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Comparisons of water activity and temperature impacts on growth of *Fusarium langsethiae* strains from northern Europe on oat-based media

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Running title: Environmental factors over the *F. langsethiae* ecology

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Abstract

This study has examined the effect of water activity (a_w, 0.995-0.90) and temperature (10-37^{o}C) on the lag phases prior to growth, growth rates and used models to develop two dimensional profiles for optimum and marginal conditions for two strains of *Fusarium langsethiae* from four northern European countries (UK, Norway, Sweden, Finland) on an oat-based medium. Results showed that the optimum a_w for growth was at 0.98-0.995 and 25^{o}C. The limit for growth of the strains was at 0.92-0.93 a_w with minima of 10^{o}C. No growth occurred at 37^{o}C. The lag phases prior to growth were lowest under optimum conditions and extended to >10 days at marginal conditions. Statistical analyses of intra and inter-strain differences in terms of both lag phases prior to growth and growth rates were not statistically significant. However, a_w and temperature were statistically significant factors. Two dimensional profiles for strains from each country of origin were built to identify optimum and marginal conditions for *F. langsethiae* for the first time. These environmental profiles will be beneficial for improving the ecological knowledge of this species which is able to produce trichothecene mycotoxins in a range of temperate cereals.

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Introduction

Fusarium species are important pathogens and cause significant losses in quality of cereals and concomitant contamination with mycotoxins. Studies in Norway on the occurrence of *Fusarium* spp. in cereal grain (Kosiak et al., 1997 and Langseth and Rundberget, 1999) led to the description of a new "powdery *Fusarium poae*" isolate which produced the mycotoxins T-2 and HT-2 (Torp and Langseth, 1999). This led to the description of a new species *Fusarium langsethiae* (Torp and Nirenberg, 2004). This species produces type A trichothecenes, especially the highly toxic T-2 and HT-2 toxins, and diacetoxyscirpenol (DAS) (Thrane et al., 2004).

T-2 toxin, the most toxic Type A trichothecene, is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunosuppressive and cytotoxic effects both *in vitro* and *in vivo* (Visconti, 2001 and Canady et al., 2001). Data collected to evaluate the risk of dietary exposure to *Fusarium* toxins by the populations of EU member states showed that T-2 and HT-2 toxins are quite common contaminants in cereals in the EU (Schothorst and van Egmond, 2004). *F. langsethiae* has been implicated in the occurrence of these toxins in surveys of cereals in temperate/cool regions such as Norway (Langseth and Rundberget, 1999) and in oats in the UK (Edwards, 2007).

F. langsethiae has been isolated from infected oats, wheat and barley in central and northern Europe (Torp and Adler, 2004; Torp and Nirenberg 2004 and Hudec and Rohácik, 2009). One problem is that this species can be readily isolated from small-grain cereals from symptomless oats and wheat grains. Its pathogenicity on these cereals has been demonstrated recently (Imathiu et al., 2009). Indeed, a Real-time PCR system has been developed to try to aid the detection of this species in Swedish oats (Fredlund et al., 2010).

At present there is no information on whether infection by this species may be important predominantly pre-harvest or whether poor post-harvest storage enables growth and contamination with T-2 and HT-2 toxins. This is important as the EU is considering setting

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legislative limits for contamination of cereals with the combined total of the two toxins. There is no detailed knowledge on the ecology of *F. langsethiae* and whether strains from different northern European countries respond to changes in interacting environmental factors in a similar manner or differently.

The objectives of this study were to compare the effect of the key ecological parameters of a_w and temperature and their interactions on (a) lag times prior to growth, (b) minimum and optimum conditions for growth and (c) develop two dimensional contour maps of the profiles for growth of two strains each of *F. langsethiae* from the UK, Norway, Sweden and Finland on an oat-based medium. Statistical comparisons were also made to identify intra- and inter-strain similarities and differences.

