



Acclimatisation of *Fusarium langsethiae*, *F. poae* and *F. sporotrichioides* to elevated CO₂: Impact on fungal growth and mycotoxin production on oat-based media

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ABSTRACT

Oats are highly susceptible to infection by *Fusarium* species, especially *F. langsethiae*, *F. poae* and *F. sporotrichioides* which contaminate the grain with mycotoxins. Climate change is expected to affect fungal colonisation and associated mycotoxin production. The objective of this study was to examine the effect of acclimatisation to elevated CO₂ on the growth and mycotoxin production capacity of these fungal species. Strains of *F. langsethiae* (FL; seven strains), *F. poae* (FP; two strains) and *F. sporotrichioides* (FS; one strain) were acclimatised by sub-culturing for 10 generations at either 400 or 1000 ppm CO₂ under diurnal temperature conditions. At each sub-culturing, the effect of acclimatisation to elevated CO₂ on (a) lag phase prior to growth, (b) growth rate on oat-based media was assessed. Additionally, the production of type A trichothecenes and related toxic secondary metabolites of sub-cultures after 1, 7 and 10 generations were assessed using LC-MS/MS qTRAP. The results showed that *Fusarium* strains had an increased lag time and growth rate in response to the combined effect of sub-culturing and elevated CO₂ levels. T-2 + HT-2 production was affected by elevated CO₂ in strain FL4 (7.1-fold increase) and a decrease in strain FL1 (2.0-fold decrease) at the first sub-culturing and FS (1.3-fold decrease) after 7 sub-cultures compared to ambient conditions. The effect of sub-culturing on T-2 + HT-2 production varied depending on the fungal strain. For strain FL4, significantly less T-2 + HT-2 toxins were produced after 10 generations (4.4-fold decrease) as compared to that under elevated CO₂ conditions after one sub-culture, and no change was observed under ambient conditions. The FS strain showed significant stimulation of T-2 + HT-2 toxin production after 10 sub-cultured generations (1.1-fold increase) compared to the initial sub-culture of this strain under elevated CO₂ conditions. The production of other toxic secondary metabolites was generally not impacted by elevated CO₂ conditions or by sub-culture for 10 generations, with the exceptions of FL1 and FP1. FL1 produced significantly more neosolaniol after 10 generations, when compared to those after 1 and 7, regardless of the CO₂ conditions. For FP1, elevated CO₂ significantly triggered beauvericin production after an initial sub-culture when compared to ambient conditions at the same sub-culture stage (29-fold). FP1 acclimatisation to elevated CO₂ led to a decrease of beauvericin production after 10 generations when compared to 1 (6-fold). In contrast, sub-culturing for 10 generations compared to 1 under ambient CO₂ conditions resulted in an increase in this toxin (12-fold).

1. Introduction

Fusarium head blight (FHB) is one of the most devastating diseases of cereals, causing both reduced crop yield and production of mycotoxins

in the grain (Mielniczuk and Skwaryło-Bednars, 2020). The most common causal *Fusarium* species to infect oats are *Fusarium graminearum*, *F. sporotrichioides*, *F. poae* and *F. langsethiae* (Fredlund et al., 2013; Hofgaard et al., 2016; Imathiu et al., 2009). Infection of oats by

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F. langsethiae, *F. poae* and *F. sporotrichioides* does not result in visual disease symptoms. However, healthy-looking grain can contain high amounts of mycotoxins, particularly the type A trichothecenes T-2 and HT-2 (Edwards et al., 2012; Kokkonen et al., 2012b; Martin et al., 2018; Opoku et al., 2018).

T-2 + HT-2 are the most commonly detected mycotoxins in oats (De Colli et al., 2021; Edwards, 2009; Hietaniemi et al., 2016; Schöneberg et al., 2018). These are type A trichothecenes, with HT-2 toxin being the deacetylated derivative of T-2 toxin (Nathanail et al., 2015). As reviewed by Adhikari et al. (2017), T-2 inhibits protein, inducing DNA damage, altering membrane function, impairing the production of antibodies, and inducing oxidative stress causing apoptosis. T-2 + HT-2 are stable compounds and remain in cereals even after food processing (EFSA Panel on Contaminants in the Food Chain CONTAM, 2011). Therefore, it is crucial to limit the concentration of these toxins in food and feed pre-harvest. The European Commission (EC) set indicative levels recommended (2013/165/EC) of these mycotoxins in food and feed; the maximum recommended level of T-2 + HT-2 for direct human consumption of oats is 200 µg/kg in the European Union (EU) (EC, 2013).

Other related type A trichothecenes, such as neosolaniol (NEO) and diacetoxyscirpenol (DAS) produced by *F. langsethiae* and *F. sporotrichioides* are of equal or higher anorectic potency than T-2 in mice (Zhang et al., 2018) but are not regulated in the EU or the United States of America. These mycotoxins have been detected in oats both in vitro and in field studies (De Colli et al., 2021; Kokkonen et al., 2012a). Furthermore, Schöneberg et al. (2018) showed that NEO content in oats was correlated with T-2 + HT-2 contamination. Beyond trichothecenes, beauvericin (BEA) is an emerging mycotoxin of interest that is produced by *Fusarium* species such as *F. poae* and *F. sporotrichioides*. It was detected in oat samples collected from the field, and its levels were correlated with *F. poae* infection levels (De Colli et al., 2021; Fredlund et al., 2013; Kokkonen et al., 2012a). EFSA has limited data available on the general toxicity of BEA and its mode of action. Feudjio et al. (2010) showed its cytotoxicity in mammalian cell cultures and its ability to activate programmed cell death.

