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Solute and matric potential stress on *Penicillium verrucosum*: impact on growth, gene expression and ochratoxin A production

S. Abdelmohsen, C. Verheecke-Vaessen, E. Garcia-Cela, A. Medina and N. Magan
Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Beds. MK43 0AL, UK

Corresponding author: Prof. N. Magan, Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Beds. MK43 0AL, UK. E.mail: n.magan@cranfield.ac.uk

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Abstract

Penicillium verrucosum survives in soil and on cereal debris. It colonises grain during harvesting, drying and storage. There is no information on the relative tolerance of *P. verrucosum* to solute and matric stress in terms of colonisation, or on the biosynthetic toxin gene clusters or ochratoxin A (OTA) production. The objectives were to examine the effect of ionic and non-ionic solute and matric potential stress on (a) growth, (b) expression of two toxin biosynthetic genes *otapks*PV and *otanrps*PV, and (c) OTA production by a strain of *P. verrucosum*. 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 (Ψ_s) or matric (Ψ_m) stress was imposed. *P. verrucosum* 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 non-ionic solute (glycerol) and matric stress treatments. Relative gene expression of the biosynthetic genes using PCR (RT-qPCR) showed that the *otapks*PV gene was expressed over a wide range of ionic/non-ionic solute stress conditions (-1.4 to -14.0 MPa; =0.99 - 0.90 a_w). The highest expression was in the non-ionic Ψ_s stress treatments at -7.0 MPa (=0.95 a_w). However, the *otanrps*PV gene was significantly up regulated under Ψ_m stress, especially with freely available water (-1.4 MPa = 0.99 a_w). OTA production was significantly decreased as Ψ_s or Ψ_m stress were imposed. Limited OTA production occurred in the direst treatments under Ψ_s and Ψ_m stress respectively. The impact of these two types of stresses on the growth of *P. verrucosum* was quite different from that for OTA production. The results are discussed in the context of the

life cycle and ecological characteristics of this species in contaminating cereals with OTA in the post-harvest phase of the cereal chain.

1. Introduction

Penicillium verrucosum is an important xerotolerant species which is responsible for contaminating cereals, especially in temperate climates with ochratoxin A (OTA), during the harvesting and storage phases (Lund and Frisvad, 2003; Linbald et al., 2004). OTA is a nephrotoxin and there are legislative limits for the maximum limits in cereals destined for food and feed use (European Union, 2006). Post-harvest, uneven or poor drying of cereal grain can result in pockets of wet grain which can initiate spoilage and OTA contamination. In other commodities such as coffee and cocoa, OTA contamination is usually caused by species which can grow in more tropical conditions and include species from the *Aspergillus* section *Circumdati*, especially *A. westerdijkiae* (Abdel-Hadi and Magan 2009). It has been shown that water availability, temperature and inter-granular atmosphere and their interactions are critical ecological considerations and determine the optimum and boundary conditions for growth and OTA production (Cairns *et al*., 2005).

Since *P. verrucusum* contaminates cereals during the harvesting and storage phases then tolerance to matric potential stress (Ψ_m) in soil and solute potential stress (Ψ_s) in crop debris may be important factors determining survival and the inoculum potential for contamination of cereal grain. Studies of *P. verrucosum* population in soil suggested that this was between 100-300 CFUS/g soil and that this species was quite competitive both in soil and on crop debris against other resident mycobiota although the water potential of the soils were not measured (Elmholt, 2003; Elmholt and Hostbjerg, 1999). **T**he relative tolerance to Ψ_s and Ψ_m stress components of the total water potential (Ψ_T) is thus important to understand the species ecological competence and in terms of potential for toxin production. In soil, the total Ψ_T is the measure of water availability for microbial growth which is commonly used. In contrast, in the food industry, water activity (a_w) is used. There are some benefits from using Ψ_T as it allows the impact of different key water stress components which are important ecologically to be examined in more detail. Thus, total Ψ_T is the sum of (i) the osmotic or solute potential due to presence of ions or other solutes (Ψ_s), (ii) the matric potential (Ψ_m) due directly to forces required to remove water bound to the matrix (e.g., soil) and (iii) the turgor potential (Ψ_t) which measures the endogenous contents of the cells which helps to enable the cells to withstand external stress (Magan, 2007). A_w does not distinguish between these components, and thus it is more difficult to study the tolerance to different forms of water

stress. There is no detailed knowledge of the impact of Ψ_s (both ionic and non-ionic) and Ψ_m stress on growth and secondary metabolite production by *P. verrcuosum*.

