Fungal Ecology, Volume 24, Part A, 2016, Pages 44-52,
DOI: 10.1016/j.funeco.2016.05.010

# Relationship between environmental conditions, carbon utilisation patterns and Niche Overlap Indices of the mycotoxigenic species *Fusarium verticillioides* and the biocontrol agent *Clonostachys rosea*

**Nik Iskandar Putra SAMSUDIN[1,2], Angel MEDINA[1], Naresh MAGAN[1*]**

[1]Applied Mycology Group, Cranfield Soil and AgriFood Institute,
Cranfield University, MK43 0AL Cranfield, Bedfordshire, UNITED KINGDOM

[2]Department of Food Science, Faculty of Food Science and Technology,
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, MALAYSIA

*Corresponding author: Prof. N. Magan, D.Sc., Applied Mycology Group, Cranfield Soil and AgriFood Institute, Cranfield University, MK43 0AL Cranfield, Bedfordshire, UK.
E-mail: n.magan@cranfield.ac.uk

Key words: biocontrol, water potential, carbon utilisation patterns, niche overlap indices, maize, mycotoxins

## Abstract

Recently, it was shown that a strain of the fungal antagonist *Clonostachys rosea* 016 was able to inhibit fumonisin $B_1$ mycotoxin production by *Fusarium verticillioides* FV1 when using different ratios of spores of each species *in vitro*. The objectives of the present work were therefore to: (a) compare the nutritional utilisation patterns and rates of uptake of key C-sources in maize by the antagonist *C. rosea* 016 and that by the pathogen *F. verticillioides* FV1; (b) examine their Niche Overlap Indices (NOI) under different interacting environmental conditions; and (c) evaluate whether the rate of utilisation of key maize C-sources influenced the competitiveness of either species using the Bioscreen®. It was found that water potential ($\Psi$)$\times$ temperature interactions had significant impacts on C-source utilisation patterns by *C. rosea* 016 and the pathogen. The NOIs, based on the utilisation of the C-sources by each strain divided by those utilised in common, showed that the antagonist and the pathogen occupied similar niches at -0.70 MPa $\Psi$+30°C and -2.8 MPa $\Psi$+25°C. Under the other conditions tested, they appeared to occupy separate niches suggesting niche exclusion. Temporal C-source utilisation patterns were then compared under different $\Psi \times$ temperature treatments. This showed that the dominant maize-based C-sources utilised by the pathogen and the antagonist were different. The pathogen *F. verticillioides* FV1 utilised carbohydrates rapidly followed by amino acids and then one fatty acid, palmitic acid. The antagonist *C. rosea* 016 utilised both carbohydrates and amino acids at a similar rate but more slowly than the pathogen. There were also differences in the utilisation of some individual amino acids and carbohydrates which might explain the occupation of different niches under some interacting environmental conditions. These findings are discussed in the context of why some competitors are able to inhibit mycotoxin production while others cannot.



Published by Elsevier. This is the Author Accepted Manuscript issued with:
Creative Commons Attribution Non-Commercial No Derivatives License (CC:BY:NC:ND 4.0).
The final published version (version of record) is available online at DOI:10.1016/j.funeco.2016.05.010
Please refer to any applicable publisher terms of use.

## 1. Introduction

In nature, the modes of inhibition of pathogens by microbial antagonists are wide ranging (e.g., competition for nutrients, synthesis of cell-wall-degrading extracellular enzymes/antibiotics/secondary metabolites, induction of plant resistance; Benítez et al., 2004; Magan and Aldred, 2007). One of the most important attributes of potential biocontrol agents is the ability to compete effectively for the available nutrients in the ecological niche, and in this way colonise and exclude the pathogen. Thus, the range of nutrients which can be utilised by a potential biocontrol agent when compared to the pathogen may provide a competitive advantage and perhaps result in exclusion from a specific ecological niche. It has been suggested that the nutritional partitioning of resources in different terrestrial ecosystems (e.g., phyllosphere, rhizosphere) may influence the ability of both biocontrol agents and pathogens to either co-exist or dominate in a particular niche (Wilson and Lindow, 1994; Arroyo et al., 2008; Cray et al., 2013). In addition, the prevailing abiotic factors (e.g., temperature, water potential, pH) may also affect the interaction and competitiveness of both antagonist and pathogen in the occupation of an ecological niche (Magan and Aldred, 2007). Lee and Magan (1999) showed that it was important to use a range of relevant carbon sources (CSs) to the matrix of interest as this gives much better information on the relative CS utilisation patterns between competing microbial species.

In the laboratory, niche overlap can be studied through the utilisation pattern of relevant CSs related to those utilised by each species and those utilised in common. This is translated into a Niche Overlap Index (NOI) which in turn reflects whether two strains co-exist (NOI > 0.9) or occupy separate ecological niches (NOI < 0.9). The resulting NOI helps to explain the co-existence or niche exclusion by different microbial species. This has been suggested as a method for choosing appropriate potential biocontrol agents. However, the use of NOI alone is insufficient for understanding the nutritional patterns of competing strains. Bioscreen® to monitor the temporal rates of CS utilisation by specific biocontrol and pathogen strains has recently been used to try and understand the mechanism of action for mycotoxin control by antagonists (Mohale et al., 2013). Such information may help in understanding the reasons for the capability of biocontrol strains to compete effectively with the pathogen, and better understand the nutritional resource partitioning under different environmental conditions in utilising relevant CSs.

Samsudin and Magan (2016) screened a range of potential microbial antagonists for competion with and controlling growth and mycotoxin (fumonisin $B_1$; $FB_1$) production by *Fusarium verticilliodes* FV1. It was found that control of $FB_1$ production was influenced by water potential ($\Psi$) and the inoculum ratio of pathogen to antagonist. One of the best antagonists was a strain of *Clonostachys rosea* 016. However, its efficacy was influenced by $\Psi$ and temperature. Thus, there was interest in understanding the mechanism of action of the antagonist *C. rosea* 016 in inhibiting *F. verticillioides* FV1 and $FB_1$ production.

Therefore, the objectives of this study were to: (a) compare the nutritional utilisation patterns and rates of uptake of key CSs in maize by the antagonist *C. rosea* 016 and that by the pathogen *F. verticillioides* FV1; (b) examine their Niche Overlap Indices (NOI) under different interacting environmental conditions; and (c) evaluate whether the rate of utilisation of key maize CSs influenced the competitiveness of either species using the Bioscreen®.

## 2. Materials and methods

### *2.1 Microorganisms*

A fumonisin-producing strain of *Fusarium verticillioides* FV1 which was isolated from Malaysian maize kernels and identified morphologically and molecularly was used in the present work. *Clonostachys rosea* 016 which inhibited production of $FB_1$ by *F. verticillioides* FV1 *in vitro* was also used (Samsudin and Magan, 2016). The strain of FV1 is held in the Applied Mycology Group culture collection. The *C. rosea* strain was isolated from crop debris and was kindly supplied by Dr. Jürgen Köhl, Bio-interactions and Plant Health Unit, Wageningen University and Research Centre, The Netherlands.