There are few studies that have investigated the impact of future climate change on the resilience of mycotoxigenic fungi and their production of mycotoxins. There is a lack of knowledge as to how fungi will acclimatise to the environmental changes in order to maintain fitness, and whether this will impact growth and toxin production. To the best of our knowledge, there are no studies on the effects of climate change on *Fusarium* infection of oats or associated mycotoxin levels, there has been a study on wheat that showed acclimatisation to elevated CO₂ enhanced FHB disease development. Váry et al. (2015) demonstrated that FHB disease levels caused by *F. graminearum* and associated yield losses were highest on wheat when both the fungus (×10 sub-cultures) and the plants were acclimatised under elevated CO₂ levels (780 ppm), as compared to when both were grown under existing ambient CO₂ (390 ppm). Bencze et al. (2017) showed that DON levels due to *F. culmorum* infection were higher in wheat grain under elevated CO₂ as compared to ambient conditions. However, the increased susceptibility of maize to *F. verticillioides* under higher CO₂ did not result in enhanced levels of fumonisins (Vaughan et al., 2014), except when combined with drought stress (Vaughan et al., 2016).

In vitro studies have shown that toxin production by *Fusarium* species pathogenic to oats can be significantly affected by changes in environmental conditions. The production of T2 + HT-2 toxins by *F. langsethiae* and *F. sporotrichioides* was significantly affected by the interacting abiotic factors of temperature x water activity (a_w) (Kokkonen et al., 2010; Verheecke-Vaessen et al., 2019; Verheecke-Vaessen et al., 2021). A recent in vitro study showed that while the growth rate of *F. langsethiae* on oat-based media did not change under elevated CO₂ (1000 ppm) conditions at 25 °C and freely available water (a_w 0.995), T-2 + HT-2 production was increased significantly, when compared to ambient CO₂ (400 ppm) conditions (Verheecke-Vaessen et al., 2019).

Colonisation of oat grains by *F. langsethiae* under elevated CO₂ conditions at 25 °C and intermediate water stress (0.98 a_w) for this species resulted in higher levels of T-2 + HT-2, as compared to ambient CO₂ conditions (Verheecke-Vaessen et al., 2019). Verheecke-Vaessen et al. (2021) evaluated the response of different *F. langsethiae* strains to climate change conditions (temperature x a_w x CO₂). Growing these strains under increased CO₂ levels (1000 ppm) resulted in a reduced lag phase and reduced growth rate irrespective of the a_w, while growing them under elevated CO₂ levels + high temperature significantly reduced the growth rate, regardless of the a_w level. However, there was intra-species variation in T-2 production, with only one of three strains showing a decreased T-2 production in response to elevated CO₂. There have been no previous studies on whether acclimatisation of *F. langsethiae* for a number of sub-cultures to elevated CO₂ will have an impact on its colonisation and toxin production on oat-based media or indeed in oats. It will also be interesting to determine whether elevated CO₂ concentrations, in combination with temperature and water stress, can change the competitiveness of oat-colonising *Fusarium* species such as *F. langsethiae* with other fungi, as has previously been shown for other *Fusarium* species which have been geographically shifted or replaced due to climate change (Beccari et al., 2017; Valverde-Bogantes et al., 2020).

The aim of this study was to examine the effect of acclimatisation to elevated CO₂ on the growth and mycotoxin production by oat-colonising *Fusarium* species. Strains of *F. langsethiae* (seven strains), *F. poae* (two strains) and *F. sporotrichioides* (one strain) were grown and sub-cultured for 10 generations under diurnal temperature conditions (21/12 °C for a 16/8 h, under dark conditions) and either 400 or 1000 ppm CO₂. Using an oat-based growth medium, we investigated the effect of elevated versus ambient CO₂ on growth (lag phases prior to growth and growth rate) after each of the 1–10 sub-cultures and on the production of type A trichothecenes and related toxic secondary metabolites after 1, 7 and 10 sub-cultures.

2. Materials and methods

2.1. Fungal strains

The *Fusarium langsethiae*, *F. sporotrichioides* and *F. poae* strains used in this study are detailed in Table 1. Fungal strains were stored at –80 °C and prior to use, were incubated on oats-based media at 25 °C for 7 days.

2.2. Medium and inoculation

Oat grains were milled using a coffee grinder which then were used to make up oat agar media using 2 % (w/v) oat flour and 2 % (w/v) agar (Technical agar No. 2, Oxoid) mixed in distilled water. The water activity (a_w) of the media was adjusted to an optimal growth condition of 0.995 a_w by adding glycerol (Medina et al., 2010). The medium was

Table 1

Fusarium species, strains and origin. Abbreviations: BEA: Beauvericin, DAS: Diacetoxyscirpenol, NEO: Neosolaniol, FERA: Food and Environment Research Agency in the United Kingdom, NIBIO: Norwegian Institute of Bioeconomy Research. All the strains were isolated from oat grains.