Studies of non-xerophilic and xerophilic or xerotolerant mycotoxigenic fungi, such as *Fusarium graminearum* and *Aspergillus ochraceus* (= *A. westerdijkiae*) showed that for the former species both macroconidial germination and growth were very sensitive to Ψ_m stress while for the latter species growth was relatively similar in both Ψ_m and Ψ_s stress (Magan, 1988; Magan et al., 1995; Ramos et al., 1999; Ramirez et al., 2004). Subsequently, Jurado *et al*. (2008) showed that *F. verticillioides* grew relatively similarly under both ionic and non-ionic Ψ_s stress, but was also more sensitive to Ψ_m stress. This was supported by molecular ecology studies which showed that a key biosynthetic gene involved in production of fumonisins, the *FUM1* gene, reflected these differences.

OTA is a polyketide mycotoxin and the biosynthetic pathway has been predominantly now elucidated in *Penicillium nordicum* (Wang et al., 2016). In *P. nordicum*, the OTA biosynthetic cluster so far identified includes biosynthetic genes encoding for (i) a PKS (*otapks*PN) and (ii) a non-ribosomal peptide synthetase (NRPS) (*otanrps*PN). Another putative genes such as *otachI*PN potentially encoding a chlorinating enzyme, and (*otatra*PN) potentially encoding a transporter protein, which is involved in OTA export (Geisen et al., 2006; Karolewiez and Geisen, 2005). Geisen et al. (2004) was able to correlate the relative expression of the *otapks*PN from *P. nordicum* with the OTA production. There is a good similarity between the OTA biosynthetic pathways in both *P. nordicum* and *P. verrucosum*, with some differences related to the function of the polyketide synthase gene (*otapks*) (Geisen et al., 2006; Wang et al., 2016). However, there have been no previous studies to examine the relationship between different types of water stress on growth, expression of some of the key biosynthetic genes involved in OTA production and the correlation with toxin production.

Thus, the key objectives were to examine the effect of ionic and non-ionic solute (Ψ_s) and matric potential (Ψ_m) stress on (a) growth, (b) relative expression of the toxin biosynthetic genes *otapks*PV and *otanrps*PV, and (c) OTA production by a toxigenic strain of *P. verrucosum* on a conducive medium.

2. Materials and Methods

Fungal strains used in this study

One strain of *P. verrucosum* (OTA11) isolated from wheat was used in this study. The OTA11 strain is a known producer of OTA and has received detailed study previously (Linblad et al., 2004; Cairns et al., 2005; Aldred et al., 2008) and is a good representative of this species. The

strain was kindly supplied by Dr M. Olsen, Swedish National Food Authority, Uppsala, Sweden.

Preparation of media for effects of solute (NaCl, glycerol) and matric stress (PEG 8000) on growth and OTA production by *P. verrucosum.*

The basic medium used in this study was a YES medium. The Ψ_s was modified with the ionic solute NaCl (Lang, 1967), and the non-ionic solute glycerol (Dallyn and Fox, 1980; to -1.4, -7.0, -9.8, 14.0 and -19.6 MPa (=0.99, 0.95, 0.93, 0.90, and 0.86 a_w).

For the Ψ_m treatments, the agar was omitted and the yeast extract with the sucrose were added together with known amounts of PEG 8000 to obtain the following matric potentials: -1.4 MPa (=0.99 a_w), -7.0 MPa (=0.95 a_w), -14.0 MPa (=0.90 a_w) and -19.60 MPa (=0.86 a_w). The solute and matric potentials of the media were checked using the Aqualab 4TE (Decagon Devices. Pullman, WA99163 USA). Previous studies have shown that the water potential generated by PEG 8000 is predominantly (99%) due to matric forces (Steuter *et al*., 1981). The Ψ_m media were prepared by using 8.5 cm sterile circular discs of capillary matting (Harrod Horticultural, Lowerstoft, Suffolk, U.K). 15 ml of the sterile cooled medium was pipetted into each 9 cm Petri plate, this was then overlayed with sterile polyester taffeta lining (8.5 cm diameter; William Gee Ltd, Dalston, London, U.K.) and finally a sterile cellophane disc (8.5 cm diameter; Innovia Films Ltd, Wigton, Cumbria, U.K.).