### *2.2 Effect of different ratios of pathogen:antagonist on fumonisin $B_1$ production*

Following the positive findings from the *in vitro* assays (Samsudin and Magan, 2016), a similar experiment was then conducted on stored maize kernels. Gamma-irradiated maize kernels (12 kGys, Isotron PLC; Swindon, UK), with retained germinative capacity were used. The $\Psi$s of the maize kernels (10 g) were modified by the addition of known amounts of deionised sterile water ($dH_2O$) to obtain -2.8 MPa $\Psi$ (0.98 $a_w$) and -7.0 MPa $\Psi$ (0.95 $a_w$) by means of a moisture adsorption curve. The hydrated maize kernels were stored overnight at 4°C for equilibration after which they were inoculated with different ratios of pathogen:antagonist spores (100:0; 75:25; 50:50; 25:75; 0:100). This inoculum was taken into account to maintain accurate $\Psi$s of the treatments. The inoculation was carried out in sterilised glass culture vessels equipped with a microporous lid which allowed air and humidity exchange (Magenta, UK). The treatments were mixed well and then stored in environmental chambers under similar $\Psi$s by placing 2 x 500 mL beakers of glycerol/water solutions with $\Psi$s corresponding to the treatments. The treatments and replicates were stored for 14 d at 25°C after which they were dried at 50°C overnight, milled and extracted using the Fumonistar™ immunoaffinity columns (Romer, Austria). Samples were quantified for $FB_1$ by HPLC-FLD using methanol:acetonitrile:water (1:1:2, v/v) as described in Samsudin and Magan (2016).

### *2.3 Carbon source (CS) utilisation and Niche Overlap Index (NOI) studies*

***Microtitre plate preparation:*** For CS utilisation patterns and determination of the NOIs for *C. rosea* 016, and for *F. verticillioides* FV1*,* sterile 24-well microtitre plates (1 mL volume; Nunc, Denmark) were used. A minimal medium comprising (w/v) 0.23% $NaNO_3$, 0.06% $MgSO_4 \bullet 7H_2O$, 0.17% $K_2HPO_4$, and 0.13% $KH_2PO_4$ was prepared and modified to -2.8 MPa $\Psi$ and -7.0 MPa $\Psi$ by adding 30 and 40% (v/v) polyethylene glycol 600 (PEG 600; Fisher, UK) respectively. Glycerol was not used to modify the $\Psi$ as it is also a CS. A total of 24 CSs (Table 1) which are the principal chemical constituents of maize kernels (Giorni et al., 2009) were incorporated separately into the minimal medium at a final concentration of 9.1 mg carbon $mL^{-1}$ (carbon equivalent to 2% (w/v) glucose). The pH was regulated to 6.0 using phosphate buffer (10nm, Sigma; Dawson et al., 1987). The 24 CS solutions (CS + minimal medium) were then autoclaved (Meadowrose, UK) at 121°C for 15 min. After cooling, the 24 CS solutions were aseptically pipetted (700 µL) into each of the 24 wells.

***Inoculum preparation and inoculation:*** Spores from 7 d old axenic cultures of *C. rosea* 016 and *F. verticillioides* FV1, grown on malt extract agar (MEA), were separately harvested with approximately 20 mL $dH_2O$ and aseptically transferred into separate sterile centrifuge tubes

before being centrifuged for 15 min at 3,500 xg. After discarding the supernatant, they were washed three times with 20 mL $dH_2O$ before being suspended in sterile buffer solutions where the spore concentration was diluted to obtain ≈$10^6$ spores $mL^{-1}$ by means of a Helber haemocytometer (depth 0.02 mm; Marienfeld, Germany). Each of the wells was inoculated with 100 µL of the spore suspension. Microtitre plates without inoculum were prepared and incubated as non-growth controls. All inoculated plates were covered with lids and sealed with Parafilm® and incubated at 25°C and 30°C. The presence/absence of fungal growth was checked with a dissecting microscope (Olympus, Japan) at 12-h intervals for 120 h.

***Calculation of Niche Overlap Index (NOI):*** The results of CS utilisation patterns were used to calculate the Niche Overlap Index (NOI; Wilson and Lindow, 1994). The NOI was obtained by comparing the CS utilisation patterns by the pathogen and the antagonist as shown below:

$$NOI = \frac{\text{Number of common CS used by both pathogen and antagonist}}{\text{Number of CS used by antagonist}}$$

The NOI values obtained are between 0 and 1, and they define whether competing strains co-exist (NOI > 0.9) or occupy separate niches (NOI < 0.9; Wilson and Lindow, 1994; Arroyo et al., 2008; Mohale et al., 2013).

### *2.4 Temporal Carbon Utilisation Sequence studies (TCUS)*

A Bioscreen-C® Microbiological Growth Analyzer (Labsystems, Finland) was used following the procedures described by Mohale et al. (2013). The spore suspension of the antagonist, *C. rosea* 016*,* and the pathogen *F. verticillioides* FV1 were prepared as described previously. A 100-well Bioscreen® microtitre plate was aseptically loaded with 200 µL of each of the 24 CS solutions. Each CS solution was loaded in four different wells (Σ96 wells) before being separately inoculated with 10 µL of spore suspension of either the antagonist or the pathogen. The remaining four wells in each plate were inoculated with spore suspensions and $dH_2O$ to act as negative controls. Experiments were conducted at 25°C and 30°C with no agitation. The optical density (OD) was automatically recorded at 20-min intervals using the 600 nm filter over a 7 d (10,080 min). Data were recorded using the software Easy Bioscreen Experiment (EZExperiment).

Before analyses, the average of the measurements for each well during the first 60 min was calculated and automatically subtracted from all subsequent measurements to remove the different signal backgrounds obtained for the different CS solutions. The "Time To Detection" (TTD) for 0.1 OD were measured at 25°C and 30°C at -2.8 MPa $\Psi$ and -7.0 MPa $\Psi$ by using a linear interpolation between successive OD readings to predict the TTD for 0.1 OD. TTD for each replicate of each CS was recorded. The average of these values, expressing the mean time that the strains needed to reach 0.1 OD with a particular CS was computed. Data for pathogen and antagonist were then organised according to these TTD values, and thus the TCUS were obtained (Mohale et al., 2013).

### *2.5 Statistical analysis*

All experiments were carried out with three replicates for CS utilisation patterns and for determining the NOI; and four replicates for TCUS per treatment (CS $\times$ $\Psi$ $\times$ temperature) per species. All experiments were carried out twice. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets was checked by

the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significant difference. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significant difference between means of treatment water potential, temperature, and carbon source, using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

## 3. Results

### *3.1 Background data on control of fumonisin $B_1$ production by* **F. verticillioides** *FV1 using the antagonist* **C. rosea** *016*

*In vitro* studies on milled maize medium showed that *C. rosea* 016 could effectively reduce $FB_1$ production regardless of the $\Psi$s and ratios of antagonist to pathogen (Figure 1a). However, *in situ* on stored maize kernels the antagonist was only effective at -2.8 MPa $\Psi$, not at -7.0 MPa $\Psi$ (Figure 1b). These differences may be due to the ability to access and utilise nutrients from milled maize agar media more rapidly than from whole maize kernels under the environmental conditions examined.

