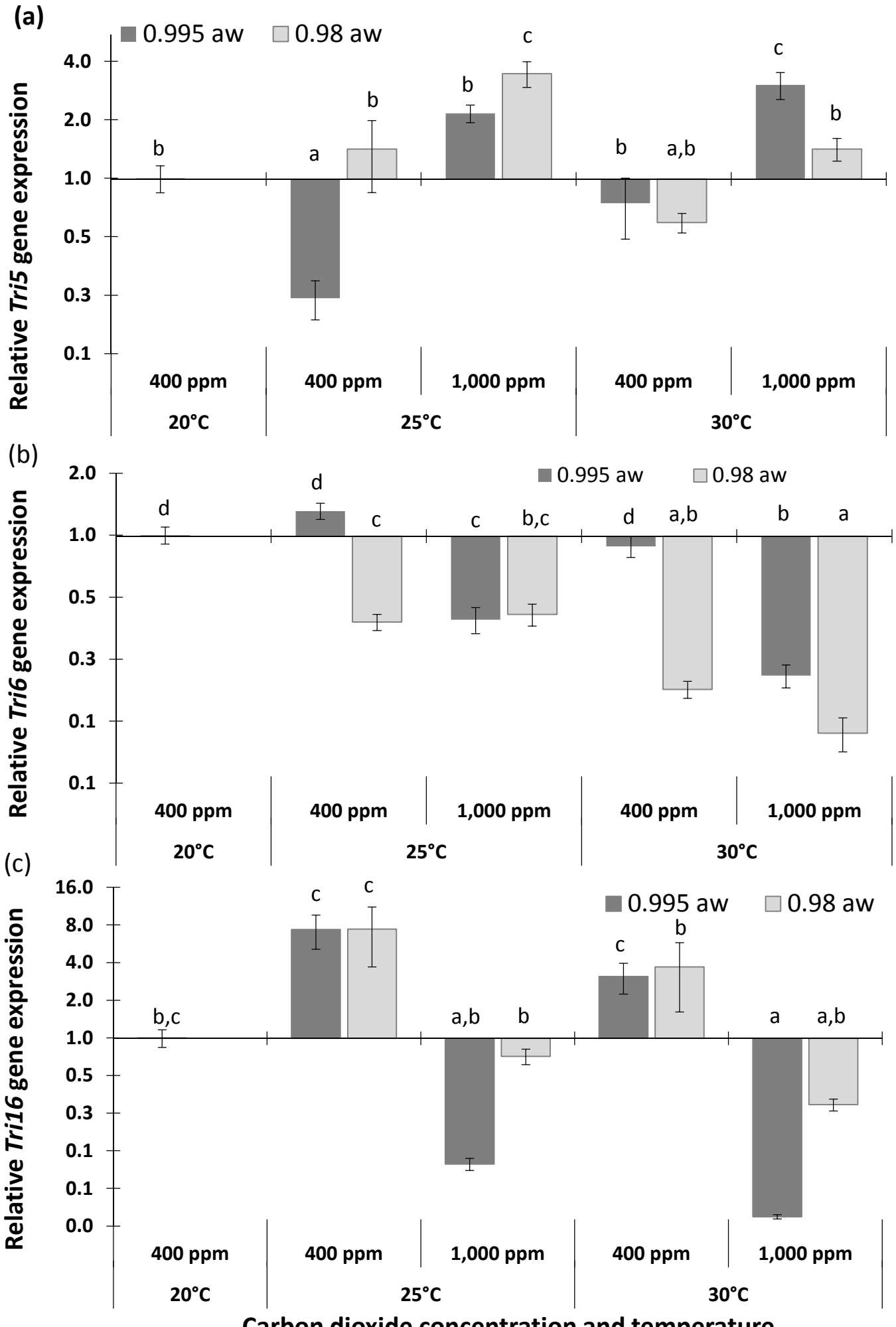

Figure 1: Verheecke-Vaessen et al.