2. Material and methods

2.1 Strains

Eight *F. langsethiae* strains from different origins were used. These were: U.K. (strains 2004/57, 2004/59); Norway (44P, 88E); Finland (05010, 05014) and Sweden (560, 562). These strains are available via the Culture Collections of Harper Adams University College, UK; Agricultural University As, Norway; Finnish Food Safety Authority (Evira, Helsinki, Finland); Swedish Food Authority, Uppsala, Sweden.

2.2 Medium preparation and inoculation

Milled whole oats were prepared by milling for 5 mins in a Waring Laboratory Science blender model 7009G (Waring Laboratory Science, CT, USA). Mixtures of 2% (w/v) oat flour in water were prepared and 2% (w/v) agar (Technical agar No. 2, Oxoid) added. Water used to prepare the medium was modified with glycerol to the required a_w levels (0.995, 0.98, 0.95, 0.93, 0.90 and 0.88). The culture media were autoclaved for 20 minutes at 121 ^{o}C. The molten media were vigorously shaken and poured into 9 cm Petri dishes (15 ml per plate). The final pH in all

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media was 6.2. In all cases 7 day old cultures were used to inoculate the different a_w x temperature treatments. For inoculation of each strain of *F. langsethiae* agar discs (4 mm diameter) were taken from the margin of the growing colonies and placed in the centre of the treatment Petri plates. These were incubated at different temperatures (10, 15, 20, 25, 30, 37^{o}C) for 10 days. All experiments were carried out with three replicates per treatment and repeated twice.

2.3 Calculation of lag phases and mycelial growth rates

Primary modelling was carried out on the colony radii data. In general, data plots showed, after a lag phase, a linear trend with time. Only in a few cases were the curves asymptotic. Due to this, data was fitted using the Baranyi biphasic model (Baranyi et al., 1993 and Baranyi and Roberts, 1994) and a linear model obtained by plotting the results against time. The linear parts were used. From these primary models the maximum lag phases (λ) and growth rates (μ_m) were obtained.

2.4 Statistical analysis and profiling

Statistical analysis was performed using the package JMP® 8 (SAS Institute Inc., 2008. Cary, NC, USA) and STATISTICA 8 (StatSoft® Inc., 2007. Tulsa, OK, USA). Only cases where growth actually occurred during the 10 day incubation period were used in the statistical analysis. Data on relative growth rates obtained from the different primary models were tested for normality using the Shapiro-Wilk test. Due to non-normality of the data, growth rate data was transformed using $\log_{10}(x)$. Homoscedasticity was checked using Levene's and Brown-Forsythe's tests. Finally, prior to analysis possible outliers were identified using Mahalanobis distances test. Subsequently, Analysis of Variance (ANOVA) was applied in order to find differences between origin of strains, temperatures, water availabilities and strains. The

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Tukey-Kramer significant difference (Tukey-Kramer HSD) multiple range test at 95% confidence level was used to group the cases into homogeneous groups with regard to the different parameters used. T-tests were used in order to compare intra-strain differences. Contour maps were developed using Sigma Plot v.10.0 (Systat Software Inc. Hounslow, London, UK).

3. Results

3.1. Initial primary modelling

Two different models were considered initially and growth curves were fitted using the Baranyi biphasic model and a linear model obtained by plotting the results against time. Results obtained by both approaches were very similar and based on the studies of Marin et al. (2008) the Baranyi biphasic model was selected to analyse all the experimental data because this minimised subjectivity in the calculation of the lag phase (λ) and growth rate (μ_m).

3.2. Effect of water activity and temperature on lag phases prior to growth

Generally, the lag phase increased when temperature and a_w stress were imposed in the oat-based culture media (Table 1). Both temperature (p=<0.0001) and water availability (p=<0.0002) had significant effects on the lag phases prior to growth (Table 2). These data are means for two strains although in some cases one of the two strains did not grow at 0.93 a_w. At this marginal a_w level and under some temperatures the lag times were >10 days, which was the maximum experimental period used in this study.