### *3.2 Carbon source (CS) utilisation patterns and Niche Overlap Indices (NOI) between* **C. rosea** *016 and* **F. verticillioides** *FV1*

For the pathogen at 25°C, an equal number of CSs (19) were utilised irrespective of the $\Psi$, while at 30°C, the highest number of CSs (21) was utilised at -7.0 MPa $\Psi$, with less utilised at -2.8 MPa $\Psi$ (18 CSs; Figure 2). For *C. rosea* 016, an equal number of CSs (10) were utilised at 30°C irrespective of the $\Psi$. A lower number of CSs were utilised at 25°C at -7.0 MPa $\Psi$ (8) and -2.8 MPa $\Psi$ (5). Overall, *C. rosea* 016 utilised almost half the number of CSs when compared to the pathogen *F. verticillioides* FV1.

Statistically, for the pathogen *F. verticillioides* FV1, there was no significant difference between the treatments (Table 2). For the antagonist *C. rosea* 016, there was a significant difference between treatments at -2.8 MPa $\Psi$+25°C where the lowest number of CSs were utilised. Comparison between the pathogen and the antagonist showed a significant difference (interaction $p = 0.0002$) with the competitor utilising significantly less CSs than the pathogen at all $\Psi \times$ temperature conditions tested.

The relative NOI values for the antagonist and the pathogen at different $\Psi \times$ temperature conditions changed. At 25°C + -2.8 MPa $\Psi$ and 30°C + -7.0 MPa both antagonist and pathogen occupied similar niches (co-existed) with NOIs of 0.90 respectively. However, under the other conditions tested, they occupied different niches with NOIs of 0.88 (25°C +-7.0 MPa $\Psi$) and 0.80 (30°C+-2.8 MPa $\Psi$).

The differential utilisation patterns of the different groups of CSs by the antagonist and the pathogen is shown in Table 3. Overall, the pathogen utilised a higher percentage of the amino acids than carbohydrates whilst the antagonist utilised more carbohydrates and less amino acids under the ecophysiological conditions tested. Fatty acids were the least preferred CSs by both antagonist and pathogen.

### *3.3 Temporal Carbon Utilisation Sequence Studies (TCUS)*

The temporal utilisation patterns of the CSs by the antagonist and the pathogen changed with the interacting environmental conditions (Figures 3 and 4). In general, the Time to Detection (TTD) to reach a 0.1 OD for *F. verticillioides* FV1 was shorter than that for

the antagonist. It is also noteworthy that all the carbohydrate sources were utilised by the pathogen by 5,500 min in all the treatments with the single exception of amylose at -7.0 MPa Ψ+30°C. Amino acid sources were utilised continuously for up to 9,000 mins. The only fatty acid utilised was palmitic acid at -7.0 MPa Ψ+30°C which was utilised by 9,800 min. On average, -2.8 MPa Ψ+25°C gave 5,500 ± 500 TTD (max. 9,500). When water stress was imposed at -7.0 MPa Ψ+25°C, the average TTD was reduced to 5,000 ± 425 (max. 9,100). At 30°C, however, the opposite occurred with utilisation rates at -7.0 MPa Ψ giving longer TTD at 4,800 ± 433 (max. 9,800) as compared to -2.8 MPa Ψ at 4,000 ± 300 (max. 6,500). Furthermore, across the temperatures, 30°C gave shorter TTD when compared to 25°C at both Ψs tested. It was also apparent that the acidic amino acid (glutamic acid, aspartic acid) utilisation was affected by the treatment regimes used. At -2.8 MPa Ψ at both temperatures, glutamic acid was the first amino acid to be utilised while aspartic acid gave the longer TTD. However, when water stress was imposed (-7.0 MPa Ψ), aspartic acid was utilised earlier at both temperatures while glutamic acid took almost double (30°C) to triple (25°C) the time to reach 0.1 OD. Similar occurrence was also observed in the utilisation of starch sources. At -2.8 MPa Ψ at both temperatures, amylose was the first carbohydrate to be utilised while amylopectin remained unutilised. However, when water stress was imposed (-7.0 MPa Ψ), amylopectin was the first carbohydrate to be utilised while amylose remained unutilised (25°C) or took triple the time (30°C) to reach 0.1 OD. For amino acids where both enantiomers were used (D- and L-alanine, D- and L-serine), it was observed that the D-isomers were utilised faster (shorter TTD) than the L-isomers under all treatment regimes.

For *C. rosea* 016, although more carbohydrates were utilised more rapidly when compared to amino acids, the TTD for both types of CSs were up to 9,500 min. No fatty acid was utilised by the antagonist during the incubation period. Furthermore, while dextrin was not utilised by the pathogen at all, the antagonist was able to assimilate this type of starch. The preference over amylose-amylopectin too was shown to be the opposite of that for the pathogen. *C. rosea* 016 preferred amylose under water stress conditions (-7.0 MPa Ψ). The disaccharide sucrose and D-melibiose were utilised in all treatment regimes. In addition, at -7.0 MPa Ψ the monosaccharide D-glucose and the disaccharide D-maltose were also utilised.

To better visualize the increase/decrease of TTD for each CS across the treatment regimes, the TTD in ascending order (shorter to longer time) for the pathogen and the antagonist are shown in Tables 4 and 5. This clarifies the differences and similarities in utilisation patterns between the pathogen and antagonist.

## 4. Discussion

Comparisons of the relative utilisation of carbon sources (CSs) by the fungal biocontrol agent and the pathogen showed distinct differences in the total number and the types used under the different Ψ × temperature conditions examined. These differences might be explained by the fact that *C. rosea* 016 is known to colonise matrices relatively slowly (≈40 mm Ø after 7 d) when compared to the growth of *F. verticillioides* FV1 (≈80 mm Ø after 7 d).

It is interesting to note that while the pathogen *F. verticillioides* FV1 predominantly utilised higher numbers of amino acids, *C. rosea* 016 utilised higher numbers of carbohydrates. The subsequent NOI values generated, based on the number of common CSs utilised by both species, showed that they occupied the same nutritional niche at -2.8 MPa

$\Psi$+25°C and -7.0 MPa $\Psi$+30°C. When the treatment regime was changed, the NOI values were modified, suggesting occupation of separate niches. The present findings are different from those of Giorni et al. (2009) who investigated the CS utilisation patterns of maize nutrients for a strain of *F. verticillioides* (ITEM 1744) at 25 and 30°C. They found that the fumonisin-producing strain utilised a significantly higher number of CSs at 25°C than at 30°C. However, this difference might be attributed to strain differences. Furthermore, the study by Giorni et al. (2009) was not aimed at comparing potential biocontrol agents but the relative occupation of the maize niche by different mycotoxigenic fungi under different ecophysiological conditions. The present study has thus provided data on the ability of the antagonist *C. rosea* 016 to utilise different CSs relevant to maize for the first time.

In the natural maize agro-ecosystem, temperatures of 20-30°C are typical between flowering and harvest, and $\Psi$ values of -18.2–2.8 MPa $\Psi$ (0.87–0.98 $a_w$) can occur in maize cobs during early dough to full ripe stage (Zorzete et al., 2008; Battilani et al., 2011). Both pathogen and antagonist can colonize maize substrates at more than -9.8 MPa $\Psi$ (0.93 $a_w$). Thus, there is an opportunity for establishment of the biocontrol agent to compete with the pathogen during the ripening stages of maize cobs (silking period).