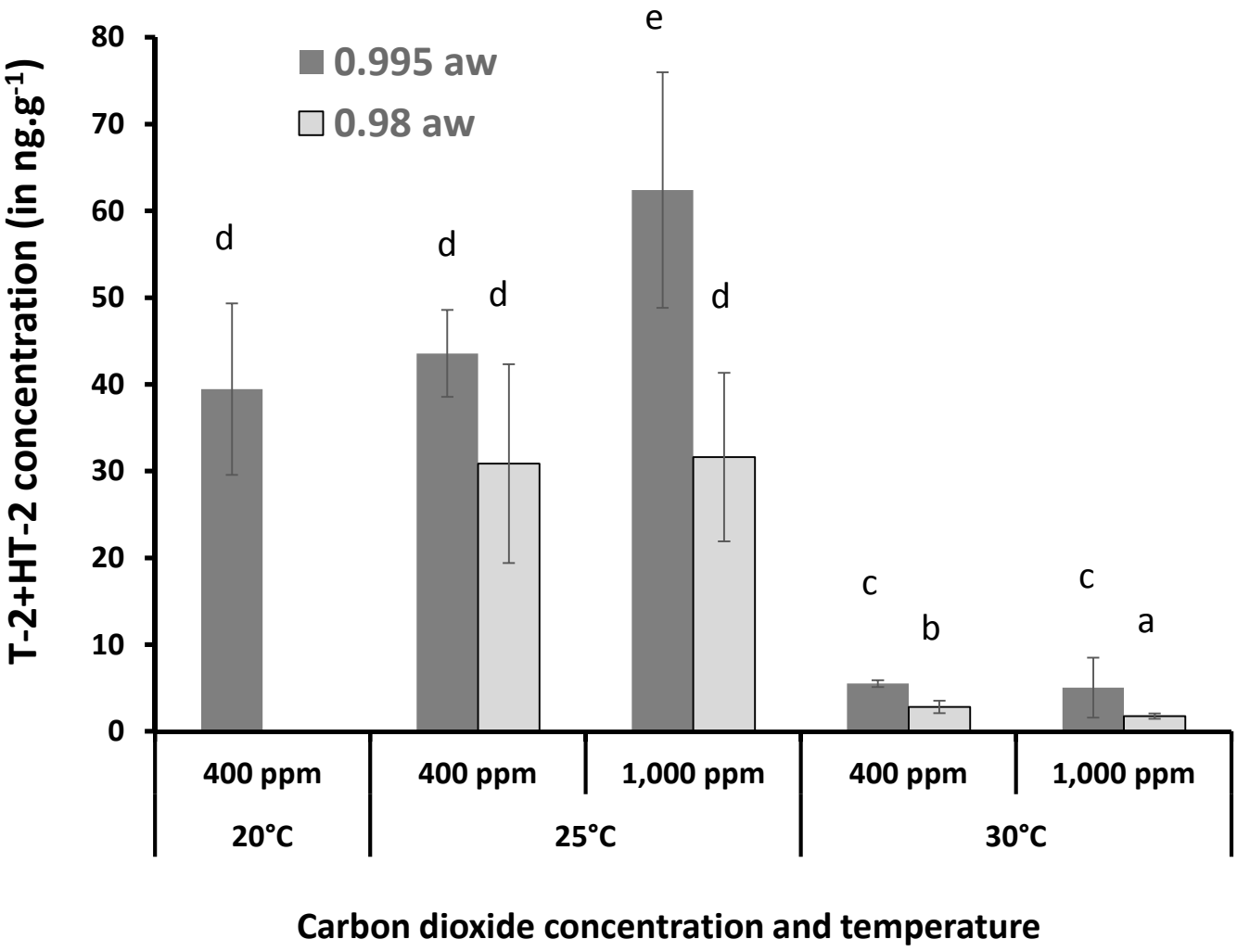

Figure 2: Verheecke-Vaessen et al.

