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Dynamics of solute/matric stress interactions with climate change abiotic factors on growth, gene expression and ochratoxin A production by *Penicillium verrucosum* on a wheat-based matrix

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ABSTRACT
Penicillium verrucosum contaminates temperate cereals with ochratoxin A (OTA) during harvesting and storage. We examined the effect of temperature (25 *vs* 30 ºC), CO_2 (400 *vs* 1000 ppm) and matric/solute stress (-2.8 *vs* -7.0 MPa) on (i) growth, (ii) key OTA biosynthetic genes and (iii) OTA production on a milled wheat substrate. Growth was generally faster under matric than solute stress at 25 ºC, regardless of CO_2 concentrations. At 30 ºC, growth of *P. verrucosum* was significantly reduced under solute stress in both CO_2 treatments, with no growth observed at -2.8 MPa (=0.98 water activity, a_w) and 1000 ppm CO_2. Overall, growth patterns under solute stress was slower in elevated CO_2 than under matric stress when compared with existing conditions. The *otapks*PV gene expression was increased under elevated CO_2 levels in matric stress treatments. There was fewer effects on the *otanrps*PV biosynthetic gene. This pattern was paralleled with the production of OTA under these conditions. This suggest that *P. verrucosum* is able to actively grow and survive in both soil and on crop debris under three way interacting climate-related abiotic factors. This resilience suggests that they would still be able to pose an OTA contamination risk in temperate cereals post-harvest.

Keywords: water availability, mycotoxin, qPCR, biosynthetic genes, climate change scenarios, abiotic stress

INTRODUCTION
Penicillium verrucosum is a predominantly soil-based xerotolerant species that also survives saprophytically on crop residue. It colonises temperate cereal grains during harvesting and delayed drying or poor post-harvest management which can lead to ochratoxin A (OTA) contamination of pockets of under-dried or moist grain (Lund and Frisvad, 2003; Lindblad et al., 2004; Magan and Aldred, 2007). Indeed, because OTA is considered to be a nephrotoxin and potentially carcinogenic for human (International Agency for Cancer Research, 1993) there

are legislative limits in cereals destined for food processing or for animal feed (European Union, 2006).

Previous ecological studies have shown that water availability, temperature and inter-granular atmosphere and their interactions have an impact on growth and OTA production *in vitro* and *in situ* in stored wheat grain and identified the optimum and boundary conditions for growth and OTA production (Cairns et al., 2005). It has also been shown that populations of *P. verrucosum* predominantly reside in soil and on crop residue which form the focal points for the development of the inoculum for contaminating cereals during harvesting and drying (Elmholt, 2003; Elmholt and Hostbjerg, 1999). Thus, an understanding of the relative tolerance of *P. verrucosum* to both soil water stress, mainly determined by the soil matric potential, and solute stress in crop residue is important. Abdelmohsen et al. (2020) recently showed that optimum growth and OTA production were at -7.0 MPa (=0.95 water activity, a_w) and -1.4 MPa (=0.99 a_w) respectively, regardless of whether solute or matric stress were imposed on *P. verrucosum.* However, this species was more sensitive to ionic solute stress (NaCl) with no growth at -19.6 MPa (=0.86 a_w) while growth still occurred in the presence of the non-ionic solute (glycerol) and matric stress treatments.

Previous studies with non-xerophilic toxigenic fungi such as *Fusarium graminearum*, and xerophilic/xerotolerant species such as *Aspergillus ochraceus* (= *A. westerdijkiae*) and *A. flavus* have examined the relative tolerance to matric *vs* solute stress (Ramos et al., 1999; Ramirez et al., 2004; Giorni et al., 2008). These showed that for the non-xerophilic species both macroconidial germination and growth were more sensitive to matric than solute stress. In contrast, the xerotolerant/xerophilic species were more resilient and able to tolerate both matric and solute stress (Magan, 1988; Magan et al., 1995; Ramos et al., 1999; Ramirez et al., 2004). Subsequently, Jurado et al. (2008) showed that the non-xerophilic mycotoxigenic species *F. verticillioides,* a pathogen of maize, grew relatively similarly under both ionic and non-ionic solute stress, but was also more sensitive to matric stress. The relative expression of the *FUM1* gene involved in fumonisins biosynthesis reflected these differences.