Previously, the use of *C. rosea* strains as a mycoparasite of several strains of economically important fungal phytopathogens has been described. For example, it has been shown to control the growth of *F. culmorum* and *F. graminearum* on wheat, *F. culmorum*, *F. graminearum* and *F. proliferatum* on maize (Palazzini et al., 2013; Luongo et al., 2005), *Sclerotinia sclerotiorum* on soybean and lettuce (Rodríguez et al., 2011), *Moniliophtora roreri* and *Phytophtora* spp. on cocoa (Mejía et al., 2008), *Alternaria* spp. on carrot (Jensen et al., 2004), *Bipolaris sorokiniana* on barley (Jensen et al., 2002), and *Botrytis cinerea* on rose (Morandi et al., 2001). Several known mechanisms employed by the soil-dwelling saprotrophic *C. rosea* has been proposed including mycoparasitism, substrate/nutrient competition, enzymatic activity and induced resistance in host plants (Lübeck et al., 2002). The Niche Overlap Index (NOI) experiments performed in the present work thus provides additional data that support the substrate/nutrient competition hypothesis and might shed light on antagonistic action of *C. rosea* 016 on *F. verticillioides* FV1 under some environmental conditions on maize. In the present work, the $\Psi$ was modified with PEG 600. In reality, there are a number of other compounds (e.g., sugar alcohols; phenolic compounds; sugars) that can contribute to the modification of $\Psi$s and hence different preference/mechanisms could be adopted by individual microorganisms. Therefore, while the *in vitro* results obtained in the present work may serve as an indicator of the possible mechanism of action, more detailed analyses on the effect of molecular mass of CSs, and also the effect of other $\Psi$-modifying solutes may be useful to try to understand the *in situ* biocontrol mechanisms.

To better understand the detailed utilisation patterns of CSs by the antagonist and the pathogen, the Temporal Carbon Utilisation Sequence (TCUS) was determined using the Bioscreen-C®. In terms of CS utilisation by *F. verticillioides* FV1, although the percentages of amino acids utilised were higher than that of carbohydrates in all treatments, the Time to Detection (TTD) for carbohydrates was, in fact, shorter when compared to amino acids in all treatments. Although the molecular weights of carbohydrates (180-500 mw; variable for polysaccharides) in the present work are more than double that of amino acids (90-165 mw), the preferential utilisation of carbohydrates observed over amino acids in terms of TTD might be explained by the fact that carbohydrates are the primary sources for energy/fuel required for cellular metabolism in almost all organisms. Amino acids also

contribute to cellular energy metabolism especially when the primary source of energy is scarce, or when cells undergo metabolic stress (Hothersall and Ahmed, 2013). Similar behaviour was also observed in *Aspergillus flavus* strains and *F. verticillioides* ITEM 1744 (Giorni et al., 2009), as well as in *Penicillium* spp. and *A. ochraceus* (Arroyo et al., 2008). As for the starch CSs, the longer TTD for amylose as compared to amylopectin might be explained by the fact that by having few α-(1→6) glycosidic bonds, amylose usually takes the form of a long chain which is difficult to hydrolyze and hence longer TTD were observed when compared to amylopectin which has short but highly branched chains [having both α-(1→4) and α-(1→6) bonds]. Therefore, amylopectin is easier to breakdown as it has many end points where enzymes can attach (Birt et al., 2013). In addition, amylopectin, which represents about 70-80% of kernel dry matter, is relevant with regard to fumonisins production (Bluhm and Woloshuk, 2005). The preference in glutamic and aspartic acids might be explained by the fact that these amino acid sources have also been shown to be essential in fumonisin production (ApSimon, 2001) and mycelial formation in several strains of the *Giberella fujikuroi* complex (*F. verticillioides*, *F. proliferatum*) as demonstrated by Jiménez et al. (2003).

For *C. rosea* 016, apart from several similarities to *F. verticillioides* FV1 (i.e., carbohydrates preferred over amino acids, amylopectin preferred over amylose), TCUS revealed slightly different patterns of CSs utilisation. In contrast to *F. verticillioides* FV1, the majority of the carbohydrate sources (70%) were preferentially utilised by *C. rosea* 016 at -7.0 MPa $\Psi$ as compared to -2.8 MPa $\Psi$. However, the utilisation of dextrin under all treatment regimes by the antagonist, with none being utilised by the pathogen, shows a clear difference between them as both were able to assimilate amylose and amylopectin (albeit at different rates under different ecophysiological conditions). The relative preference for monosaccharides and disaccharides observed might be explained by the effects of water stress. Under low water stress conditions, disaccharides were preferred by *C. rosea* 016. As water stress was imposed, the monosaccharide that was easier to breakdown was utilised in addition to the disaccharide. However, it is also noteworthy that the different growth rates between the two competing species in the present work might indirectly give rise to the results obtained, and that the zero minute (0 min) in TTD should not be construed as “un-utilised”, rather it indicated that certain CSs were perhaps utilised later outside the experimental time frame of 7 d (10,080 min). Of course, as this antagonist is usually a soil-borne saprotroph, it can effectively survive on crop residue, and compete with *F. verticillioides* by reducing the inoculum load of the pathogen. This could occur by: (a) minimising spore production reducing spore movement via the soil and roots to the foliar plant parts; and (b) by reducing the amounts of spores splashed onto the aerial plant surfaces to minimise infection via bird/insect damage.

The changes in biocontrol potential under different environmental conditions has been seen previously both for fungal and bacterial biocontrol agents (Mohale et al., 2013; Al-Saad et al., 2016; Cary et al., 2016). The findings in the present work differ from those of Mohale et al. (2013) who found that irrespective of temperature, at higher $\Psi$ (-1.4 MPa; -5.6 MPa) atoxigenic biocontrol strains and aflatoxigenic *Aspergillus flavus* strains co-existed. Subtle changes in environmental fluxes may thus have a significant effect on relative efficacy of a biocontrol agent. Recent work by Cary et al. (2016) suggests that under marginal conditions for the activity of bacterial biocontrol inoculants they can stimulate fungal pathogen growth. Similarly, Al-Saad et al. (2016) found that while some bacterial antagonists effectively controlled growth of *A. flavus*, under water stress aflatoxin $B_1$ production was

stimulated. Thus, environmental fluxes may influence the interaction between the antagonist and fungal pathogen and that different species might adopt different mechanisms of action which in turn can contribute to the different exclusion competence of individual species or strains. Furthermore, the present work only used similar concentrations of CSs, as described by Wilson and Lindow (1994). The actual amount of each CS in maize kernels might be different and this may further influence the utilisation patterns (Lee and Magan, 1999; Mohale et al., 2013; Al-Saad et al., 2016).

## 5. Conclusions

Overall, the CS utilisation patterns, NOI and TCUS studies carried out in the present work provided additional information on the relative competitiveness of both the antagonist and the pathogen. The ability of the two competing species to utilise different CSs demonstrated in the present work reflects their competitiveness under different ecophysiological conditions. By occupying similar niches under several ecophysiological conditions, *C. rosea* 016 appeared to have antagonistic properties which may provide an ecological advantage in controlling growth of *F. verticillioides* and reducing fumonisin production.