<i>Fusarium</i> species	Isolate code	Origin	Supplied by
<i>F. langsethiae</i>	Fe2390 (FL1)	UK	FERA
<i>F. langsethiae</i>	Fe2391 (FL2)	UK	FERA
<i>F. langsethiae</i>	Fe2392 (FL3)	UK	FERA
<i>F. langsethiae</i>	Fl11010 (FL4)	UK	Harper Adams
<i>F. langsethiae</i>	Fl11011 (FL5)	UK	Harper Adams
<i>F. langsethiae</i>	Fl11018 (FL6)	UK	Harper Adams
<i>F. langsethiae</i>	201,059 (FL7)	Norway	NIBIO
<i>F. poae</i>	202,172 (FP1)	Norway	NIBIO
<i>F. poae</i>	202,189 (FP2)	Norway	NIBIO
<i>F. sporotrichioides</i>	202,176 (FS)	Norway	NIBIO

autoclaved for 15 min at 121 °C. Sterile cellophane membranes (8.5 cm; A.A Packaging Limited, UK) were carefully overlaid on the surface of the oat media avoiding any air bubbles using sterilised round ended forceps (Verheecke-Vaessen et al., 2019).

For fungal growth measurement, each fungal strain was inoculated with a 4 mm diameter agar plug taken from the growing margins of the stock culture colony (2 cm from the growth edges) using a surface-sterilised cork-borer, with the discs carefully placed centrally in 9 cm Petri plates (Verheecke-Vaessen et al., 2019). Each Petri plate was sealed with leukopor® tape (BSN medical, UK) to maintain humidity while allowing gas exchange. The a_w of the plates were measured before and after incubation to assure constant a_w throughout the experiment. Three replicates were used of each species and strain.

2.3. Sub-culturing for 10 generations, measurements and quantification of lag phases and growth rate

Initially climate change growth chambers BDW-40 (Winnipeg, Canada) were set up for a 24 h cycle including 16 h at 21 °C followed by 8 h at 12 °C under dark conditions, 75 % relative humidity, under either ambient CO₂ (400 ppm) or elevated CO₂ (1000 ppm) conditions. The initial cultures of all strains/species were placed in the environmental growth chambers for 10 days. They were then sub-cultured onto fresh media under the treatment conditions for the 2nd sub-culture after another 10 days. This was repeated for a total of 10 sub-culture generations.

At each sub-culture, the diametric growth was measured in two directions at right angles to each other on days 2, 3, 4 and 5. The growth data were recorded in excel and the results were plotted against time. A linear model was used to calculate and obtain the lag time prior to growth (λ) and growth rate ($\mu_{\max}$) (Medina et al., 2010).

2.4. Sample collection for toxin analysis

On day 10, nine plugs (4 mm diameter) were collected in 2 mL Eppendorf tubes and stored at −20 °C until toxin analysis from the plates used for measurement. And the fungal biomass was collected on day 10 for sub-cultures 1, 7 and 10 by removing the cellophane membrane carefully (where the fungal mycelia is growing) which was then flash frozen in liquid nitrogen after being wrapped in aluminium foil and stored at −80 °C. The collected cellophane membrane then were freeze-dried using a FreeZone12 vacuum freezer drier (Labconco, USA), and the dry samples weighed using an Ohaus Explorer® Precision digital balance (USA).

2.5. Toxin extraction and quantification using LC-MS/MS

The plugs were extracted with 500 μ L of acetonitrile: water: formic acid (79:20.9:0.1, v:v:v) and shaken for 90 min (300 rpm; 25 °C) before centrifugation for 10 min at 22600g. The supernatants were transferred into vials and kept at −20 °C. Appropriate shaking was applied before analysis. Measurements were performed by a qTRAP-LCMS 6500+ system (Exion Serie) coupled with IonDrive™ Turbo Spray (both Sciex Technologies, Warrington, UK). An ACE 3-C18 column (2.1 $\times$ 100 mm, 3 μ m particle size; Hichrom) equipped with a C18 security guard cartridge (4 $\times$ 3 mm, Gemini, Agilent) kept at 40 °C was used for chromatographic separation with the following gradient. Solvent A: water: acetic acid (v:v, 99:1) and Solvent B: methanol: acetic acid (v:v, 99:1) both supplemented with 5 mM ammonium acetate were applied for 15 min long with at a gradient of: 0 min, 10 % B; 0–2.0 min, 10–40 % B; 2.0–10.0 min, 100 % B; 10.0–11.50 min, 100 % B; 11.50–12.0 min, 5 % B; 12.00–15.0 min, 5 % B. The flow rate of the mobile phase was 0.3 mL/min, and the injection volume was set to 3 μ L.

An unscheduled Multiple Reaction Monitoring (MRM) mode was used to perform ESI-MS/MS in both positive and negative modes with a 10-millisecond dwell time per Q3 analysed. The source conditions were

set as follows: Curtain gas 40 %, Collision Gas Medium, IonSpray voltage −4500 V (in negative mode) and 5500 V (in positive mode), Temperature 400 °C, ion source gas 1 60 psi and ion source gas 2 60 psi. The Entrance Potential for all compound was fixed at 10 V. Two MRM per analyte were acquired to confirm the identity of the identified metabolite. The data were acquired using Analyst® Data Acquisition version 1.6.3, and the data quantification was performed using MultiQuant™ version 3.0.3. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated following the recommendations in Malachová et al. (2014). The media contained seven mycotoxins, including Beauvericin, Diacetoxyscirpenol, Fusarenon-X, 15-Monoacetoxyscirpenol, Neosolaniol, T-2 + HT-2.