OTA is a polyketide mycotoxin, with the biosynthetic pathway predominantly elucidated in *P. nordicum* (Wang et al., 2016). In this species, the gene cluster for OTA includes those encoding for a polyketide synthase (PKS) (*otapks*PN) and non-ribosomal peptide synthetase (NRPS) (*otanrps*PN). Geisen et al. (2004) correlated the relative expression of the *otapks*PN from *P. nordicum* with OTA production. There is a good homology between the OTA biosynthetic pathways in both *P. nordicum* and *P. verrucosum,* with some differences related to the function of the PKS gene (*otapks*) (Geisen et al., 2006; Wang et al., 2016). Abdelmohsen et al. (2020) were able to show that *P. verrucosum* was able to express the *otapks*PV over a wide range of ionic/non-ionic solute stress conditions (-1.4 to -14.0 MPa; =0.99 - 0.90 a_w). Interestingly, the *otanrps*PV gene was significantly up-regulated under matric stress, especially with relatively freely available water (-1.4 MPa = 0.99 a_w). These studies focused on solute/matric stress and did not examine the effects of interactions with temperature or other abiotic factors.

There is now interest in the resilience of mycotoxigenic fungi to climate-related abiotic factors and whether this will stimulate or inhibit mycotoxin production. Such interacting factors have been shown to result in stimulation of biosynthetic genes involved in mycotoxin production and phenotypic toxin production including aflatoxins by *A. flavus*, OTA by *A. westerdijkiae* and T-2/HT-2 toxin by *F. langsethiae* (Akbar et al., 2016, 2020; Medina et al., 2017; Verheecke-Vaessen et al., 2019; Cervini et al., 2020). However, no studies have previously examined solute *vs* matric stress when combined with changes in temperature and exposure to existing or elevated CO_2 may have on growth, biosynthetic genes involved in toxin production and the amounts of toxin production. This may be important in understanding the potential changes in the life cycle and ecological characteristics of this species especially in

soil and on crop debris which will influence the inoculum potential for contamination of cereals with OTA, especially in the harvesting, drying and post-harvest phases.

Thus, the objectives of this work were to examine the effect of solute or matric stress (-2.8 or -7.0 MPA (=0.98 and 0.95 a_w), temperature (25 or 30 ^oC) and exposure to CO_2 (400 *vs* 1000 ppm) on: (a) growth, (b) relative expression of two key biosynthetic genes (*otapks*PV, *otanrps*PV) involved in OTA biosynthesis and (c) OTA production by *P. verrucosum* on a milled wheat matrix.

MATERIALS AND METHODS

Fungal strain

A strain of *P. verrucosum* (OTA11) was used in these studies. This was isolated from wheat grain and is a known producer of OTA (Cairns et al., 2005; Abdelmohsen et al., 2020). We are grateful to Dr. Monica Olsen (National Food Authority, Sweden) for the supply of the strain.

Inoculum preparation and inoculation

The fungal strain was sub-cultured on malt extract agar (30.0 g L^{-1} malt extract, 5.0 g L^{-1} peptone and 15.0 g L^{-1} agar) at 25 °C in the dark for up to 10 days. The spores were gently dislodged from the colony surface by using a surface sterilised loop and placing them into suspension in 9 ml sterile distilled water containing 0.05% (v/v) Tween-80 in 25 ml Universal bottles. The suspensions were shaken and then the spore concentration determined using a haemocytometer and adjusted to 10^6 spore ml^{-1}. This was used for inoculation by taking 0.1 ml of an inoculum and spreading onto a 2% milled wheat agar medium which was incubated overnight at 25 °C. The germlings were then used as the inoculum and 4 mm agar discs were taken with a surface sterilised cork borer and used to centrally inoculate the treatment plates.

Solute and matric potential modified media

A basal 2% (w/v) milled wheat agar medium was used in this study. This medium was modified to -2.8 (=0.98 a_w) and -7.0 MPa (=0.95 a_w) water potentials by using mixtures of the non-ionic solute glycerol + water (Abdelmohsen et al., 2020). The media were autoclaved at 121 ℃ and poured into 9 cm Petri plates (approx. 15 ml) and kept at 4 ℃ in separate plastic bags until used. The final a_w levels were checked with an Aqua Lab TE4 (Decagon Devices. Pullman, WA, 99163, USA).