3.3. Comparison of effects of water activity and temperature on mycelia growth of *F. langsethiae* strains

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Figure 1 shows the effect of a_w at different temperatures on the mean growth of two strains each from the four different countries. This shows that in all cases strains grew best at 20-25^{o}C at 0.98-0.995 a_w. After 10 days, only strains from Finland, Norway and Sweden were able to grow at 0.93 a_w with no growth being observed at 0.90 a_w regardless of the temperature. No growth was also observed at 37^{o}C during the experimental periods of this study. Statistical analyses (ANOVA) of the relative growth rates showed that there was no significant intra- and inter-strain differences, although overall, temperature ($p=0.0009$) and a_w ($p=<0.0001$) were statistically significant environmental factors (Table 3).

Using the Tukey-Kramer HSD test at 95% confidence levels, the treatments were grouped into homogeneous groups with regard to these two parameters. The a_w level provided three different non-overlapping clusters. In the first cluster 0.995 and 0.98 a_w were grouped together confirming that highest growth rates occur at these a_w levels. The other water availabilities were placed in two different groups corresponding to 0.95 and 0.93 a_w.

Analyses of the temperature effects provided two different overlapping clusters. The first cluster was composed of 15 to 25^{o}C and the second cluster included 30, 10, 15 and 20^{o}C. Thus only significant differences were observed between 30 and 10 with 25^{o}C . These results confirmed the faster growth was observed in warmer temperatures. Possible intra-strain differences were examined using T-test analysis. For each of the four pairs of strains there were no significant differences (In all cases $t> 0.18$). The most marked differences were observed between the two Swedish strains.

3.4. Two dimensional contour maps of growth profiles of *F. langsethiae* in relation to temperature and water activity

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The conditions under which equivalent growth rates occurred under different environmental conditions were joined to produce contour lines to map the relative optimum and marginal conditions for growth of the *F. langsethiae* strains from each country examined. Figure 2 compares these profiles by country of origin, based on the mean data for two strains in each case. It should be noted that in these studies no growth occurred at 37°C and thus growth may be limited to temperatures of <35°C. These contour maps show that optimum conditions for all strains, regardless of origin, is around 25°C at >0.98 a_w. Growth rates were in the range 4.5-5.0 mm day^{-1}. Marginal conditions for growth are over a wider temperature range and approx. limited at about 0.93-0.92 a_w. For comparison, 0.98 a_w represents about 28-30% moisture content in oat grains at 15-25°C (wet weight basis) and would be considered to be very wet conditions, while 0.93-0.92 a_w is about 23-24% and 0.90 a_w approx. 19-21% moisture contents.

4. Discussion

This is the first study focused on impact of important environmental factors on the ecology of *F. langsethiae*. We have analysed strains from four different European countries and the effect of temperature and water availability on the lag phases prior to growth and growth rates on a relevant oat-based medium. Similarities and differences between strains and between origins were also taken into account. The contour maps for the effect of interacting conditions of a_w x temperature on optimum and marginal conditions for growth should be very useful in determining the risk factors during harvesting and post-harvest management of grain, especially oats.

Since the differentiation between *Fusarium sporotrichioides*, *F. langsethiae* and *F. poae* was made possible by using molecular means (Wilson et al., 2004 and Yli-Mattila et al., 2004), *F. langsethiae* has become one of the important fungi to consider regarding T-2 and HT-2 contamination of cereals, especially oats in northern Europe.

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The present study has shown that environmental factors affect the lag phases (λ) prior to growth. Other studies of *Fusarium* spp. such as *F. verticilliodes* and *F. proliferatum* from the section Liseola have shown that both lag phases prior to growth and mycelial extension were maximum at marginal conditions such as 0.90-0.92 a_w and marginal temperatures (Marin et al 1995; Marín et al., 1996 and Etcheverry et al., 2002). This is important information as the addition of fungicides or other control systems can then be examined to try and increase this lag phase significantly to minimise the potential for growth and T-2/HT-2 toxin production under variable environmental conditions.