Inoculum preparation and inoculation

The fungal strain was initially sub-cultured on malt extract agar (MEA) at 25°C in the dark for 7-10 days. Using a sterile loop, the spores were gently dislodged from the colony surface into 9 ml sterile distilled water containing 0.05 % Tween-80 in 25ml Universal bottles. The fungal conidial spore suspension was shaken vigorously on a vortex mixer and then the concentration was determined using a haemocytometer and adjusted to 10^6 spores/ml. A 50µL of this spore suspension was placed in a 9 cm Petri MEA plate and spread over the surface with a surface sterilised spreader. This was incubated over night at 25°C. This allowed conidial germination to be initiated. 4 mm agar discs of the initial inoculum were made using a surface sterilised cork borer and used to carefully inoculate the treatments and replicate plates taking care not to puncture the cellophane membrane surface. The experiment was carried out with four replicates per treatment and repeated once. The treatments were all incubated separately in polyethylene bags which were kept closed to avoid moisture loss and

incubated at 25°C for 10 days. This temperature was chosen because it is in the optimum range of temperatures for both growth and OTA production (Cairns-Fuller et al., 2005). The time frame of 10 days was chosen to ensure that over the whole range of matric and solute potential conditions used that growth, fungal biomass (for molecular studies) and mycotoxin data could be obtained. In addition enough biomass under extreme conditions was required for carrying out the RT-qPCR studies.

Growth assessment

The colony diameters of the replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily for ten days and the growth rate calculated by plotting the radial mycelial growth extension against time and the slope of the linear growth phase was used to obtain the radial growth rates (mm/day, Patriarca et al., 2001).

Isolation of total RNA

The fungal biomass was harvested after 10 days of incubation in the presence of liquid nitrogen to keep the integrity of RNA and stored in -80^{o}C for molecular work and -20^{o}C for OTA analysis. The fungal cell walls were disrupted using the according to the bead-beating method recommended by Leite et al. (2012). The RNA was extracted using the Total RNA Spectrum Plant Kit Spectrum Plant (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 (A260/A280 ratio) and (A260/A230 ratio) were determined spectrophotometrically using a 2.5µL aliquot on the Picodrop (Spectra Services Inc., USA). The RNA integrity was checked using the Experion™ Automated Electrophoresis System using the Experion RNA StdSens analysis kits (Bio-Rad Laboratories Ltd., Hertfordshire, UK), where the RQI chosen as the minimum quality control value 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 as the target genes and the ß-tubulin gene used as the reference gene.

Primers

The primer pairs PV-bentaqfor/rev, previously designed from the *otanrps*PV gene involved in the OTA biosynthetic pathway (Rodríguez et al., 2014) and the β-tubulin gene (Leite, 2013) were used. Nucleotide sequences of primers used in the RT-qPCR assays are shown in Table 1.

cDNA synthesis and qPCR

The cDNA was synthesized according to the Omniscript RT kit protocol (Qiagen, UK). The qPCR was prepared with three replicates of RNA control samples together with a template-free negative control which was also included in each run. The reaction mixture consisted of 5 µL of SYBR (Sso Advanced Tm Universal Syber ® Green Supermix), 300nM of each primer, and 1 µL of cDNA template in a final volume of 10 µL. After an activation step of 10 min at 95°C, all subsequent 40 cycles were performed according to the following temperature regime: 95°C for 15s and 60°C for 30s. After the final qPCR cycle, a melting curve analysis of the qPCR products was performed. The Ct determinations was perform using Bio-Rad CFX software.

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, the Cq of the gene of interest was subtracted from the Cq of the reference gene. Subsequently, for ΔΔCq, the non-modified medium was used as the control.

Quantification of OTA production

The solute potential treatments and replicates were harvested after 10 days as described previously. Plugs were taken across the colony using a sterile 4 mm diameter cork borer. The five plugs 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 200rpm at 25°C. The plugs were discarded by centrifugation after 10 min at 15000^{x}g. The extracts were filtered through a 0.22µm (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	120CC-C18 column (Poroshell 120, length 100 mm, diameter 4.6 mm, particle size 2.7 micron;).

Temperature of column	25ºC
Excitation	330nm
Emission	460nm
Flux	1ml/min
Retention time	2.6 min
Run time	13 min

The OTA was analysed using HPLC-FLD by including OTA standards at different concentrations with each batch and their peaks were detected by Chemstation software. Comparisons were made between the standard curve and the different treatments and replicates.