For modification of the matric potential, the agar was omitted and the 2% (w/v) milled wheat was mixed with different amounts of PEG 8000 to obtain the target matric potentials detailed above. These matric potentials were checked using the Aqua Lab 4 TE. Previous studies have shown that the water potential generated by PEG 8000 is predominantly (99%) due to matric forces (Steuter et al., 1981). The media were prepared in 9 cm Petri plates that contained a sterile circular 8.5 cm diameter disc of capillary matting. After decanting 15 ml of the sterile cooled 2% (w/v) wheat broth medium into the Petri plates they were then overlayed with sterile circular layers 8.5 cm diameter of polyester fibre and then a sterile cellophane layer. This method has been detailed previously (Jurado et al., 2008). The different treatments were kept in different polyethylene bags at 4 ^oC and kept closed to avoid moisture loss and changes in solute/matric potential regimes. These were removed and equilibrate at 25 ^oC before they were centrally inoculated as described previously. The inoculated solute and matric stress treatments and replicates were incubated at either 25 or 30 ^oC in the environmental chambers.

Effect of interacting climate-related abiotic conditions on* P. verrucosum *growth/OTA production in relation to solute and matric imposed water stress.

The different treatments and replicates were placed in separate 13 L plastic environmental chambers (Verheecke-Vaessen *et al.,* 2019). These chambers also contained inlet and outlet valves at each end. The chambers contained glycerol/water solutions (2 x 500 mls) to maintain the equilibrium relative humidity (erh) of the atmosphere within the individual chambers at the target water potential levels. The chambers were flushed with either synthetic air or 1000 ppm CO_2 daily for 10 days. The gas cylinders contained either 400 ppm CO_2 (ambient air) or a speciality gas of 1000 ppm CO_2 (certified gas; British Oxygen Company, Guildford, Surrey, U.K.). The environmental chambers were flushed at 3 L min^{-1} to replace 3x the volume of the incubation chamber every 24 hrs and incubated at the target temperatures.

Growth assessment

Colony diameters of 4-5 replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily or as required for up to ten days. The growth rate was calculated by plotting the radial mycelial growth against time and the linear regression of the slope of the linear growth phase was used to obtain the radial growth rates (mm day^{-1}, Medina and Magan, 2010).

Isolation of total RNA

The fungal biomass was harvested after 10 days incubation in the presence of liquid nitrogen to keep the integrity of the RNA and stored at -80 °C for molecular work, and -20 °C for OTA analysis.

The fungal cell walls were disrupted using the bead-beating method recommended by Leite *et al*. (2012). The RNA was extracted using the Total RNA Spectrum Plant Kit (Sigma, UK) following the manufacturers protocol. To remove genomic DNA contamination, samples were treated with an on-column DNase digestion using the RNase-Free DNase Set Kit (Qiagen, UK). The RNA concentration and purity (A_{260}/A_{280} ratio) & (A_{260}/A_{230} ratio) were determined spectrophotometrically using a 2.5 µL aliquot on the Picodrop (Spectra Services Inc., USA). For checking the RNA integrity, the Experion™ Automated Electrophoresis System using the Experion RNA StdSens analysis kits (Bio-Rad Laboratories Ltd., Hertfordshire, UK) was used, where the RQI that the minimum quality control was set at RQI >7.

RT-qPCR assays and relative quantification

RT-qPCR assays were used to amplify the *otapks*PV and *otanrps*PV genes, with the ß-tubulin gene used as the reference.

(a) ***Primers:*** The primer pairs PV-bentaqfor/rev, previously designed from the *otanrps*PV gene involved in the OTA biosynthetic pathway (Rodríguez *et al*., 2011) and the β-tubulin gene (Leite, 2013) were used. Nucleotide sequences of primers used in the RT-qPCR assays are detailed in Table 1.