When the results for water relations of growth of *F. langsethiae* strains are compared with data of other *Fusarium* species, the overall profiles are relatively similar to those for *F. culmorum* and *F. graminearum* and perhaps *F. avenaceum* (Magan and Lacey, 1984). However, *F. langsethiae* strains appear to be slightly more sensitive to a_w than these others. No growth was observed in the present study at 0.90 a_w regardless of strain or temperature. Previous studies have shown that these aforementioned species were able to grow at 0.90 a_w, with germination occurring at a_w conditions of 0.88-0.90 (Magan and Lacey, 1984; Marin et al., 1995 and Ramirez et al., 2006).

Our data shows that, generally, maximum growth rates were obtained at 25ºC. No differences were found when statistical comparisons were made for intra and inter-strain differences. Certainly this appears to be a consistent trait. For example, studies with different strains of *F. graminearum* have shown optimum growth at 25^{o}C, for *F. culmorum* and for *F. poae,* 20-25^{o}C. The latter species is closely related to *F. langsethiae* (Cook and Christen, 1976; Pettitt et al., 1996; Brennan et al., 2003; Doohan et al., 2003 and Ramirez et al., 2006).

Few detailed studies have been carried out to examine the effect of these important environmental factors on pathogenicity of *F. langsethiae*. One recent study has concluded that, at 5 and 15^{o}C, under "in vitro" conditions, *F. langsethiae* appears not to be responsible for *Fusarium* seedling blight (FSB) in oats and wheat (Imathiu et al., 2010). Although this

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species does not produce any disease symptom in the seeds, molecular studies have shown that *F. langsethiae* DNA is present in ripening oats between flowering and harvest (Parikka et al., 2007; Yli-Mattila et al., 2008; Fredlund et al., 2010). Furthermore, good correlations have been obtained between total *F. langsethiae* DNA and the presence of T-2 and HT-2 toxins (Yli-Mattila et al., 2008; Fredlund et al., 2010).

Some studies have suggested that higher contamination with *F. langsethiae* and T-2/HT-2 toxin has been found in conventional than organically produced oats (Edwards, 2009). Differences in toxin contamination depending on the origin have been also found. Scudamore et al. (2009) in a recent four year study of contamination of oats with T-2/HT-2 found highest contamination in samples from the UK and Ireland while levels from Scandinavia were usually lower. Certainly, our studies suggest that there is little difference between strains from different northern European countries in terms ability to grow under interacting extrinsic environmental factors.

There has been little information on whether growth and toxin production may occur during damp conditions at harvest or during poor drying. Since oats are often harvested late in the season damp conditions during harvesting and post-harvest drying and storage can occur. With the EU considering legislative limits on combined T-2 and HT-2 content in oats and derived products, it is critical to understand and identify high and low risk ecological conditions in relation to colonization by *F. langsethiae*. Our results show that *F. langsethiae* species grow best at temperatures around 25^{o}C and at > 0.98 a_w where water is more available. Studies are now required to determine the effect of these interacting environmental factors on T-2/HT-2 toxin production to understand how production profiles compare with those for growth. While there was little intra and inter-strain differences in growth, we now need knowledge of whether changes in environmental stress has a similar effect on toxin production by these strains.

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Table 1. Mean lag time (λ), in days ± standard error, for two strains of *F. langsethiae* in relation to temperature x water activity treatments.