Statistical analyses of data

Each treatment was carried out with at least three to four replicates for the growth rate assessment, gene expression and OTA production. The normality was checked using the Shapiro test and homoscedasticity was checked using the Leven test (Shapiro and Wilk, 1965; Levene, 1960). The factors and response and their interactions were checked for normality. As this was the case for all sets of data they were analysed using ANOVA (JMP software). The statistical significant level was set at $p<0.05$ for all single and interacting factors.

3. Results

Comparison of solute and matric potential water stress on growth of *P. verrucosum*

Figure 1 compares the effect of different Ψ_s potential (ionic and non-ionic) with Ψ_m stress on growth of the toxigenic *P. verrucosum* strain (OTA11). This shows that -7.0 MPa (=0.95 a_w) was optimal for growth, when exposed to non-ionic Ψ_s and Ψ_m stress. However, under ionic Ψ_s stress this was at -1.4 MPa (0.99 a_w). Overall, growth was faster under Ψ_m stress. Growth of the *P. verrucosum* strain was more sensitive to ionic Ψ_s stress, with no growth observed at the lowest water potential (Ψ_T) tested -19.6 MPa (= 0.86 a_w). Statistically, all three types of water stress had a significant effect on the growth rate of the *P. verrcuosum* strain (Table 2).

Comparison between the effect of solute and matric stress on *otapksPV* and o*tanrpsPV* gene expression by *P. verrucosum*

Figure 2. shows the impact of the interaction between the Ψ_s (Glycerol, NaCl) and Ψ_m (PEG 8000) stress on the relative *otapks*PV expression in relation to the different treatments. The pattern of production is clear where Ψ_s stress was imposed with maximum expression at -7.0 and -1.4 MPa (=0.95 and 0.99 a_w) and a decrease at -14.0 MPa (=0.90 a_w) respectively. Very low expression was observed where Ψ_m stress was imposed. The ANOVA test revealed a significant difference in relative expression of this biosynthetic gene in response to the imposed types of water stress (Table 3).

The influence of the different water stress treatments on the second gene examined, *otanrps*PV is shown in Figure 3. The results were markedly different. There was a very high expression of this gene when water was relatively freely available (-1.4 MPa). Under imposed Ψ_m stress this was significantly decreased (Table 4). For this gene, in the non-ionic glycerol-imposed Ψ_s stress conditions, the expression was generally very low. However, for ionic-imposed Ψ_s stress, there was some increase in expression at -14.0 MPa (=0.90 a_w). There was less of a pattern of expression of this gene in relation to the different imposed types of water stress.

Comparison between the effect of solute and matric potential stress on OTA production by *P. verrucosum*

For OTA production, the *P. verrucosum* strain produced significantly less toxin under Ψ_m stress at -1.4 MPa and -7.0 MPa (=0.99, 0.95 a_w) when compared with imposed ionic and non-ionic Ψ_s stress conditions (Figure 4; Table 5). Optimum OTA production was produced under Ψ_s stress when imposed with glycerol or NaCl, especially under freely available water conditions (-1.4 MPa=0.99 a_w). Very little OTA was produced under imposed Ψ_m or Ψ_s stress conditions at -14.0 or 19.6 MPa (=0.90, 0.96 a_w). This was fourfold more than under moderate water stress (-7.0 MPa = 0.95 a_w).

4. Discussion and Conclusions

This is the first study to examine the impact of different Ψ_s and Ψ_m stress effects on growth, biosynthetic gene expression and OTA production by *P. verrucosum*. This certainly suggests that *P. verrucosum* is probably well suited to survival in soil and also on crop residues based on the colonisation over a range of water stress regimes, including Ψ_m stress which is

considered more difficult to overcome (Griffin, 1981; Magan, 2007). Overall, ionic Ψ_s stress using NaCl was more toxic when high concentrations were used at -19.6 MPa (=0.86 a_w), completely inhibiting growth. This contrasts with the activity of *P. nordicum,* which predominantly occupies the very high salt ecological niches of cured meats where it is able to grow better under extreme ionic stress (Rodriguez *et al*, 2014).

P. verrucosum was able to grow optimally at -7.0 MPa (=0.95 a_w) which represents a significant level of water stress conditions when considering soil moisture relations as the wilting point of plant is around -1.4 MPa (=0.99 a_w). The tolerance to Ψ_m stress would suggest that this species has evolved to successfully not just to survive, but actively grow in such stressed conditions. These results are very different from those observed for *Fusarium* species such as *F. verticillioides* and *F. graminearum*, where mycelial growth was shown to be significantly more sensitive to Ψ_m than Ψ_s stress (Jurado *et al*., 2008; Ramirez *et al*., 2004). However, *Fusarium* species are not xerotolerant and more sensitive to such changes. In contrast, studies with strains of the xerophilic *A. ochraceus* and *A. flavus* isolated from cereals had similar tolerances to both solute and matric stress (Ramos et al., 1999; Giorni et al., 2008).