2.6. Statistical analysis

Statistical analysis were performed using the package JMP® 14 (SAS Institute Inc., 2016. Cary, NC, USA) and Minitab® (Minitab, Inc. 2010. State College, PA, USA). All the data for growth and mycotoxin production were tested for normality by the Shapiro-Wilk test and homoscedasticity by the Levene's test. If both normality and homoscedasticity were validated than an ANOVA coupled with a student *t*-test was applied. If one was missing, a Kruskal-Wallis analysis was performed for single-factor analysis and an effect test was performed for multiple factors analysis. For mycotoxin production, if the data was not normal Johnson transformation was used within Minitab, then using the transformed data to perform a Tukey's pairwise comparison test at the 5 % level of significance within Minitab.

3. Results

3.1. Impact of elevated CO₂ level on the lag phases prior to growth and colonisation rates on oat-based media by non-acclimatised and acclimatised strains of the *Fusarium* sp.

Table 2 compares the effect of ambient vs elevated CO₂ levels on both lag phases and growth rates of non-acclimatised and acclimatised strains/species of *Fusarium* after 10 sub-cultures. The mean of the ten-generation lag phase and growth rate observed per strain in ambient and elevated CO₂ conditions are also presented in Table 2. This indicates that the lag times for all the strains tested was either unaffected (strains FL2, FL5) or statistically longer under elevated CO₂ conditions independently of the strain origin or species ($P < 0.05$). Fig. 1 compares the effect of acclimatisation to elevated CO₂ or ambient conditions on the lag phases and growth rates of strain FL7 as an example for sub-cultures 1–10. There was a trend of increased lag phase periods observed for generations 4–9. The fastest growth rates were mainly observed at elevated CO₂ conditions (e.g., acclimatised sub-culture 9).

3.2. Effect of acclimatisation for different sub-cultures under elevated CO₂ levels on T-2 + HT-2 production by strains of *F. langsethiae* and *F. sporotrichioides*

There was no conclusive evidence that CO₂ affected T-2 + HT-2 production except for FS where T-2 + HT-2 production was reduced when exposed to elevated CO₂ (Table 2). For four strains, an effect was observed in one of the three sub-cultures studied (Fig. 2, Fig. S1). For FL2, T-2 + HT-2 production was not detected under elevated CO₂ conditions after one sub-culture, but it was under ambient conditions, while at this same sub-culture, T-2 + HT-2 levels for FL4 were 7.1-fold higher under elevated CO₂ conditions when compared to ambient conditions ($P < 0.05$). After 7 sub-cultures, T-2 + HT-2 production in FL1 and FS was respectively 2- and 1.3-fold lower at elevated CO₂ conditions when compared to ambient conditions ($P < 0.05$).

An inter-strain variation in the different sub-cultures was observed in the production T-2 + HT-2 in *F. langsethiae*, which were categorised into three different patterns (Fig. 2; Fig. S1). The first pattern showed that T-

Table 2

Comparison of the lag time prior to growth (λ ; in days) and the mean growth rate (mm/day) and the T-2 + HT-2 production for different *Fusarium* species and strains in response to the ambient (400 ppm) and elevated (1000 ppm) CO₂ levels at 21/12 °C for a 16/8 h under dark conditions on oat medium. Values with * are statistically different from the elevated CO₂ conditions by ANOVA (lag time prior to growth and growth rate) and Kruskal Wallis (T-2 + HT-2 production). $\pm$ standard deviation. ND: Not detected.

Strain	Lag time (λ)				Diametric growth rate				T-2 + HT-2 production in ng/g of agar			
	Ambient CO ₂		Elevated CO ₂		Ambient CO ₂		Elevated CO ₂		Ambient CO ₂		Elevated CO ₂	
FL1	0.4	± 0.1	0.5	$\pm 0.1^*$	9.2	± 0.2	9.5	± 0.2	738.4	± 232.7	724.0	± 274.7
FL2	0.7	± 0.1	0.6	± 0.1	10.3	± 0.3	10.2	± 0.3	1494.0	± 670.0	1454.8	± 1426.8
FL3	0.4	± 0.1	0.5	$\pm 0.1^*$	9.0	± 0.3	9.0	± 0.2	811.2	± 195.4	971.1	± 460.5
FL4	0.3	± 0.1	0.4	$\pm 0.1^*$	8.7	± 0.4	8.9	± 0.4	70.8	± 67.8	172.2	± 148.0
FL5	0.4	± 0.1	0.3	$\pm 0.1^*$	9.3	± 0.3	9.8	$\pm 0.2^*$	902.2	± 200.3	804.0	± 200.7
FL6	0.5	± 0.1	0.6	$\pm 0.1^*$	9.6	± 0.6	10.2	$\pm 0.4^*$	1086.5	± 590.8	1073.0	± 525.1
FL7	0.3	± 0.1	0.5	± 0.1	9.3	± 0.6	9.9	$\pm 0.4^*$	1578.5	± 726.7	1280.6	± 244.1
FP1	0.2	± 0.1	0.4	$\pm 0.1^*$	11.0	± 0.2	11.7	$\pm 0.4^*$	ND		ND	
FP2	0.2	± 0.1	0.3	$\pm 0.1^*$	11.0	± 0.3	11.2	± 0.5	ND		ND	
FS	0.4	± 0.1	0.6	$\pm 0.1^*$	12.7	± 0.3	13.3	$\pm 0.2^*$	2417.8	± 254.6	2017.7	$\pm 554.8^*$