## Acknowledgements

The authors would like to acknowledge the financial support received from the Malaysian Ministry of Higher Education (MOHE) under the Academic Training Scheme 2012-2015. We are very grateful to Dr. Jürgen Köhl of Wageningen University and Research Centre, The Netherlands, for the provision of the antagonist strain.

## References

Al-Saad, L., Al-Badran, A. I., Al-Jumayli, S. A., Magan, N., Rodriguez, A. 2016. Impact of bacterial biocontrol agents on aflatoxin biosynthetic genes, *aflD* and *aflR* expression, and phenotypic aflatoxin $B_1$ production by *Aspergillus flavus* under different environmental and nutritional regimes. Int. J. Food Microbiol. 17, 123-129.

ApSimon, J. W. 2001. Structure, synthesis, and biosynthesis of fumonisin $B_1$ and related compounds. Environ. Health Perspec. 109, 245-249.

Arroyo, M., Aldred, D. C. N., Magan, N. 2008. Environmental factors and preservatives affect carbon utilisation patterns and niche overlap of food spoilage fungi. Fungal Ecol. 1**,** 24-38.

Battilani, P., Formenti, S., Ramponi, C., Rossi, V. 2011. Dynamic of water activity in maize hybrids is crucial for fumonisin contamination in kernels. J. Cereal Sci.54**,** 467-472.

Benítez, T., Ana, M., Rincón, M., Carmen, L. A., Codón, C. 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 7, 249-260.

Birt, D. F., Boylston, T., Hendrich, S., Jane, J. L., Hollis, J., Li, L., McClelland, J., Moore, S., Phillips, G. J., Rowling, M., Schalinske, K., Scott, M. P., Whitley, E. M. 2013. Resistant starch: promise for improving human health. Adv. Nutri. 4, 587-601.

Bluhm, B. H., Woloshuk, C. P. 2005. Amylopectin induces fumonisin $B_1$ production by *Fusarium verticillioides* during colonisation of maize kernels. Mol. Plant-Microbe Inter. 18, 1333-1339.

Cray, J. A., Bell, A. N. W., Bhagama, P., Mswaka, A. Y., Timson, D. J., Hallsworth, J. E. 2013. The biology of habitat dominance: can microbes behave as weeds. Microbial Biotechnol. 6, 453-492.

Cray, J. A., Connor, M. C., Stevenson, A., Houghton, J. D. R., Rangel, D. E. N., Cooke, L. R., Hallsworth, J. E. 2016. Biocontrol agents promote growth of potato pathogens, depending on environmental conditions. Microbial Biotechnol. 10.1111/1751-7915.12349

Dawson, R.M.C, Elliott, D.C., Elliot, W.H., Jones, K.M. 1987. Ph, buffers and physiological media. Data for Biochemical Research. Oxford University Press, New York, pp. 418-448.

Giorni, P., Magan, N., Battilani, P. 2009. Environmental factors modify carbon nutritional patterns and niche overlap between *Aspergillus flavus* and *Fusarium verticillioides* strains from maize. Int. J. Food Microbiol. 130, 213-218.

Hothersall, J. S., Ahmed, A. 2013. Metabolic fate of the increased yeast amino acid uptake subsequent to catabolite depression. J. Amino Acids, 1-7.

Jensen, B., Knudsen, I. M. B., Jensen, D. F. 2002. Survival of conidia of *Clonostachys rosea* on stored barley seeds and their biocontrol efficacy against seed-borne *Bipolaris sorokiniana*. Biocon. Sci. Technol. 12, 427-441.

Jensen, B., Knudsen, I. M. B., Madsen, M., Jensen, D. F. 2004. Biopriming of infected carrot seed with an antagonist, *Clonostachys rosea*, selected for control of seedborne *Alternaria* spp. Phytopath. 94, 551-560.

Jiménez, M., Mateo, J. J., Hinojo, M. J., Mateo, R. 2003. Sugars and amino acids as factors affecting the synthesis of fumonisins in liquid cultures by isolates of the *Gibberella fujikuroi* complex. Int. J. Food Microbiol. 89, 185-193.

Lee, H. B., Magan, N. 1999. Environmental factors and nutritional utilisation patterns affect niche overlap indices between *Aspergillus ochraceus* and other spoilage fungi. Lett. Appl. Microbiol. 28, 300-304.

Lübeck, M., Knudsen, I. M. B., Jensen, B., Thrane, U., Janvier, C., Funck-Jensen, D. 2002. GUS and GFP transformation of the biocontrol strain *Clonostachys rosea* IK726 and the use of these marker genes in ecological studies. Mycol. Res. 106, 815-826.

Magan, N., Aldred, D. 2007. Chapter 2: Environmental fluxes and fungal interactions: maintaining a competitive edge, in Van West, P., Avery, S. and Stratford, M. (eds.). *Stress in Yeasts and Filamentous Fungi*. Amsterdam, the Netherlands: Elsevier Ltd., pp. 19-35.

Mohale, S., Magan, N., Medina, A. 2013. Comparison of growth, nutritional utilisation patterns, and niche overlap indices of toxigenic and atoxigenic *Aspergillus flavus* strains. Fungal Biol. 117, 650-659.

Luongo, L., Galli, M., Corazza, L., Meekes, E., De Haas, L., Van Der Plas, C. L., Köhl, J. 2005. Potential of fungal antagonists for biocontrol of *Fusarium* spp. in wheat and maize through competition in crop debris. Biocont. Sci. Technol. 15, 229-242.

Mejía, L. C., Rojas, E. I., Maynard, Z., Van Bael, S., Arnold, A. E., Hebbar, P., Samuels, G. J., Robbins, N., Herre, E. A. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol. Control 46, 4-14.

Morandi, M. A. B., Maffia, L. A., Sutton, J. C. 2001. Development of *Clonostachys rosea* and interactions with *Botrytis cinerea* in rose leaves and residues. Phytoparasitica 29**,** 103-113.

Palazzini, J. M., Groenenboom-de Haas, B. H., Torres, A. M., Köhl, J., Chulze, S. N. 2013. Biocontrol and population dynamics of *Fusarium* spp. on wheat stubble in Argentina. Plant Pathol. 62**,** 859-866.

Rodríguez, M. A., Cabrera, G., Gozzo, F. C., Eberlin, M. N., Godeas, A. 2011. *Clonostachys rosea* BAFC3874 as a *Sclerotinia sclerotiorum* antagonist: mechanisms involved and potential as a biocontrol agent. J. Appl. Microbiol. 110, 1177-1186.

Samsudin, N. I. P., Magan, N. 2016. Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* and fumonisin $B_1$ under different environmental conditions. World Mycot. J. 9, 205-213.

Wilson, M., Lindow, S. E. 1994. Co-existence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl. Environ. Microbiol. 60, 4468-4477.

Zorzete, P., Castro, R. S., Pozzi, C. R., Israel, A. L. M., Fonseca, H., Yanaguibashi, G., Correa, B. 2008. Relative populations and toxin production by *Aspergillus flavus* and *Fusarium verticillioides* in artificially inoculated corn at various stages of development under field conditions. J. Sci. Fd. Agricul. 88, 48-55.