This study has shown a good correlation between the relative gene expression of the *otapks*PV gene in the biosynthetic cluster and OTA production, especially under Ψ_s stress. Previously, Geisen et al. (2011) had suggested that the *otapks*PV gene is a key gene in the biosynthesis of OTA by *P. verrucosum* and that abiotic factors significantly influence both gene expression and toxin production. In the present study, conditions for high OTA production was mainly observed under non-ionic Ψ_s stress, which occurred under conditions which also resulted in a high relative *otapks*PV expression. However, under ionic Ψ_s stress at -14.0 MPa (=0.90 a_w) a considerable amount of toxin was produced at higher concentrations than in the other ionic solute treatment conditions. This suggests that perhaps two different regulatory pathways for OTA production may occur, one acting under optimal, and another under imposed stress conditions as postulated by Geisen *et al*. (2006). Previously, Jurado et al. (2008) showed that ionic Ψ_s stress had a significant impact on the induction of the *FUM1* gene, considered to be a key regulatory gene in fumonisins biosynthesis, although growth of *F. verticillioides* was inhibited by about 50%. In the present study the expression of the *otapks*PV gene did not correlate directly with growth. Perhaps a more detailed temporal kinetic study would be needed to correlate the gene expression with the OTA production levels observed. However, Schmidt-Heydt et al. (2007) did examine the effect of solute stress imposed with either NaCl or glycerol on growth, *otapks*PV expression and OTA production in the presence and absence of sub-optimal preservative concentrations of aliphatic acids. In

this study the kinetics of *otapks*PV was examined and found to be optimum after 8-9 days at 25^{o}C by *P. verrucosum*. Based on these studies they suggested that *otapks*PV expression profile and OTA production by this species was activated under two conditions, i.e., under conditions optimal for growth and under stress conditions. This was also shown for mycotoxin production by other mycotoxigenic fungi by Schmidt-Heydt et al. (2008).

The *otanrps*PV expression pattern was different from that of the *otapks*PV and expression was not consistent with toxin production. Previously, Geisen *et al*. (2004) demonstrated that the *otapks*PN gene expression paralleled the induction of toxin production during the time course of their experiments. However, these studies were carried out with freely available water conditions. Under these conditions, initial mRNA could be detected after 4 days incubation (growth phase), when initial traces of OTA also occurred. They observed maximum *otapksPN* mRNA after 6-8 days when the OTA concentration remained relatively constant. Subsequently, after 10-11 days no *otapks*PN mRNA was observed. However, under matric and solute stress conditions marginal for growth this may change.

The results for the other gene assayed, *otanps*PV, showed that this was not significantly upregulated when compared to the *otapks*PV, which was. This may partially be because of the time frame of our experiments as these genes must functionally be expressed for OTA production. Perhaps this gene is thus switched on earlier that the *otapks*PV gene resulting in much lower detection levels at the endpoint of our experiments. In addition, the NRPSs exhibits a modular structure, with one module being a semi-autonomous unit that recognizes, activates, and modifies a single residue of the final peptide by means of different domains including the adenylation domain (A), the peptidyl carrier domain (P), and the condensation domain (C) (Finking and Marahiel, 2004; Schwarzer *et al*., 2003; Weber and Marahiel, 2001). More studies are required to understand the role of this gene and these domains in the biosynthetic pathway for OTA production by *Penicillum* and *Aspergillus* species.

The profile of OTA production under different Ψ_s stress was significantly different from those for growth. Optimum OTA production occurred at -1.4 MPa (=0.99 a_w) when water was relatively freely available regardless of the solute used. At -7.0 MPa (0.95 a_w) there was a significant decrease in OTA with both Ψs treatments. In contrast, some other toxigenic *Penicillium* species such as *P. nordicum* are ecologically specialised to grow in high ionic Ψs environments (Rodriguez et al., 2014). This species which colonises cured meats has evolved to colonise high ionic solute environments more rapidly than low salt environments. However, sometimes *P. verrucosum* has also sometimes been found in NaCl-rich products and may

contaminate cured meats with OTA over a narrower ionic solute range than *P. nordicum* (Rodriguez et al., 2014).