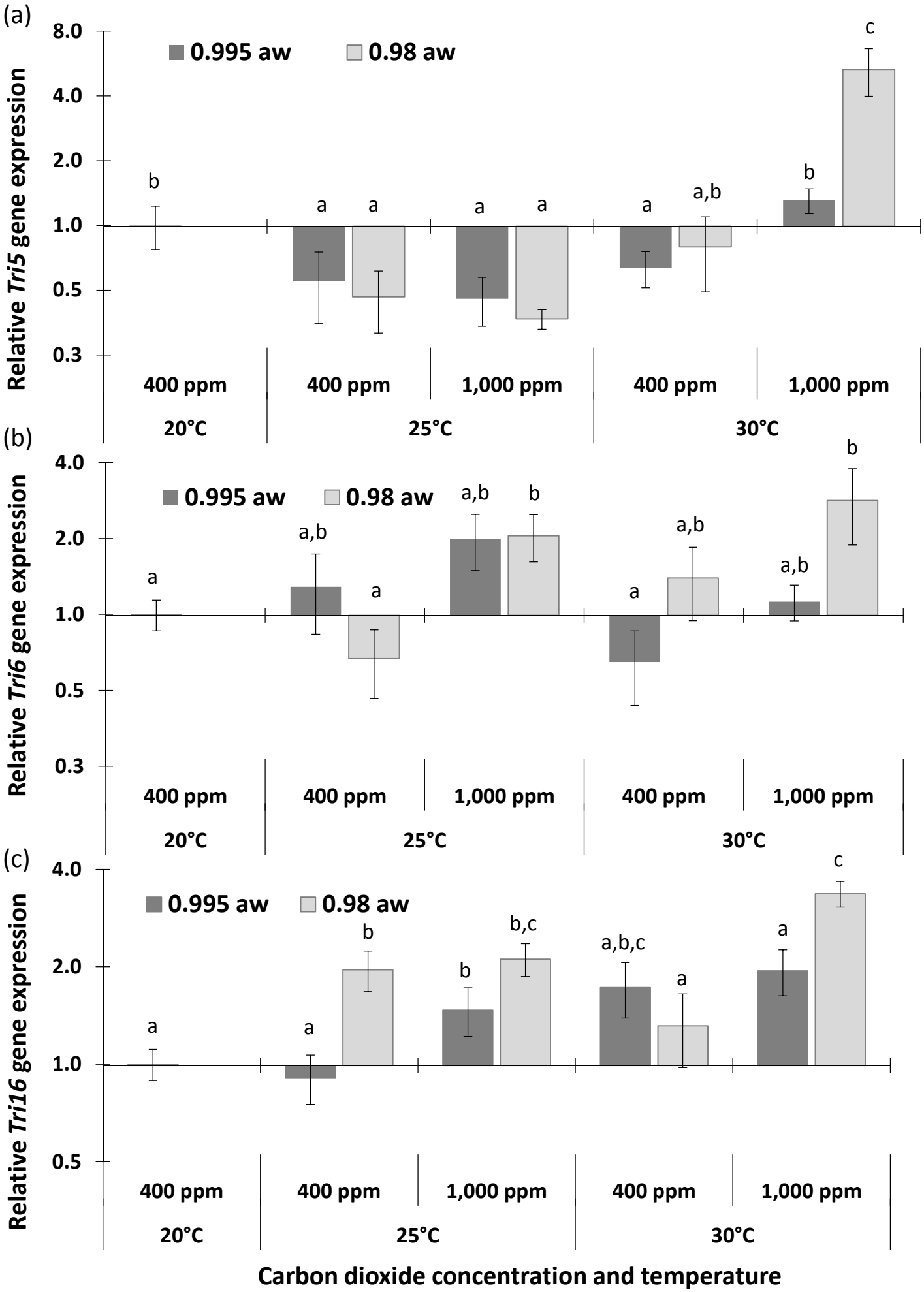

Figure 3: Verheecke-Vaessen et al.