**Table 1. The major maize-derived carbon sources and the percentage added into each well.**

| Carbon source | % Compound (w/v) |
|---|---|
| **Amino acids** | |
| L-Leucine | 1.65 |
| L-Alanine | 2.25 |
| D-Alanine | 2.25 |
| D–L-Threonine | 2.25 |
| L-Serine | 2.68 |
| D-Serine | 2.68 |
| L-Histidine | 1.96 |
| L-Proline | 1.74 |
| L-Phenylalanine | 2.00 |
| L-Aspartic acid | 2.00 |
| L-Glutamic acid | 2.00 |
| **Carbohydrates** | |
| D-Galactose | 2.28 |
| D-Raffinose | 2.50 |
| D-Glucose | 2.28 |
| D-Maltose | 2.28 |
| D-Fructose | 2.28 |
| Sucrose | 2.16 |
| D-Melibiose | 2.28 |
| Dextrin | 2.00 |
| Amylopectin | 2.00 |
| Amylose | 2.00 |
| **Fatty acids** | |
| Oleic acid | 2.00 |
| Linoleic acid | 2.00 |
| Palmitic acid | 2.00 |

**Table 2. The *p*-values for the effects of species and treatments on the number of carbon sources utilisation as analysed by Analysis of Variance (ANOVA).**

***F. verticillioides* FV1**

| Source of variation | SS | df | MS | F | *p*-value | F crit |
|---|---|---|---|---|---|---|
| Between treatments | 8.25 | 3 | 2.75 | 3.4737 | 0.0506 | 3.4903 |
| Within treatments | 9.5 | 12 | 0.7917 | | | |
| Total | 17.75 | 15 | | | | |

***C. rosea* 016**

| Source of variation | SS | df | MS | F | *p*-value | F crit |
|---|---|---|---|---|---|---|
| Between treatments | 62.75 | 3 | 20.92 | 50.2 | 0.0000 | 3.4903 |
| Within treatments | 5 | 12 | 0.4167 | | | |
| Total | 67.75 | 15 | | | | |

***F. verticillioides* FV1 × *C. rosea* 016**

| Source of variation | SS | df | MS | F | *p*-value | F crit |
|---|---|---|---|---|---|---|
| Species | 1152 | 1 | 1152 | 1906.76 | 0.0000 | 4.2597 |
| Treatments | 52.75 | 3 | 17.5833 | 29.1035 | 0.0000 | 3.0088 |
| Interaction | 18.25 | 3 | 6.0833 | 10.069 | 0.0002 | 3.0088 |
| Within | 14.5 | 24 | 0.6042 | | | |
| Total | 1237.5 | 31 | | | | |

**Table 3. The mean percentage of carbon sources utilisation according to type by the pathogen *Fusarium verticillioides* FV1 and the antagonist *Clonostachys rosea* 016 grown at different water potentials and temperatures.**

| | ***Fusarium verticillioides* FV1** | | | ***Clonostachys rosea* 016** | | |
|---|---|---|---|---|---|---|
| | **AA[1]** | **CH[2]** | **FA[3]** | **AA** | **CH** | **FA** |
| **-7.0 MPa, 25°C** | 100 | 80 | 0 | 9.1 | 70 | 0 |
| **-7.0 MPa, 30°C** | 100 | 90 | 33.3 | 27.3 | 70 | 0 |
| **-2.8 MPa, 25°C** | 100 | 80 | 0 | 18.2 | 30 | 0 |
| **-2.8 MPa, 30°C** | 90.9 | 80 | 0 | 45.5 | 50 | 0 |

***[1]AA = amino acid (11 sources)***
***[2]CH = carbohydrate (10 sources)***
***[3]FA = fatty acid (3 sources)***

**Table 4. Visualization of time to detection (TTD) in ascending order for the 24 carbon sources (CSs) under different water activity × temperature conditions tested for growth of *Fusarium verticillioides* FV1 after 7 d incubation.** Key: Colour code: yellow = amino acids, blue = carbohydrates, red = fatty acids. Zero TTD (0) signifies TTD above the timeframe (> 10,800 min).

| -2.8 MPa, 25°C | | -7.0 MPa, 25°C | | -2.8 MPa, 30°C | | -7.0 MPa, 30°C | |
|---|---|---|---|---|---|---|---|
| L-Glutamic acid | 970 | Amylopectin | 2145 | L-Glutamic acid | 1465 | L-Leucine | 1555 |
| L-Aspartic acid | 2015 | L-Leucine | 2275 | L-Leucine | 2060 | Amylopectin | 2185 |
| L-Leucine | 2455 | L-Aspartic acid | 2550 | Amylose | 2885 | D-Glucose | 2990 |
| L-Serine | 4680 | D-Raffinose | 4305 | D-Raffinose | 3080 | L-Aspartic acid | 3375 |
| Amylose | 4747 | Sucrose | 4310 | D-Maltose | 3165 | D-Maltose | 3505 |
| D-Fructose | 5180 | D-Glucose | 4320 | L-Aspartic acid | 3555 | Sucrose | 3640 |
| D-Glucose | 5230 | D-Melibiose | 4430 | D-Glucose | 3675 | D-Raffinose | 3780 |
| D-Raffinose | 5325 | D-Maltose | 4455 | L-Serine | 3685 | D-Fructose | 3865 |
| D-Melibiose | 5345 | D-L-Threonine | 4475 | D-Melibiose | 3855 | D-Melibiose | 4035 |
| D-Galactose | 5480 | D-Fructose | 4535 | D-Fructose | 4090 | L-Serine | 4255 |
| Sucrose | 5535 | D-Galactose | 4975 | Sucrose | 4120 | D-Galactose | 4590 |
| D-Maltose | 5595 | L-Serine | 5215 | L-Proline | 4555 | D-Serine | 4770 |
| L-Proline | 6370 | L-Alanine | 5345 | D-Galactose | 4650 | D-L-Threonine | 4925 |
| L-Alanine | 6395 | L-Proline | 5800 | D-Serine | 4775 | L-Alanine | 5385 |
| D-L-Threonine | 6567 | D-Serine | 5850 | D-L-Threonine | 5175 | L-Histidine | 5520 |
| D-Serine | 7000 | L-Histidine | 6200 | L-Alanine | 5180 | L-Proline | 5550 |
| L-Histidine | 8330 | L-Glutamic acid | 6640 | L-Histidine | 5755 | L-Glutamic acid | 6025 |
| L-Phenylalanine | 9073 | L-Phenylalanine | 8805 | L-Phenylalanine | 6535 | L-Phenylalanine | 6745 |
| D-Alanine | 9550 | D-Alanine | 9193 | | | Amylose | 7780 |
| | | | | D-Alanine | 0 | D-Alanine | 7893 |
| Dextrin | 0 | Dextrin | 0 | Dextrin | 0 | Palmitic acid | 9820 |
| Amylopectin | 0 | Amylose | 0 | Amylopectin | 0 | | |
| Oleic acid | 0 | Oleic acid | 0 | Oleic acid | 0 | Dextrin | 0 |
| Linoleic acid | 0 | Linoleic acid | 0 | Linoleic acid | 0 | Oleic acid | 0 |
| Palmitic acid | 0 | Palmitic acid | 0 | Palmitic acid | 0 | Linoleic acid | 0 |