Schmidt-Heydt *et al*. (2011) suggested that *P. verrucosum* may have evolved to produce modified patterns of secondary metabolites, including OTA, on substrates with increased concentrations of NaCl. They also showed that production of OTA under high NaCl conditions was an adaptive response. They did not consider Ψ_m stress, where the amount of OTA was significantly decreased when compared with the other two Ψs treatments in the present study. This suggests that Ψ_m stress may be more inhibitory than both ionic and non-ionic Ψ_s imposed stress in terms of the metabolic requirements for the biosynthesis of OTA. Thus, while growth was relative unaffected by changes in Ψ_m stress, toxin production was significantly impacted.

Previous studies have attempted to examine the population ecology of *P. verrucosum* in field soil and how these may be affected by different harvesting and drying practices (Elmholt and Hostbjerg, 1999; Elmholt, 2003). This showed that *P. verrucosum* was present in less than 25% of soils and in relatively low populations (100-300 CFUs g/dry soil). The presence of grain in soil, which acted as a nutritional source, significantly increased the populations in soil, probably allowing better colonisation under matric potential stress. However, the soil types and water potentials of the soils were not monitored although some fluctuations were recorded in *P. verrucosum* populations over the season (Elmholt and Hostbjerg, 1999). These factors may be critical in the competitiveness of *P. verrucosum* in both soil and on crop debris.

In conclusion, this study has provided new data on the ability of this mycotoxigenic species (*P. verrucosum*) to grow and produce OTA under different types of Ψ_s and Ψ_m stress. Expression of a key gene involved in the biosynthetic pathway, *otapks*PV, was significantly upregulated over a range of Ψ_s stress conditions, whether ionic or non-ionic. However, under Ψ_m the relative gene expression was different. Overall, the expression of this gene paralleled the OTA production. The *otanrps*PV gene expression was not significantly activated at the 10 day time frame of our experiments. It may be useful to have a temporal kinetic study under matric and solute stress to identify the relative fluxes in expression of these two genes involved in OTA production. However, the data obtained provides useful molecular and ecological data on the resilience of this species over a range of relevant conditions which occurs in soil and in crop residue. This type of data may help to better understand the inoculum potential of *P. verrucosum* in soil and on crop debris. This may also help explain

why this species is predominantly responsible for post-harvest contamination of temperate cereals, especially when inefficient drying and/or poor storage management occurs.

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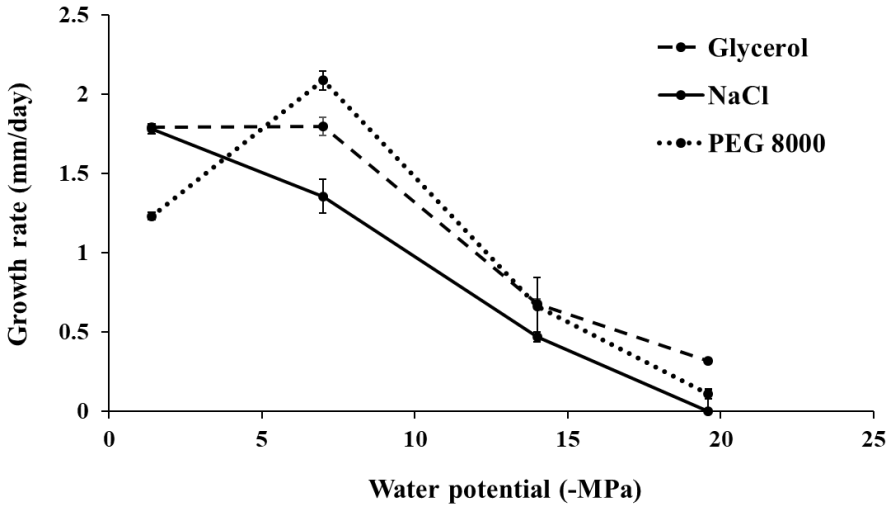

Figure 1. Comparison of the effect of ionic (NaCl) and non-ionic (glycerol) solute potential and matric potential (PEG 8000) stress on growth rate of *P. verrucosum* on a YES medium at 25ºC. Bar indicates Least Significant Difference (P=0.05).