(b) ***Relative Gene Expression:*** Relative quantification of the expression of *otapks*PV and *otanrps*Pv genes was performed using the reference ß-tubulin gene. To calculate the ΔCq, Cq of the gene of interest was subtracted from the Cq of the reference gene (Rodriguez et al., 2014). Subsequently, for ΔΔCq, the non-modified medium used as a control.

Quantification of OTA production

The treatments were harvested after 10 days. For solute stress treatments 5 (5 mm diameter) plugs were taken across the colony using a sterile cork borer. For matric stress treatments biomass was taken from the cellophane surface and combined with 1-2 mls of medium below the colony area. The samples were placed into 2 mL Eppendorf tubes and weighed. OTA was extracted by adding 1 mL HPLC grade methanol and shaken for 1 hour at 200 rpm at 25 °C. The medium and biomass were separated from the extraction solvent by centrifugation for 10 min at 15000 x g. The extracts were filtered through a 0.22 µm (type PTFE) filter directly into amber HPLC vials. The conditions for OTA detection and quantification were as follows:

Mobile Phase	Acetonitrile (57%):Water (41%):Acetic acid (2%)
Column	C_{18} column (Poroshell 120, length 100 mm, diameter 4.6 mm, particle size 2.7 micron).
Temperature of column	25 ºC
FLD Excitation wavelength	330 nm
FLD Emission wavelength	460 nm
Flow rate	1 ml min^{-1}
Retention time	2.6 min
Run time	13 min
Limit of Detection:	2.83 ng g^{-1}
Limit of Quantification:	9.43 ng g^{-1}

The OTA was analysed using HPLC-FLD by including OTA standards at different concentrations with each batch and their peaks detected by Agilent Chem-Station software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA). Comparisons were made between the standard curve and the different treatments and replicates (Abdelmohsen et al., 2020).

Statistical analysis

Each treatment was carried out with 4-5 replicates for growth rate assessment, gene expression and OTA production and repeated once. The normality was checked using the Shapiro test and homoscedasticity was checked using the Levene test. The factors and responses were examined using the Kruskall-Wallis (non-parametric) when the data were not normally distributed. For normally distributed data, the data sets were analysed using ANOVA in JMP® 14 (SAS Institute Inc., 2016. Cary, NC, USA). The statistical significant level was set at $p<0.05$ for all single and interacting treatments.

RESULTS

Effect of climate change-related interacting factors on relative growth rates at 25 °C and 30 °C on wheat-based matrices

Figure 1a, b compares the effect of matric and solute stress, temperature (25 and 30 ^{o}C) and CO_2 exposure (400 or 1000 ppm) on the relative growth of the *P. verrucosum* strain. Growth was significantly affected when exposed to 30 ^{o}C and -2.8 MPa (= 0.98 a_w) and 1000 ppm CO_2 where no growth occurred in the solute stress treatment. However, at -7.0 MPa (0.95 a_w) and 1000 ppm CO_2 there was an increased growth rate when compared to existing conditions. With matric stress there was no effect on growth, with similar colonisation rates under all the treatments at 30 ^{o}C (Figure 1b).

Statistically, the impact of treatments showed that there was a significant effect of the different individual abiotic factors on growth at 25 °C. At 30 ^{o}C, with solute imposed stress, there was a significant effect when exposed to 1000 ppm CO_2 at -2.8 MPa water potential (=0.98 a_w) as no growth was observed.

Table 1. Nucleotide sequences of primers for RT-qPCR assays

Primer pairs	Gene	Nucleotide sequences (5'-3')	Product size (pb)	Publication
PV-bentaq-for	ß-tubulin	CTAGGCCAGCGCTGACAAGT	63	Leite, (2013)
PV-bentaq-rev	ß-tubulin	CTAGGTACCGGGCTCCAA	63	
*otapks*PV-for	*otapks*PV	TTGCGAATCAGGGTCCAAGTA	1080	Schmidt-Heydt et al. (2007)
*otapks*PV-rev	*otapks*PV	CGAGCATCGAAAGCAAAAACA	1080	
*otanprs*PV-for	*otanrps*PV	GCCATCTCCAAACTCAAGCGTG	699	Rodriguez et al. (2011)
*otanprs*PV-rev	*otanrps*PV	GCCGCCCTCTGTCATTCCAAG	699	