**Table 5. Visualization of time to detection (TTD) in ascending order for the 24 carbon sources (CSs) under different water activity × temperature conditions tested for growth of *Clonostachys rosea* 016 (BCA1) after 7 d incubation.** Key: Colour code: yellow = amino acids, blue = carbohydrates, red = fatty acids. Zero TTD (0) signifies TTD above the timeframe (> 10,800 min).

| -2.8 MPa, 25°C | | -7.0 MPa, 25°C | | -2.8 MPa, 30°C | | -7.0 MPa, 30°C | |
|---|---|---|---|---|---|---|---|
| L-Leucine | 5747 | Amylose | 7080 | L-Leucine | 2695 | Amylose | 5707 |
| Dextrin | 8760 | Sucrose | 7160 | Amylopectin | 2720 | L-Leucine | 6807 |
| D-Melibiose | 8910 | D-Raffinose | 7445 | D-Alanine | 6170 | Sucrose | 6930 |
| L-Serine | 9000 | Dextrin | 7693 | L-Aspartic acid | 7040 | D-Glucose | 7290 |
| Sucrose | 9495 | D-Glucose | 7860 | L-Serine | 7155 | D-Raffinose | 7865 |
| | | D-Melibiose | 8260 | Dextrin | 7500 | Dextrin | 7935 |
| L-Alanine | 0 | L-Proline | 8820 | Sucrose | 8360 | L-Proline | 8710 |
| D-Alanine | 0 | D-Maltose | 9560 | D-Melibiose | 8380 | D-Maltose | 9095 |
| D-L-Threonine | 0 | | | L-Proline | 8747 | D-Melibiose | 9535 |
| D-Serine | 0 | L-Leucine | 0 | D-Glucose | 9740 | L-Alanine | 9553 |
| L-Histidine | 0 | L-Alanine | 0 | | | | |
| L-Proline | 0 | D-Alanine | 0 | L-Alanine | 0 | D-Alanine | 0 |
| L-Phenylalanine | 0 | D-L-Threonine | 0 | D-L-Threonine | 0 | D-L-Threonine | 0 |
| L-Aspartic acid | 0 | L-Serine | 0 | D-Serine | 0 | L-Serine | 0 |
| L-Glutamic acid | 0 | D-Serine | 0 | L-Histidine | 0 | D-Serine | 0 |
| D-Galactose | 0 | L-Histidine | 0 | L-Phenylalanine | 0 | L-Histidine | 0 |
| D-Raffinose | 0 | L-Phenylalanine | 0 | L-Glutamic acid | 0 | L-Phenylalanine | 0 |
| D-Maltose | 0 | L-Aspartic acid | 0 | D-Galactose | 0 | L-Aspartic acid | 0 |
| D-Fructose | 0 | L-Glutamic acid | 0 | D-Raffinose | 0 | L-Glutamic acid | 0 |
| Amylopectin | 0 | D-Galactose | 0 | D-Maltose | 0 | D-Galactose | 0 |
| Amylose | 0 | D-Fructose | 0 | D-Fructose | 0 | D-Fructose | 0 |
| D-Glucose | 0 | Amylopectin | 0 | Amylose | 0 | Amylopectin | 0 |
| Oleic acid | 0 | Oleic acid | 0 | Oleic acid | 0 | Oleic acid | 0 |
| Linoleic acid | 0 | Linoleic acid | 0 | Linoleic acid | 0 | Linoleic acid | 0 |
| Palmitic acid | 0 | Palmitic acid | 0 | Palmitic acid | 0 | Palmitic acid | 0 |

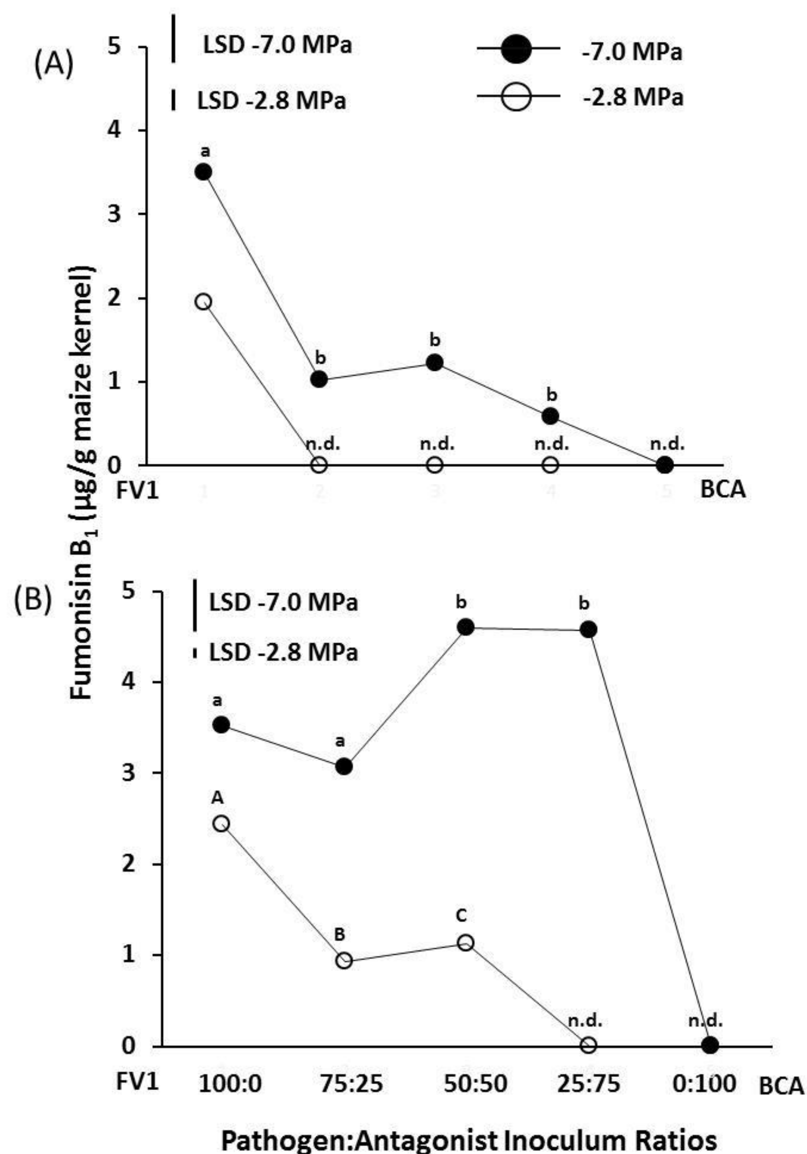


Figure 1. The effect of different *Fusarium verticillioides* FV1: *Clonostachys rosea* 016 inoculum ratios and water potentials on fumonisin B1 production on (A) milled maize medium and (B) stored maize grain. 100:0 served as positive control, and 0:100 as negative control. Data are mean of triplicate (n = 3) with bars indicating Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$. Different letters indicate significant difference ($p < 0.05$) between treatments when compared to the control. n.d.; none detected. Data for (A) from Samsudin & Magan (2016) (B) unpublished data.