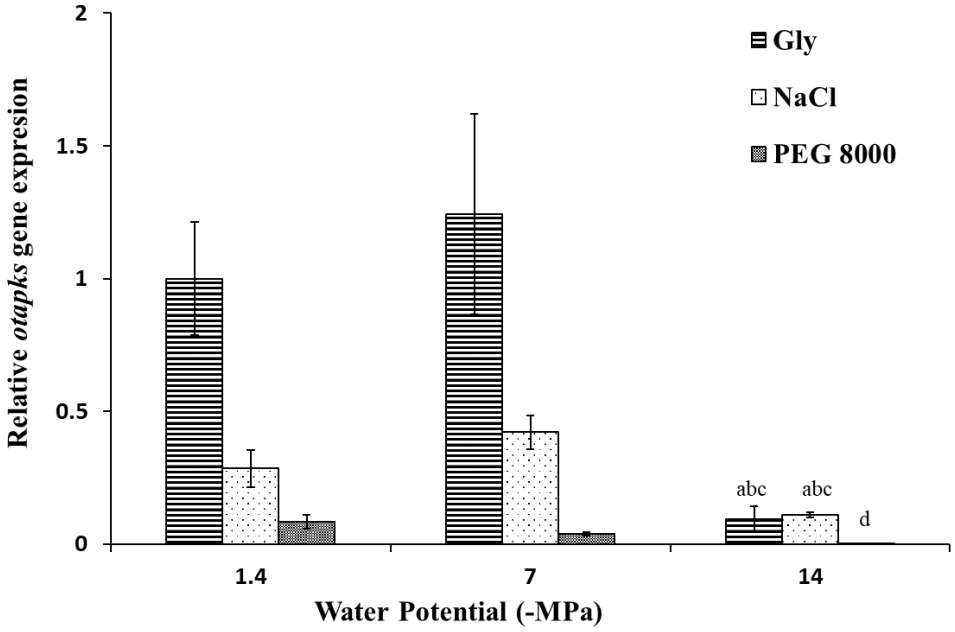

Figure 2. Comparison of the effect of ionic (NaCl) and non-ionic (glycerol) solute potential and matric potential stress (PEG 8000) on relative gene expression of *otapksPN* gene expression in cultures of *P. verrucosum* grown on YES medium at 25°C. Comparisons were made with the unmodified YES medium as the calibrator. Different letters indicate significant differences.

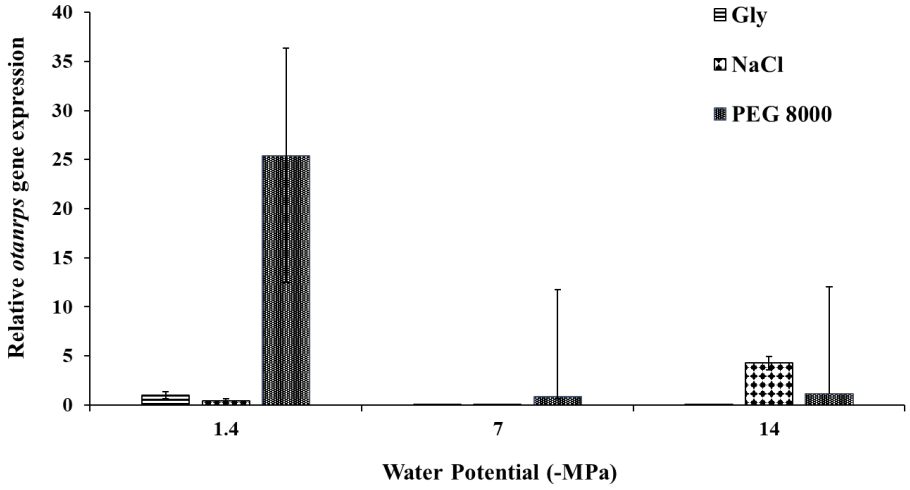

Figure 3. Comparison of the effect of ionic (NaCl) and non-ionic (glycerol) osmotic potential and matric potential stress on relative gene expression values of *otanrpsPV* gene expression in cultures of *P. verrucosum* grown on YES medium 25°C. Comparisons were made with colonies grown on the unmodified YES medium as a calibrator. Different letters indicate significant difference.