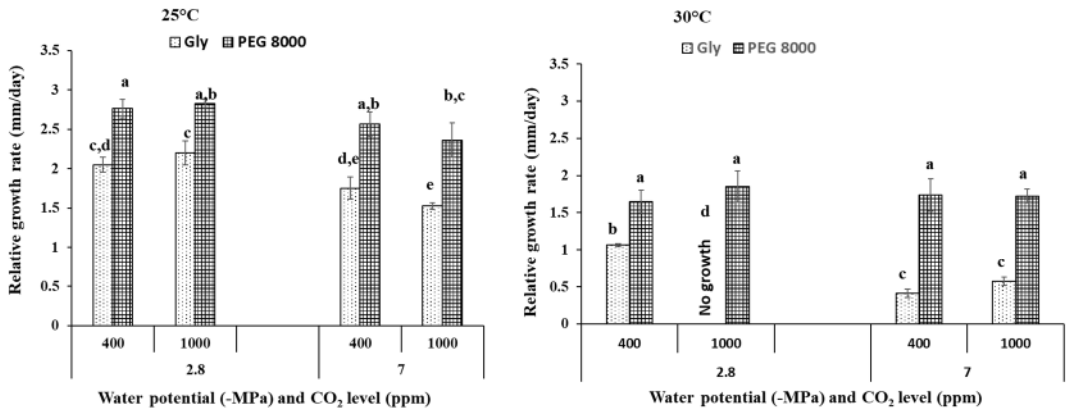

Figure 1. Effect of solute or matric potential stress x CO_2 (400 or 1000 ppm) x temperature (25 and 30 °C) on relative growth rate of *P. verrucosum* grown on wheat-based media modified with glycerol (non-ionic solute potential) or PEG 8000 (matric potential) after 10 days growth on milled wheat media. Different letters indicate significant differences between treatments.

Effect of climate change-related abiotic factors on two biosynthetic genes involved in ochratoxin A production on a wheat-based matrix

Effects on* otapks*PV gene expression: At 25 °C, the pattern of gene expression at the two water stress levels in the different media was quite different (Figure 2). Under solute stress, at -2.8 MPa water potential (= 0.98 a_w), the gene expression was increased at 1000 ppm when compared to the existing conditions. However, under matric stress, the gene expression was lower in the 1000 ppm CO_2 and water stress treatment of -7.0 MPa (= 0.95 a_w) when compared to existing conditions.

Statistically, the imposed water stress and type of solute stress had a significant effect on the relative *otapks* gene expression at 25 °C. However, exposure to elevated CO_2 levels showed no significant influence on this toxin biosynthetic gene, which remained constant, regardless of the imposed solute or matric stress (Figure 2a).

However, at 30 °C, the pattern of expression of this gene suggested more resilience and tolerance to the interacting abiotic stresses imposed. Under matric stress, especially at -7.0 MPa (=0.95 a_w) there was a significant effect on growth, especially at elevated CO_2 (1000 ppm) conditions (Figure 2b).

Effects on* otanrps*PV gene expression: The expression of the *otanrps* gene involved in OTA biosynthesis, had expression patterns consistent with that of the *otapks* gene under matric potential stress (Figure 3a). In contrast, very low gene expression occurred in the solute stress treatments. Statistically, the expression of this gene was significantly reduced when *P. verrucosum* was exposed to elevated CO_2 (1000 ppm) under water stress of -7.0 MPa (=0.95 a_w) at 25 °C. At 30 °C, the gene expression remained constant, but decreased significantly under elevated CO_2 and increased water stress, especially in the matric potential modified treatments (Figure 3b).