		Temperature									
		30°C		25°C		20°C		15°C		10°C	
	a_w	λ	± S.D.[a]	λ	± S.D.	λ	± S.D.	λ	± S.D.	λ	± S.D.
England	0.995	3.92	± 0.14	0.51	± 0.04	0.98	± 0.08	1.75	± 0.15	2.91	± 0.14
	0.98	2.27	± 0.09	0.80	± 0.09	1.24	± 0.13	2.02	± 0.06	2.57	± 0.03
	0.95	2.98	± 0.13	1.39	± 0.01	1.82	± 0.32	4.25	± 0.05	6.31	± 0.40
	0.93	6.85	± 0.15	2.95	± 0.24	4.92	± 0.22	>10		>10	
Finland	0.995	1.51	± 0.28	0.39	± 0.18	1.27	± 0.05	2.09	± 0.14	3.70	± 0.13
	0.98	0.74	± 0.12	1.04	± 0.13	1.55	± 0.03	2.47	± 0.26	4.64	± 0.07
	0.95	2.14	± 0.12	1.86	± 0.11	2.79	± 0.08	5.66	± 0.06	>10	
	0.93	3.70	± 0.29	4.65	± 0.10	9.67	± 0.15	>10		>10	
Norway	0.995	0.35	± 0.17	0.83	± 0.12	1.24	± 0.03	2.19	± 0.36	3.27	± 0.13
	0.98	0.25	± 0.27	0.88	± 0.00	1.15	± 0.09	1.96	± 0.41	4.19	± 0.63
	0.95	1.76	± 0.18	1.34	± 0.28	1.95	± 0.04	4.66	± 0.79	6.33[b]	
	0.93	3.56	± 0.24	4.35	± 0.24	5.25	± 0.25	>10		>10	
Sweden	0.995	4.07	± 0.32	1.35	± 0.03	1.60	± 0.20	2.34	± 0.02	3.51	± 0.52
	0.98	2.08	± 0.21	1.09	± 0.28	1.67	± 0.14	2.49	± 0.09	4.05	± 0.47
	0.95	3.19	± 0.23	1.71	± 0.13	3.84	± 0.01	3.71	± 0.56	7.72	± 3.77
	0.93	5.98	± 0.11	>10		>10		>10		>10	

[a] Standard deviation

[b] Only one plate grew

>10 No growth observed after 10 days

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Table 2 . The ANOVA results for comparing the effect of factors on lag times prior to growth of 8 strains of *F. langsethiae* on oat-based medium. * denotes significant values

Source	DF	F Ratio	Prob > F
Origin	3	0.7745	0.5124
Temperature	4	5.0560	0.0013*
Water availability	3	10.0804	<0.0001*

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Table 3 . The ANOVA results for comparison of effect of factors on growth of the 8 strains of *F. langsethiae* studies on oat-based medium. * denotes significant values

Source	DF	F Ratio	Prob > F
Origin	3	0.7053	0.5506
Strain	7	0.5174	0.8199
Temperature	4	5.0061	0.0009*
Water availability	3	34.1598	<0.0001*

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Figure legends

Figure 1. Effect of a_w at different temperatures on the mean growth of two strains each from the four different countries.

Figure 2. Two dimensional contour maps of growth profiles of *F. langsethiae* from different origins (A: England, B: Finland, C: Norway and D: Sweden) in relation to temperature and water activity. Numbers on the isopleths refer to similar growth rates (mm/day).