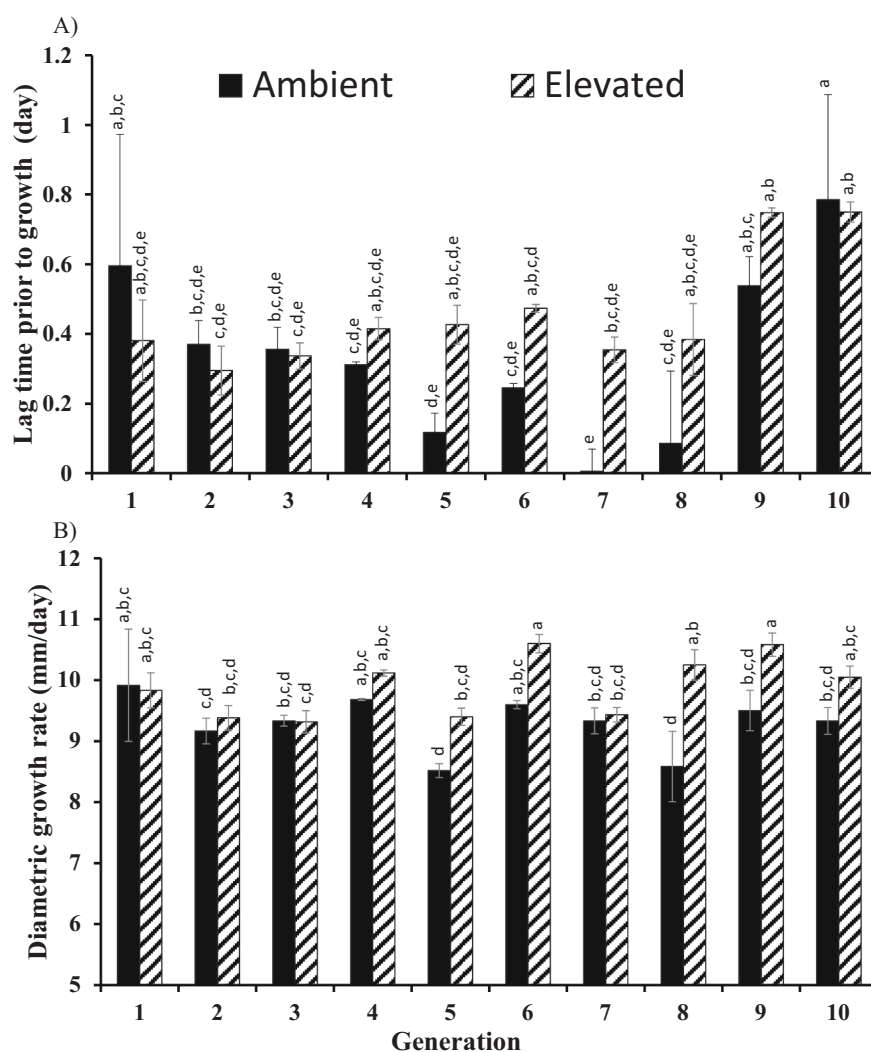


Fig. 1. Example of the effect after 10 sub-culturings of acclimatisation on (A) the lag time prior to growth and (B) growth rate of *Fusarium langsethiae* strain FL7 under ambient and elevated CO₂ conditions. Experiment done with three biological replicates. Different letters indicate significant differences between conditions ($P < 0.05$).

2 + HT-2 production in three of the seven strains of *F. langsethiae* (FL3, FL5 and FL6) was not impacted by sub-culturing for 10 generations, regardless of the CO₂ conditions ($P > 0.05$). In the second pattern, T-2 + HT-2 production in *F. langsethiae* strains FL1 and FL7 varied at sub-culture 7 when compared to the 1st and/or 10th sub-culture under

both CO₂ conditions studied ($P < 0.05$), but not when comparing sub-culture 10 versus 1. The third pattern was for strain FL4 that showed a decrease in T-2 + HT-2 production at sub-culture 10 compared to the initial one under elevated CO₂ conditions (4.4-fold) ($P < 0.05$), with no change observed under ambient conditions at the same sub-cultures