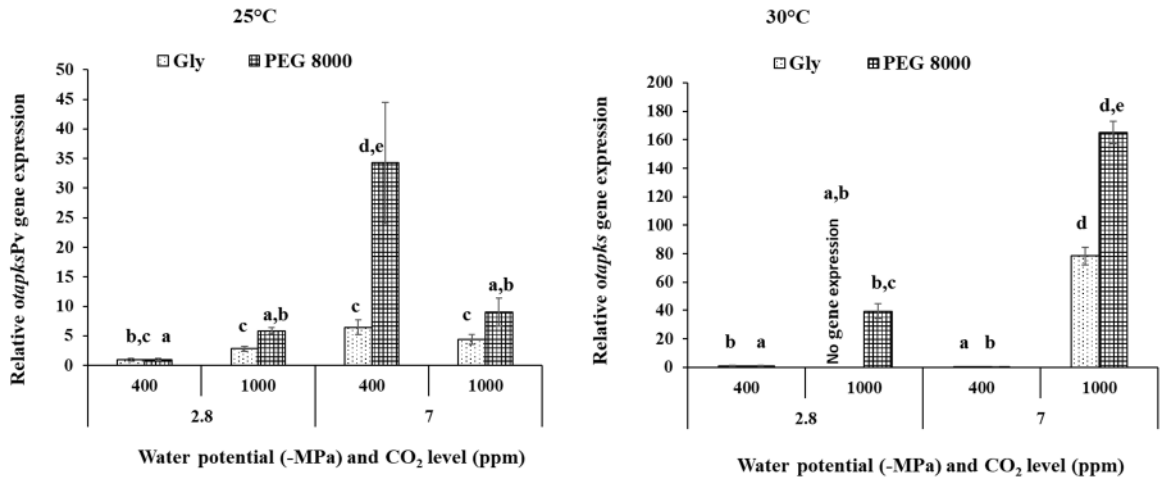

Figure 2. Abdelmohsen et. al.

Figure 2. Effect of solute or matric potential stress x CO_2 (400 or 1000 ppm) x temperature (25 and 30 ºC) on relative *otapks*PV gene expression of *P. verrucosum* grown on the milled wheat-based media for 10 days. Comparisons were made with the control treatment of 400 ppm CO_2 and -2.8 MPa (=0.98 a_w) as the calibrator for each medium separately. Different letters indicate significant differences between the treatments.

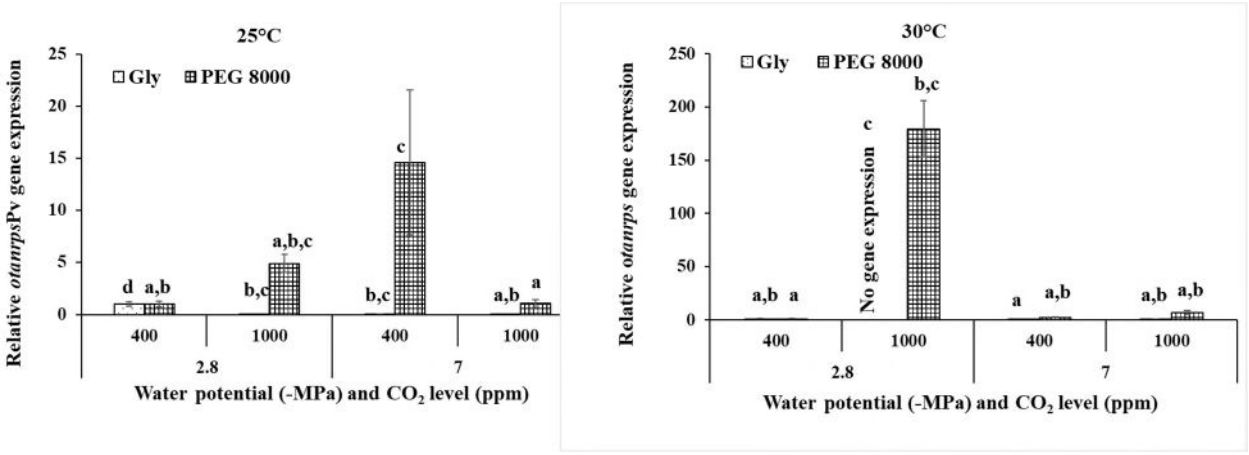

Figure 3. Effect of solute or matric potential stress x CO_2 (400 or 1000 ppm) x temperature (25 and 30 °C) on relative *otanrps*PV gene expression of *P. verrucosum* grown on the milled wheat media for 10 days. The control treatment (400 ppm CO_2, -2.8 MPa water potential (= 0.98 a_w) used as a calibrator for each medium separately. Different letters indicate significant difference between treatments.