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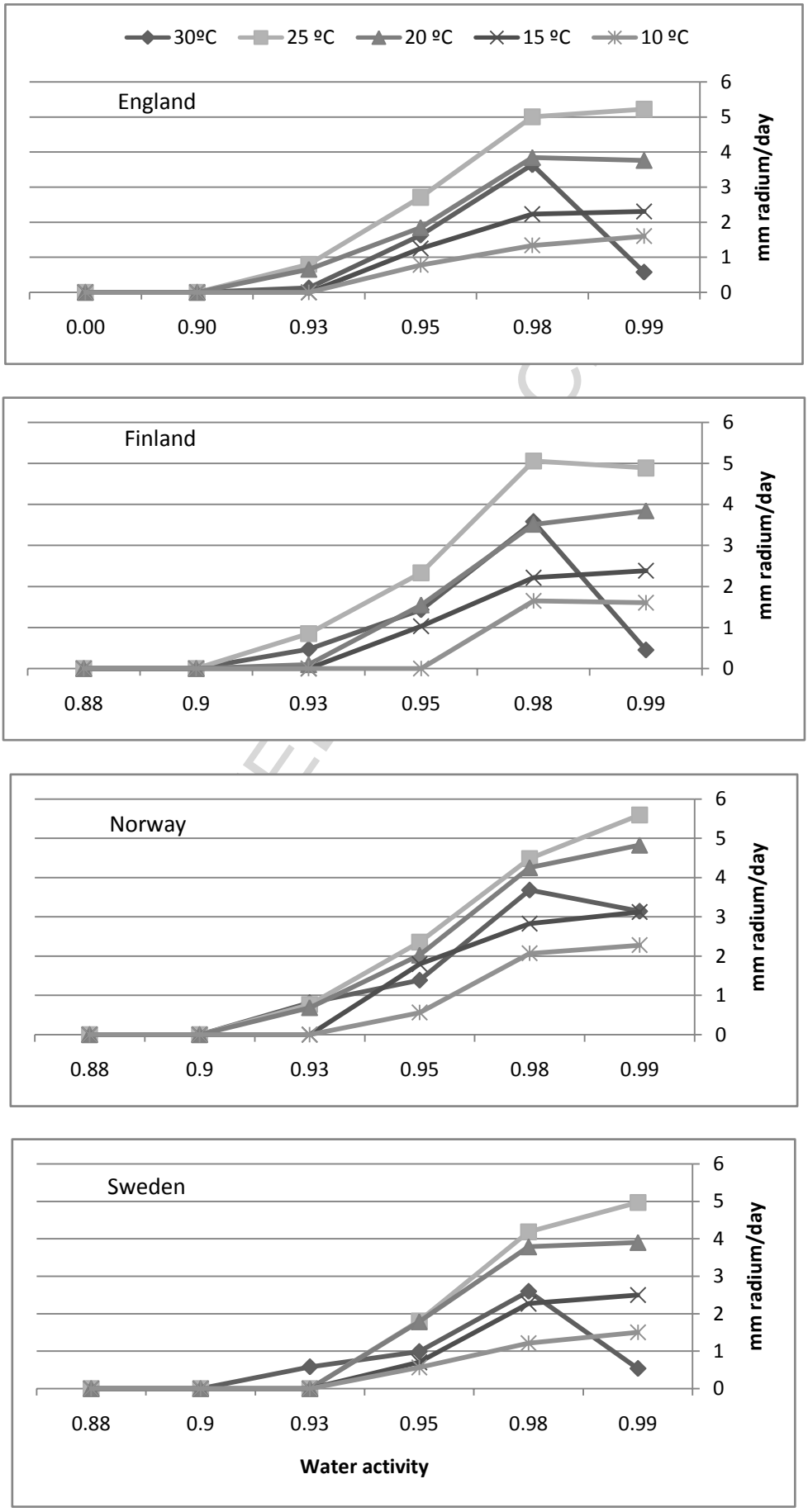