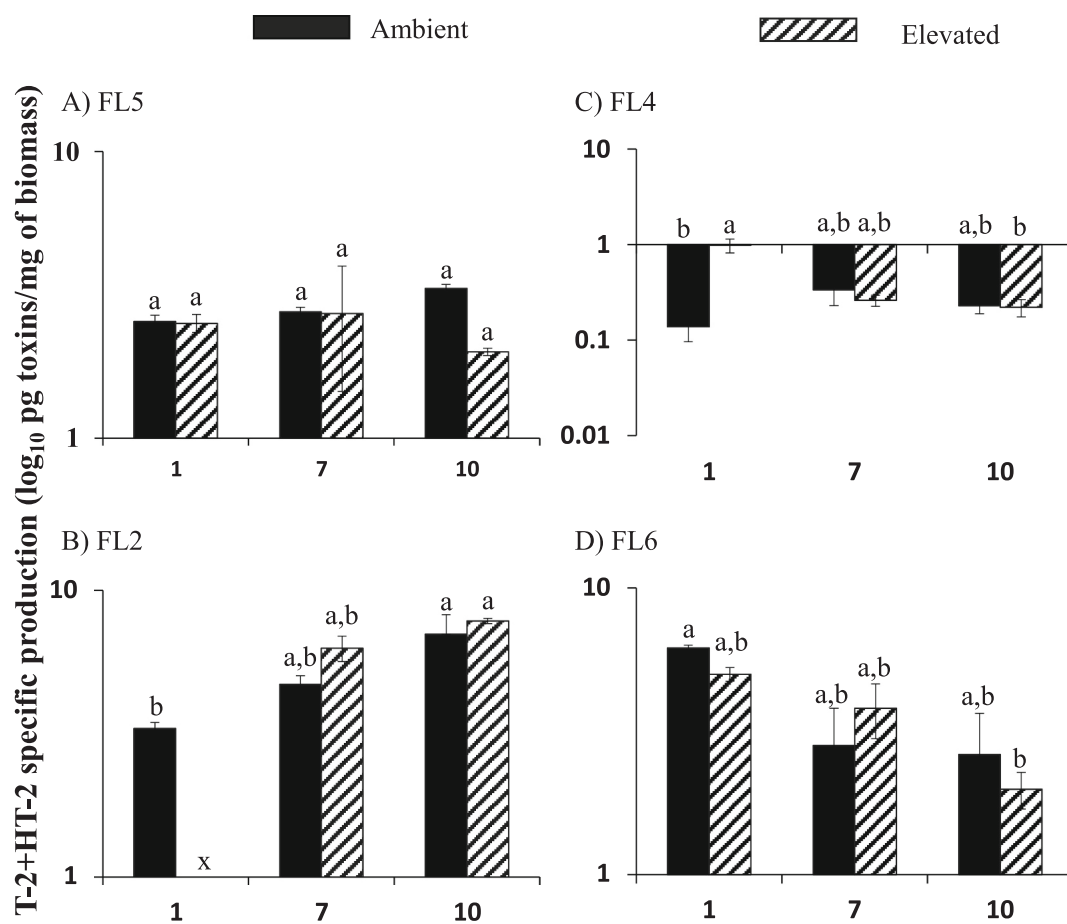


Fig. 2. The effect of 10 sub-culturings of acclimatisation on T-2 + HT-2 production by four *Fusarium langsethiae* strains (A) FL5, (B) FL2, (C) FL4, and (D) FL6 under ambient and elevated CO₂ conditions. x: only one of the triplicates was analysed and no toxins were detected. Experiment done with three biological replicates. Different letters indicate significant differences between conditions ($P < 0.05$).

(Fig. 2c; $P > 0.05$). In the one strain of FS studied, an increase of T-2 + HT-2 production was detected after 10 sub-cultures when compared to the first one under elevated CO₂ conditions (1.1-fold), compared to ambient conditions at the same sub-culture stages ($P < 0.05$).

Four different factors (species, strain, sub-culturing, and elevated CO₂), and four combinations of these factors were tested for their effect on *F. langsethiae* and *F. sporotrichioides* production of T-2 + HT-2. Statistically, for the 7 *F. langsethiae* and one *F. sporotrichioides* strain which all produced T-2 + HT-2 under the conditions tested, several of the tested factors were found to significantly impact on T-2 + HT-2 production (see Table 3). Elevated CO₂ in combination with strain and strain x sub-culture had a significant effect on T-2 + HT-2 production on the oat-based matrices.

3.3. Comparison of the effect of elevated CO₂ levels on other toxic secondary metabolites production by non-acclimatised and acclimatised strains of the *Fusarium* species

F. langsethiae, *F. poae* and *F. sporotrichioides* were collectively able to produce additional secondary toxic metabolites on oat-based medium (Table 4); beauvericin (BEA), diacetoxyscirpenol (DAS) and neosolaniol (NEO). The production of these toxic secondary metabolites was quantified and monitored after acclimatisation for 1, 7 and 10 generations. Under ambient and elevated CO₂ conditions (400 and 1000 ppm, respectively). The FS strain was able to produce all of these secondary metabolites; all FL strains except FL5 were able to produce NEO and DAS; FL5 was able to produce only NEO; FP1 was able to produce only BEA; FP2 was able to produce BEA and DAS (Table 4).

Table 3

Statistical significance of the effect of single, two and three-way interacting factors on the lag times prior to growth, growth rates and T2 + HT-2 toxin production by the strains of all three *Fusarium* species examined in this study. *Statistically significant P -values. 9/3: 9 applies to lag time and growth rate values while 3 applies to the T-2 + HT-2 data.