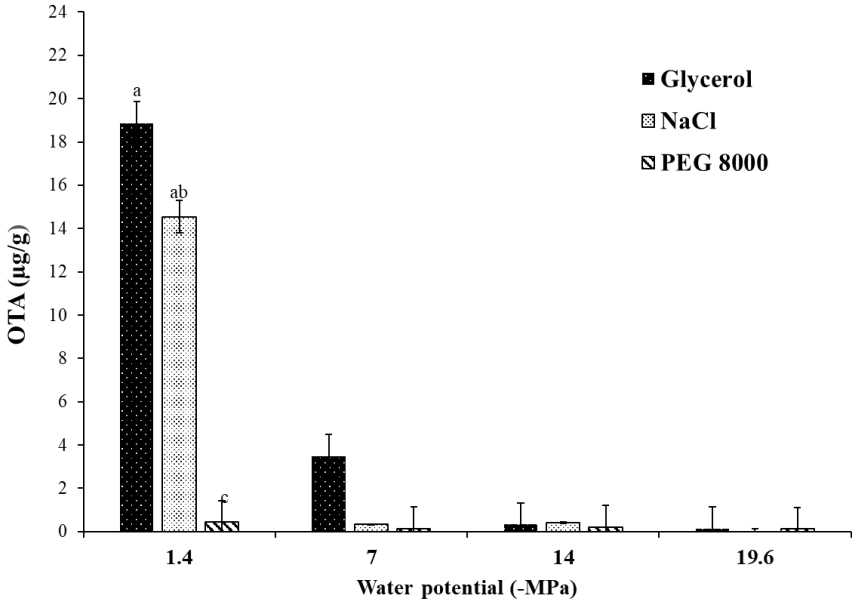

Figure 4. Comparison of the effect of ionic (NaCl) and non-ionic (glycerol) osmotic potential and matric potential stress on ochratoxin A production by colonies of *P. verrucosum* on YES medium at 25°C. Bars represent SEM. Different letters indicate significant differences.

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		
*otapks*PV-for	*otapks*PV	TTGCGAATCAGGGTCCAAGTA	51	Schmidt-Heydt*et al*., (2007)
*otapks*PV-rev	*otapks*PV	CGAGCATCGAAAGCAAAAACA		
*otanprs*PV-rev	*otanrps*PV	GCCATCTCCAAACTCAAGCGTG	117	Rodriguez *et al*., (2011)
*otanprs*PV-for	*otanrps*PV	GCCGCCCTCTGTCATTCCAAG		

Table 2. Analysis of Variance (ANOVA) of the effects of total Ψ_t (total), and type of imposed water potential stress (matric vs ionic vs non-ionic solute stress) and their interactions on growth rate at 25°C[a].

Source of variation	DF	Sum of squares	Prob>F
Ψ_t	3	65.02	<0.0001*
Ψ type	2	0.62	0.0017*
Ψ_t vs Ψ type	6	5.21	<0.0001*

[a] Shown are the ANOVA results for the effects of Ψ_t (-1.4, -7, -14.0, -19.6 MPa), type of water potential (nonionic and ionic Ψ_s and Ψ_m), and their interactions on growth rate at 25°C.
*, significant at $P<0.05$.

Table 3. Analysis of Variance (ANOVA) of the effects of Ψ_t (total), and type of water potential stress and their interactions on *otapks*PV gene expression at 25°C[a].

Source of variation	DF	Sum of squares	Prob>F
Ψ_t	2	32.82	0.0017*
Ψ type	2	50.91	0.0002*
Ψ_t vs Ψ type	4	7.15	0.4188

[a] Shown are the ANOVA results for the effects of Ψ_t (-1.4, -7, -14.0 MPa), type of water potential (nonionic and ionic Ψ_s and Ψ_m), and their interactions on gene expression at 25°C.
*, significant at $P<0.05$.

Table 4. Analysis of Variance (ANOVA) of the effects of Ψ_t (total), and type of water potential stress and their interactions on *otanrps*PV gene expression at 25°C[a].

Source of variation	DF	Sum of squares	Prob>F
Ψ_t	2	124.39	<.0001*
Ψ type	2	39.72	0.0023*
Ψ_t vs Ψ type	4	70.37	0.0011*

[a] Shown are the ANOVA results for the effects of Ψ_t (-1.4, -7, -14.0, MPa), type of water potential (nonionic and ionic Ψ_s and Ψ_m), and their interactions on gene expression at 25°C.
*, significant at $P<0.05$.

Table 5. Analysis of Variance (ANOVA) of the effects of Ψ_t (total), and type of water potential stress and their interactions on OTA production at 25°C[a].

Source of variation	DF	Sum of squares	Prob>F
Ψ_t	3	777904926	<0.0001*
Ψ type	2	32400	0.9
Ψ_t vs Ψ type	6	400092853	0.018*

[a] Shown are the ANOVA results for the effects of Ψ_t (-1.4, -7, -14.0, -19.6 MPa), type of water potential (nonionic and ionic Ψ_s and Ψ_m), and their interactions OTA production at 25°C.
*, significant at $P<0.05$.