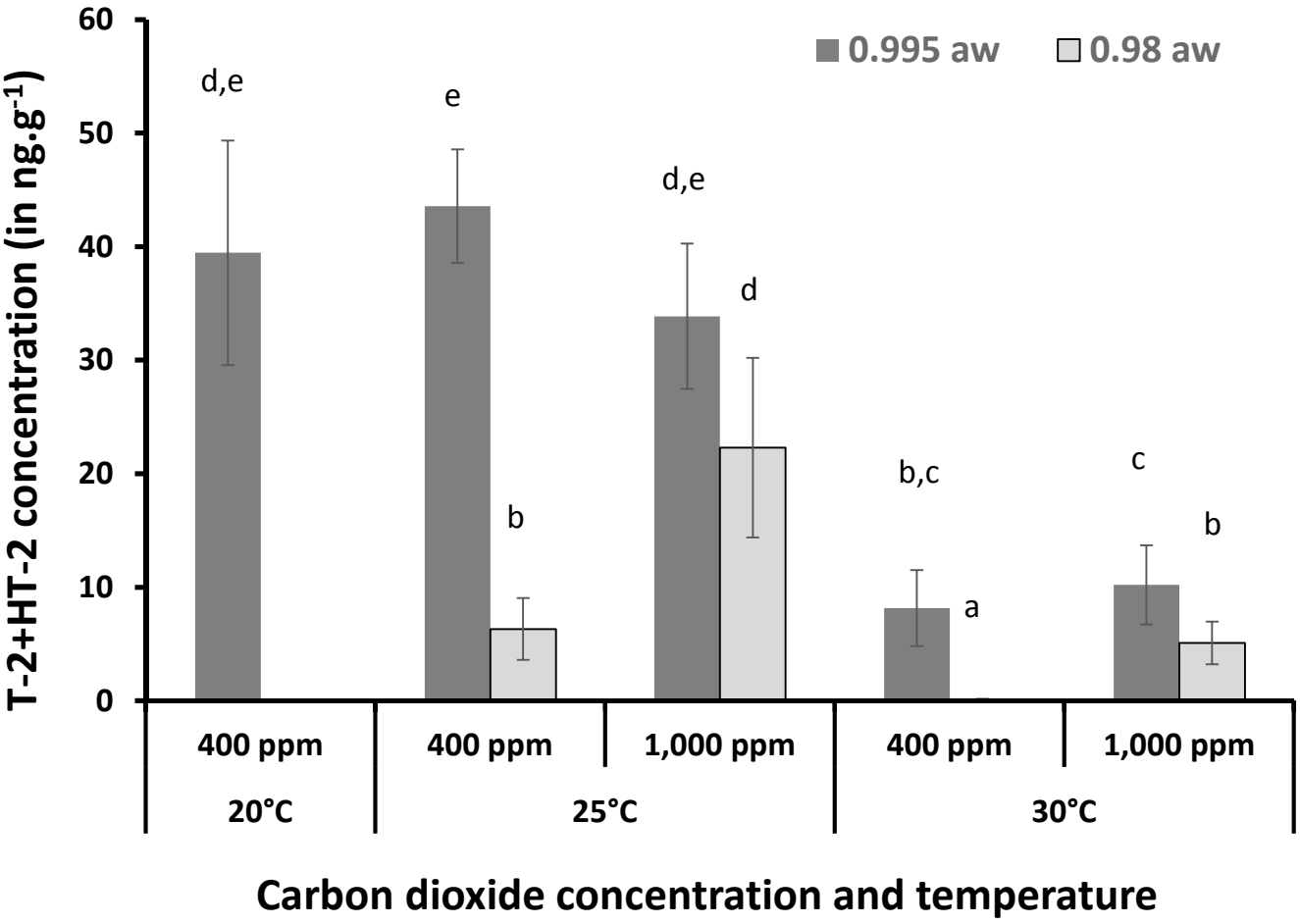

Figure 4: Verheecke-Vaessen et al.