Factors	DF	P -values		
		Lag time (days)	Growth rate (mm/day)	T-2 + HT-2 (μ g/g)
Species	2	<0.0001*	<0.0001*	<0.0001*
Strain	9	<0.0001*	<0.0001*	<0.0001*
Sub-cultures	9/3	<0.0001*	<0.0001*	0.1287
Elevated CO ₂	1	<0.0001*	<0.0001*	0.9361
Strain x sub-cultures	81	<0.0001*	<0.0001*	<0.0001*
Strain x elevated CO ₂	9	<0.0001*	0.0018*	0.0081*
sub-cultures x elevated CO ₂	9	0.0005*	<0.0001*	0.0635
Strain x sub-cultures x elevated CO ₂	81	0.0121*	<0.0001*	<0.0001*

The production of NEO and DAS were not significantly impacted by either sub-culturing or elevated CO₂ conditions for strains FL3, FL4, FL6, FL7 and FS (data not shown; Fig. S2c, Fig. S2e; $P > 0.05$). *F. langsethiae* strains FL1 (Fig. S2a) and FL5 (data not shown) both produced DAS regardless of the acclimatisation for 10 generations or the CO₂ levels. For NEO, both strains produced significantly more NEO after 10 generations when compared to those after 7 and 1, regardless of the CO₂ conditions ($P < 0.05$, except for FL1 sub-culture 10 vs 1: $P = 0.0656$).

Table 4

Range of average specific production level in ng/g of media for the secondary metabolites detected in this study produced by the different *Fusarium* species. Key to secondary metabolites: BEA, Beauvericin; DAS, Diacetoxyscirpenol; NEO, Neosolaniol. ND: Not detected.

<i>Fusarium</i> species	Toxic secondary metabolites produced				
	T-2	HT-2	DAS	NEO	BEA
<i>F. langsethiae</i> (FL1)	217.3–694.3	235.2–320.5	798.9–1304.1	1144.4–2714.3	ND
<i>F. langsethiae</i> (FL2)	ND – 813.0	ND – 1728.5	ND – 1161.1	ND – 3973.9	ND
<i>F. langsethiae</i> (FL3)	276.2–759.2	257.6–717.7	227.6–424.5	1785.2–2948.1	ND
<i>F. langsethiae</i> (FL4)	ND – 13.9	32.6–342.8	ND	ND–1472.7	ND
<i>F. langsethiae</i> (FL5)	314.8–664.9	226.8–526.4	1205.5–2003.9	1074.5–2495.6	ND
<i>F. langsethiae</i> (FL6)	335.4–1104.2	196.9–658.6	854.6–1781.6	2192.4–4853.3	ND
<i>F. langsethiae</i> (FL7)	520.7–1028.1	233.8–1321.3	1128.5–3423.3	818.5–33,445.1	ND
<i>F. sporotrichioides</i> (FS1)	1422.1–1615.0	228.3–983.0	2202.5–3491.9	5126.7–15,738.7	103.2–277.9
<i>F. poae</i> (FP1)	ND	ND	ND – 1438.7	ND	223.9–6969.3
<i>F. poae</i> (FP2)	ND	ND	ND	ND	ND – 905.5

The two strains of *F. poae* and the one of *F. sporotrichioides* both produced BEA (Table 4). The production of BEA by *F. sporotrichioides* was not significantly impacted by number of sub-cultures or acclimatisation to elevated CO₂ conditions (Fig. S2e; $P > 0.05$). However, for the two *F. poae* strains, the BEA profile was impacted. In FP1, elevated CO₂ significantly triggered BEA production after the initial sub-culture by 29-fold in comparison with BEA production under ambient CO₂ conditions at the same sub-culture stage ($P < 0.05$). FP1 acclimatisation led to a decrease of BEA production by 6-fold between the initial sub-culture and that after 10, under elevated CO₂ conditions. However, there was an increase by approx. 12-fold between the same generations (1 and 10) under ambient CO₂ conditions ($P < 0.05$). For FP2, the production was not affected under elevated CO₂ conditions compared to ambient conditions in the different sub-cultures examined ($P > 0.05$). However, BEA production decreased under ambient conditions with no detection of this compound after 10 generations when compared to the sub-cultures after 1 and 7 generations on the oat-based matrix under the same conditions (data not shown; $P < 0.05$).

4. Discussion

This study is the first to assess the impact of acclimatisation to elevated CO₂ on the growth and toxin production by *F. langsethiae*, *F. sporotrichioides* and *F. poae* strains isolated from UK and Norway oats grains on oat-based medium. This study included an assessment of the sub-culturing the species and strains for up to 10 generations to examine effects on growth and toxic secondary metabolite production. This has not been examined previously for any mycotoxigenic species. The lag phases prior to growth and subsequent colonisation of oat-based media by the strains of *Fusarium* acclimatised for different generations suggest relatively good resilience of the strains examined, especially after 10 sub-cultures acclimatisation. While in some cases acclimatisation under elevated CO₂ (1000 ppm) resulted in a delay in initial growth (lag phases) the colonisation rates were relative unaffected and, in some cases, better when acclimatised under elevated CO₂ (1000 ppm). A previous study by Verheeeke-Vaessen et al. (2019) found that at 25 °C and with freely available water (0.995 a_w), *F. langsethiae* strain FL1 had a reduced lag phase and similar growth rate when exposed to 1000 ppm CO₂ compared to the ambient conditions (400 ppm). However, this previous study was carried out under steady state temperature x a_w x elevated CO₂ conditions with a non-acclimatised strain. The present work simulated the fluxes in day/night temperature (21/12 °C for a 16/8 h, dark) during fungal cultivation in all 10 sub-cultures of the species and strains. This contrasts with some of the previous limited studies previously carried out (Medina et al., 2017; Peter Mshelia et al., 2020).