Figure 1 Medina & Magan

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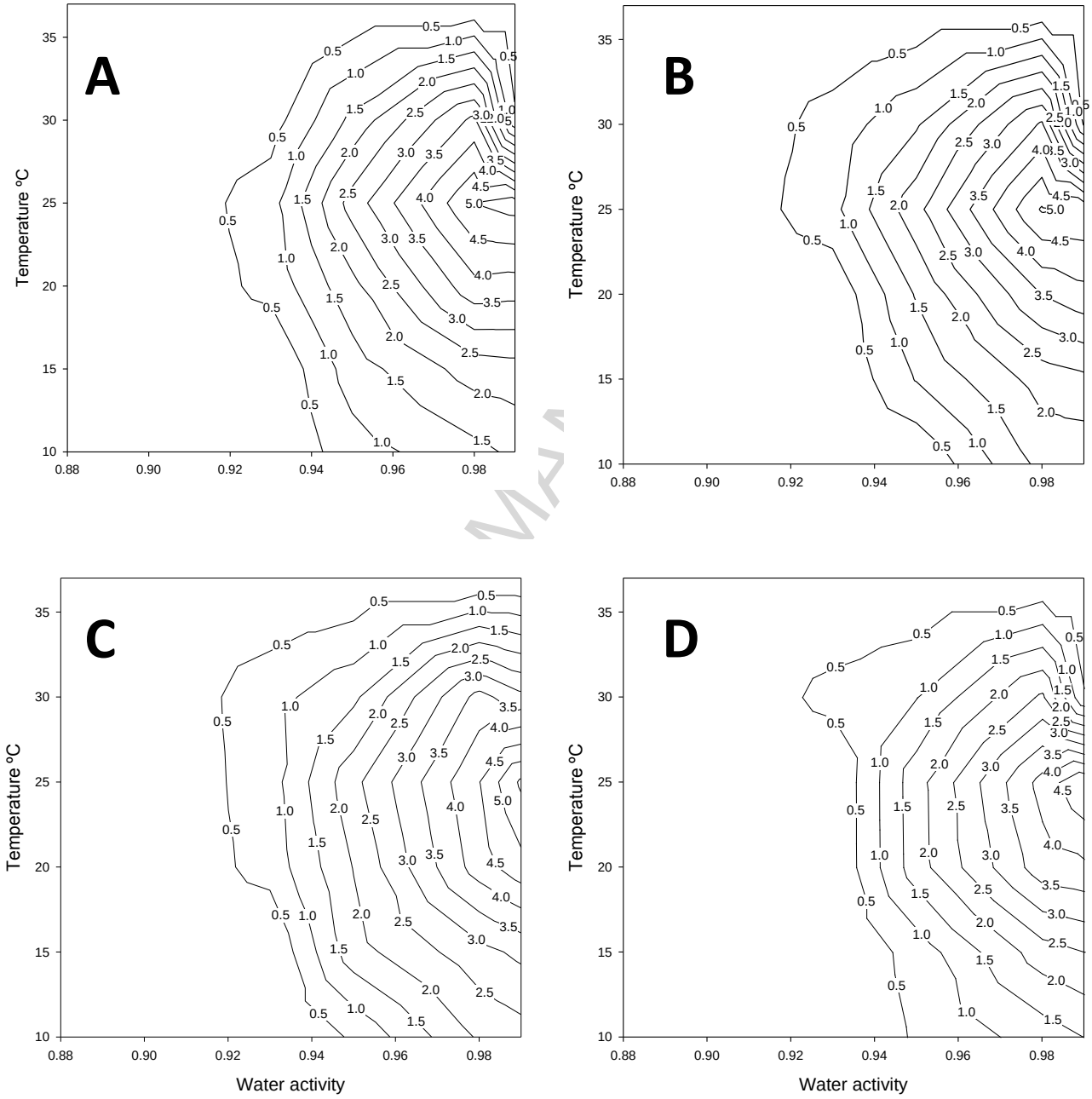


Figure 2 Medina & Magan



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Comparison of water activity and temperature impacts on growth of Fusarium langsethiae strains from northern Europe on oat-based media

Medina, Angel; Magan, Naresh

2010-09-01T00:00:00Z
Elsevier Science B.V., Amsterdam.
http://dx.doi.org/10.1016/j.ijfoodmicro.2010.07.021
A. Medina, N. Magan, Comparisons of water activity and temperature impacts on growth of Fusarium langsethiae strains from northern Europe on oat-based media, International Journal of Food Microbiology, Volume 142, Issue 3, 1 September 2010, Pages 365-369

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