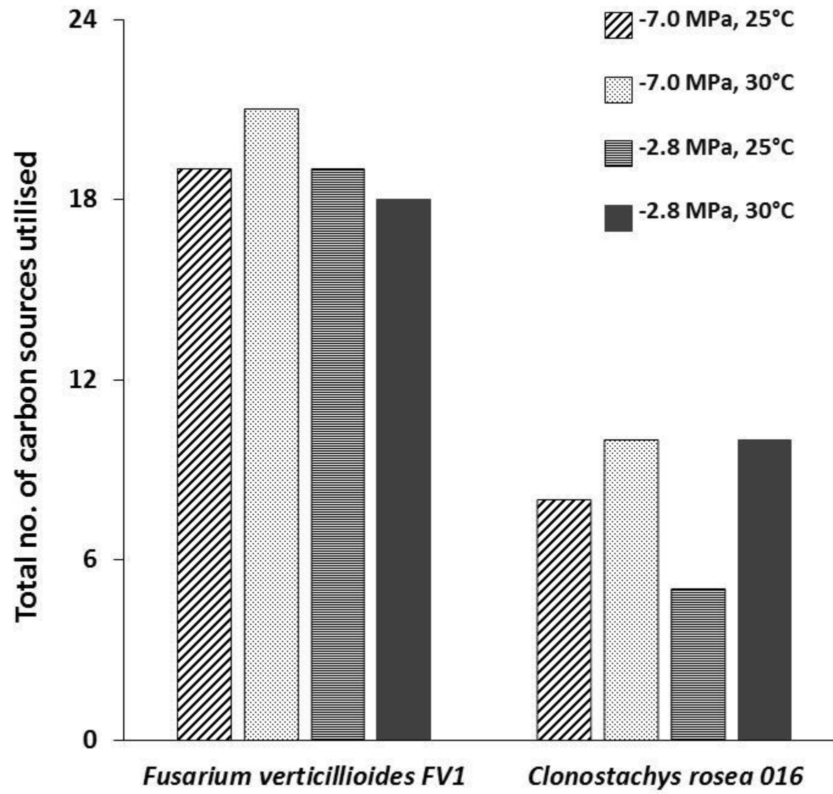


Figure 2. The effect of water potential/temperature on the total number of carbon sources (n=24) utilised by the pathogen *Fusarium verticillioides* FV1 and the antagonist *Clonostachys rosea* 016 after 7 d incubation.

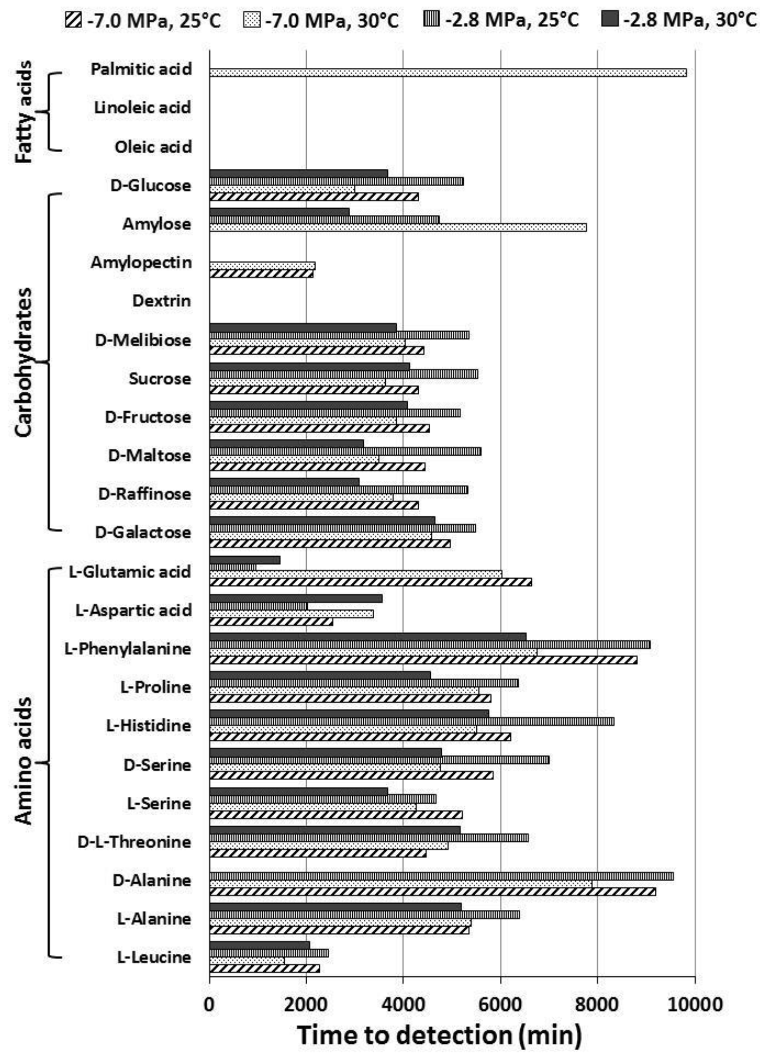


Figure 3. The effect of water potential /temperature on the time to detection (TTD) of the pathogen *Fusarium verticillioides* FV1 after 7 d incubation.

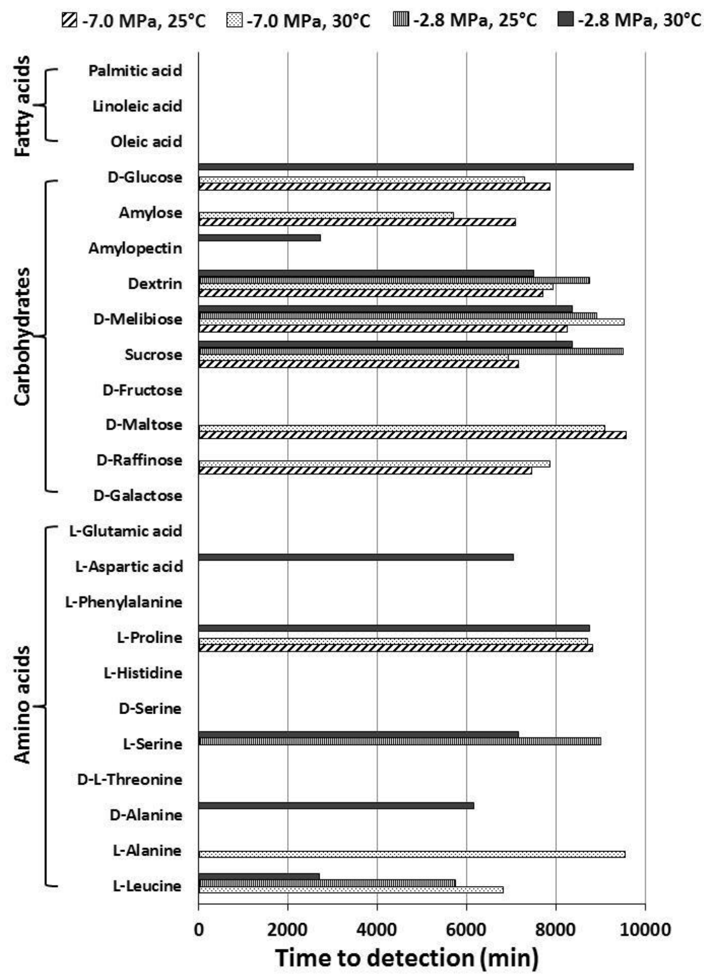


Figure 4. The effect of water potential/temperature on the time to detection (TTD) of the antagonist *Clonostachys rosea* 016 after 7 d incubation.