A range of strains of *F. langsethiae* were used to examine the consistency of effects due to acclimatisation in the present study. Indeed, this showed that there was some variation between strains in terms of effects of acclimatisation on both lag time prior to growth and on relative colonisation rates. The only comparable study was by Váry et al. (2015)

which utilised 10 sub-cultures of acclimatisation of the wheat fungal pathogen *F. graminearum*. However, they did not examine inter-strain differences. In their research, it was found that *F. graminearum* that had been exposed to high levels of CO₂ for 10 generations had a greater pathogenicity in ripening wheat ears and showed more visible symptoms. However, the impact on the contamination of wheat grain with DON was not measured. Another study was conducted on *F. verticillioides* and *F. graminearum*, in which temperature was gradually increased in 10 subcultures under 800 or 1200 ppm CO₂. This study showed that acclimatised strains were not affected in terms of growth, zearalenone, type B trichothecenes, and fumonisin production (Peter Mshelia et al., 2020). Baazeem et al. (2022) recently carried out acclimatisation studies with the xerophilic mycotoxigenic species *Aspergillus flavus* for 5 sub-cultures on a pistachio-based medium at 35 °C or 37 °C, 400 or 1000 ppm CO₂, and 0.93 or 0.98 water activity (a_w). They found little differences in colonisation of raw pistachio nuts by non-acclimatised and acclimatised strains of *A. flavus* at 37 °C and 1000 ppm CO₂ and different steady state a_w levels. However, there was some differential effects on aflatoxin B₁ contamination with one acclimatised strain showing a stimulation of toxin production and another none under climate-related abiotic factors (temperature x a_w x elevated CO₂). According to Medina et al. (2017) and Perrone et al. (2020), the combined impact of increased temperature, elevated CO₂, and drought stress on mycotoxigenic fungal pathogens may have a greater influence on their evolving resilience or fitness than any single or combination of two climate-related abiotic factors..

Few if any studies examining resilience of mycotoxigenic fungi in relation to climate change, especially for acclimatised strains, have focused on changes in toxic secondary metabolite production. The present study showed that T-2 + HT-2 production was significantly increased in response to elevated CO₂ in one out of seven *F. langsethiae* strains depending on number of acclimatisation sub-cultures made. One other strain of *F. langsethiae* (FL1) and the one *F. sporotrichioides* strain produced less T-2 + HT-2 after 7 sub-cultures under elevated CO₂ compared to ambient conditions, and after acclimatisation for 10 sub-cultures, T-2 + HT-2 production equalled or increased when compared to the initial cultures under CO₂ conditions. The rest of the isolates showed the same production of T-2 + HT-2 under elevated CO₂ compared to the ambient conditions. Recently, Verheeeke-Vaessen et al. (2021) showed that the production of T-2 + HT-2 by the strain FL2 used in our present study was not differentially impacted by elevated CO₂ when exposed to elevated temperatures of 30–34 °C in combination with water stress (0.98 and 0.95 a_w). The effect on multiple mycotoxins and emerging mycotoxins has attracted much interest as many cereals are contaminated with more than one mycotoxin (Gruber-Dorninger et al., 2019; Raj et al., 2022). The present study showed that regardless of acclimatisation for 10 generations, the patterns of production of the four type A trichothecenes, T-2, HT-2, NEO, and DAS under elevated CO₂ conditions followed the same pattern in terms of relative amounts produced. The only exception was for acclimatised sub-cultures of

F. langsethiae strains FL5 that showed no significant impact on T-2 + HT-2 production or DAS but a switch to increased production of NEO perhaps due to the acclimatisation. This may be important in developing a better understanding of synergies between related toxic compounds of the same family (Chen et al., 2020).

For the three strains producing BEA, only the two *F. poae* strains showed significant differences in toxic secondary metabolite production due to acclimatisation for 10 sub-cultures under 1000 ppm CO₂. In addition, NEO and DAS production was only impacted by elevated CO₂ conditions after one acclimatisation sub-culture for one of these two strains. This did not occur subsequently.

In conclusion, this study suggests that there may be complex interactions between the resilience of different mycotoxigenic species and their evolution during acclimatisation to climate change-related abiotic factors. It is important to understand the ecophysiology of the changes in acclimatisation for 10 or more generations and also quantify the possible changes in biosynthetic genes involved in toxic secondary metabolite production and whether this results in a switch to related toxic metabolites which could become more important in the development of resilience to climate change-related interacting abiotic conditions. This could have significant implications for the fitness of such strains and the role of secondary metabolites in pathogenicity of a range of staple cereals and other commodities.

CRedit authorship contribution statement

Amal KAHLA: resources, realisation of the fungal growth experiment, formal analysis, writing original and review, **Carol VERHEEKE-VAESSEN:** conceptualization, methodology, data analysis and statistics, writing Original and review, LC-MS/MS analysis, **Mariluz DELPINO-DEELIAS:** support of the experiment, fungal growth measurement and sample taking, **Maria GUTIERREZ-POZO:** LC-MS/MS analysis, **Angel MEDINA, Naresh MAGAN and Fiona DOOHAN:** conceptualization, reviewing, editing, supervision, and funding acquisition.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2023.110176>.

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Acclimatisation of *Fusarium langsethiae*, *F. poae* and *F. sporotrichioides* to elevated CO₂: impact on fungal growth and mycotoxin production on oat-based media

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