Effect of climate change-related abiotic factors on OTA production by P. verrucosum

The concentrations of OTA (µg g^{-1}) produced by the *P. verrucosum* cultures when grown on wheat-based media in elevated CO_2 showed differences in tolerance to the imposed types of water stress (Table 2). This strain was more tolerant of matric stress with a consistently higher toxin production pattern regardless of water potential and CO_2 concentrations at 25 °C. However, in the solute stress-modified media, very low amounts of OTA was detected.

At 30 °C, tolerance of *P. verrucosum* to matric imposed stress resulted in a stimulation of OTA in the 1000 ppm CO_2 exposure treatment when compared to that in air (400 ppm; Table 3). Statistically, single factors and some two-way interacting factors such as solute type x temperature and water potential x solute type were significant (Table 3). However, all the three-way climate-related interacting abiotic factors showed no significant effects on toxin production.

Table 2. Effect of solute/matric potential stress x elevated CO_2 x temperature on OTA production (µg/g) on wheat-based media modified with glycerol (solute) or PEG 8000 (matric potential stress) after 10 days incubation.

			25°C		30°C	
	Water potential (-MPa)	**CO_2 level (ppm)**	**Solute stress (Glycerol-amended media**	**Matric Stress (PEG 8000-amended media**	**Solute stress (Glycerol-amended media**	**Matric stress (PEG 8000-modified media**

2.8	400	0.75±0.17	55.74±39.75	0.04±0.01	2.05±1.40
	1000	0.04±0.03	83.55±14.16	ND*	1.92±0.65
7.0	400	0.06±0.01	156.00±90.90	0.16±0.03	0.93±0.46
	1000	0.08±0.01	102.43±66.95	0.10±0.01	4.27±3.24

*ND: not determined because of no growth

Table 3. Summary statistical analyses for the effect of interacting climate-related abiotic factors on ochratoxin A production in relation to single, two-, three- and four-way interactions between factors. The probability values in bold were significant (p<0.05). Based on ANOVA results for the effects of water potential, type of water potential (non-ionic solute; matric), CO_2 level (400 ppm, 1000 ppm) and temperature (25, 30 °C) were analysed.

Factor	Significance (p<0.05)
Water potential	**0.0261**
Solute type	**<0.0001**
Temperature (temp)	**0.0010**
CO_2	0.2180
Solute type x temp	**0.0010**
Water potential x solute type	**0.0240**
CO_2 x temp	0.3609
CO_2 x solute type	0.2180
Solute type x temp x CO_2	0.3609
Water potential x temp x solute type	0.1066
Water potential x temp x CO_2	0.3494
Water potential x solute type x CO_2	0.1824
Water potential x CO_2 x solute type x temp	0.3402

Discussion

This study has examined the effect of different types of water stress and their interaction with other climate change-related scenarios on the molecular ecology of *P. verrucosum.* To our knowledge, no previous studies have addressed this in the context of resilience of such mycotoxigenic fungi in relation to interacting abiotic stresses relevant to activity in soil and on crop residue. This OTA producing strain was able to grow at both the tested solute and matric imposed stress conditions (-2.8, -7.0 MPa) with no significant differences between exposure to existing and elevated CO_2 at 25 °C. However, when temperature was elevated by +5 °C, the growth pattern was different in both water stress treatments. In the solute-modified wheat media, growth was decreased significantly in the elevated CO_2 treatments with no growth observed in the 1000 ppm, and -2.8 MPa (=0.98 a_w) treatment. The general pattern of growth was lower than at 25 °C. However, under matric potential stress, growth was faster, regardless of the water stress level or CO_2 level. *P. verrucosum* is normally considered to be a problem in temperate cereals in cooler climatic regions.

The present study and previous study by Abdelmohsen *et al.* (2020) suggest that certainly at ≤25 °C this species will remain active and colonise both soil and cereal crop residue effectively because of the tolerance of both matric and solute stress. However, at 30 °C, inoculum potential may be reduced in crop residue because of the lack of resilience to solute stress shown in the present study. However, under matric stress in both existing and elevated CO_2 conditions this species is very resilient and soil may be a more important reservoir for the inoculum of this species than crop debris.

Previous studies have suggested that soil populations of *P. verrucosum* can vary between 100-300 CFUs g^{-1} soil, and is very competitive in the soil and crop residue niches (Elmholt, 2003; Elmholt and Hostbjerg, 1999). However, these studies did not examine the impact of solute and matric stress on the *P. verrucosum* populations. Studies by Magan (1988) examined both *in vitro* and *in situ* effects of solute and matric stress on germination on cereal straw certainly showed that soil fungi, including both *Fusarium* and *Penicillium* species, had a relatively good tolerance to both types of imposed water stress, although interactions with CO_2 were not investigated. The present study suggests that under climate-related abiotic factors ecological competence will be conserved better in soil than on crop residue under climate-related interacting abiotic factors.

For the biosynthetic genes involved in OTA production, the *otapks* and *otanrps* gene expression patterns appeared to be only slightly affected by the elevated CO_2 treatment, especially at 25 °C. Interestingly, under solute stress with existing or elevated CO_2 the expression of both *otapks*PV and *otanrps*PV genes were very low with no expression recorded at intermediate water stress level of -2.8 MPa (=0.98 a_w) and 1000 ppm. However, at -7.0 MPa (=0.95 a_w) relative *otapks*PV expression was significantly increased in the 1000 ppm CO_2 exposure treatment. Previously, for other mycotoxigenic fungi such as *A. flavus* it was found that for the former species the *aflD* (structural gene) and *aflR* (regulatory gene) were stimulated under elevated temperatures and CO_2 conditions in maize–based media and in stored maize (Medina *et al*., 2017; Garcia-Cela et al., 2020). Verheecke-Vaessen et al. (2019) showed that for *Fusarium langsethiae* both the *TRI5* gene and T-2/HT-2 toxin production are stimulated under interacting climate-related abiotic factors. Cervini et al. (2020) in studies with strains of *A. carbonarius*, an OTA producer in grapes and vine fruits, showed that under solute stress conditions imposed with the non-ionic solute glycerol, cycles of increased day/night temperatures and elevated CO_2 (1000 ppm) resulted in a stimulation of both structural genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal, AcOTAp450*, *AcOTAbZIP*) and regulatory genes (*LaeA*/*VeA*/*VelB*, "so called velvet complex") in the biosynthetic pathway of OTA. They suggested that this could increase the risks of OTA contamination in the wine production chain in southern Italy under climate–related abiotic changes. However, interactions with matric stress would provide more information on effects on the inoculum potential of this species, especially in soil. The stimulation observed is similar to that seen with other chemically related stresses. For example, the effect of intermediate concentrations of food grade preservatives on growth, *otapks*PV expression and OTA production found similar responses. Intermediate concentrations of calcium propionate or potassium sorbate resulted in a stimulation of *otapks*PV and OTA production under different ionic and non-ionic solute stress (Schmidt-Heydt et al., 2007; 2008).

OTA production was also influenced by the imposition of climate-related abiotic stress factors. *P. verrucosum* was stimulated to produce more OTA under interacting matric stress with CO_2 concentrations at both 25 and 30 °C. In contrast, with solute stress, especially at 30 °C *P. verrucosum* activity was inhibited by solute stress influencing OTA production. In contrast, under matric stress, especially at -2.8 MPa (=0.98 a_w) and to some extent at -7.0. MPa (=0.95 a_w) there was a stimulation of OTA. This was consistent with the effects noted in the gene expression responses, especially for the *otapks*Pv gene.

In conclusion this study has highlighted, for the first time, the impact of three-way interacting climate-related abiotic factors on growth, key OTA biosynthetic genes and OTA production by an important ochratoxigenic *Penicillium* species which contaminates temperate cereals post-harvest. This well studied strain of *P. verrucosum* was shown to be quite resistant to the imposed interacting climate-related abiotic factors in terms of growth rate and expression of OTA biosynthetic genes, especially in relation to matric stress. This type of data is important in understanding the life cycle of this species and its potential resilience under present and future climate change scenarios. This could also contribute to the development of models for the relative risks of OTA contamination in temperate cereal chains and developing effective intervention strategies to reduce inoculum potential in soil and on cereal crop residue.

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