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THE EFFECTS OF INCREASING CO_2 CONCENTRATION ON FUSARIUM SPECIES AGGRESSIVENESS ABILITY BY MONOCULTURE WHEAT VARIETY

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ABSTRACT

Increasing CO_2 concentration in the atmosphere influences on the physiology, morphology and biomass of plants and their interactions with plant pathogens. Fusarium head blight and crown rot are among the most important diseases of cereals causing high yield loss in wheat and barle in many countries and regions including Australia. Over 1390 isolates of four Fusarium species from crown tissues of two wheat varieties were obtained from a Free-Air field CO_2. Enrichment (FACE) trial from 2007 and 2008 and from a controlled environment. Five isolates from the dominant Fusarium species of each treatment were selected for the aggressiveness detection. There was a significant increase in the pathogencity of Fusarium species by increasing CO_2 level. There was a significant increase in the aggressiveness ability of F. culorum and F. pseudograminearum by increasing CO_2 concentration after five cycles of monoculture of wheat variety.

INTRODUCTION

The level of global atmospheric CO_2 has increased by 31% since pre-industrial revolution and it is projected to increase by from 445ppm to 710ppm in 2050 according to the Intergovernmental Panel on Climate Change (7, 8). The increase in CO_2 concentration in the Earth's atmosphere has a direct impact on agriculture and ecosystems, affecting crop development and productivity (3). Crop yield have been predicted to improve to 17% via rising CO_2 from a 'fertilization effect' (3). Rising CO_2 also influences physiology, morphology and biomass of plants. Increasing CO_2 concentration changes plant function especially in terms of photosynthetic capacity, water-use efficiency, growth and yield with positive impacts (3). Morphologically, a number of changes occur in plant height, branches, tillers number, leaf thickness and area, leading to enlargement of crop canopy (15). Increasing canopy size can lead to changing pathogen dynamics by providing a suitable microclimate, increasing humidity; as a result, trapping more spores by enlarging infection court size (Pangga et al., 2011). The increase in CO_2 concentration may enhance plant resistance via slowing down pathogen invasion; while, in some cases rising CO_2 level results in high sporulation rates due to the increase of pathogen fecundity (2, 5, 15). Melloy et al (12) point out that there is a significant increase of Fusarium biomass in wheat at elevated CO_2, and the low level of resistance in cultivars cannot reduce Fusarium biomass.

Fusarium spp overwinter or over summer as saprophytes in infected wheat, maize or other types of cereals and grass, producing ascospores or macroconidia that disseminate by wind, rain and insects, repeating the disease cycle when wheat is sown the following season (3).

Fusarium species cause the diseases crown rot (CR) and head blight (FHB) in wheat and barley which result in enormous yield losses in many

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countries and regions including Australia (10). FHB causes significant financial losses due to reducing yield productivity and price discounts due to its effects on grain quality; the estimated cost of these losses in USA were about 2.7$ billion between 1998 and 2000 as reviewed by Goswami and Kistler (6). CR is one of the main problems in Australian crop production causing massive damage to the entire wheat belt (1, 13, 18). Both FHB and CR have increased in importance worldwide with the adoption of reduced till farming practices and monoculture of wheat leads to severe Fusarium infections (2, 3). The effects of high CO_2 on the crown rot host-pathogen interaction with wheat has been studied since 2007 at a Free to Air CO_2 Enrichment (FACE) facility in Horsham, Victoria and under controlled environment facilities (CEF) in CSIRO laboratories in Brisbane. Fusarium species were isolated from stem base tissue from crop residue after the first (2007) and second FACE cycles (2008). Each year two wheat varieties (Tamaroi and variety 2-49) were planted at ambient and elevated (550ppm) CO_2. Soil and stubble from the 2007 FACE were brought to Brisbane and the same two varieties were grown under similar environmental conditions in a CEF, simulating wheat monoculture. Populations of Fusarium have been isolated from the CEF continuous monoculture trial after five and seven cropping cycles. The main objectives of this study are to investigate if there is any effect of high CO_2 or wheat variety on pathogen aggressiveness, and investigate if there is an effect of cycles of monoculture of wheat agricultural system. Materials and methods Samples of Fusarium isolates used in this study were obtained from a Free-Air field CO_2 Enrichment (FACE) trial from 2007 and 2008 and from a controlled environment. The FACE 2007 and 2008 field trials, Horsham, Victoria referred to as Cycle 1 and Cycle 2, respectively and control environment facility (CEF) sequential infection cycles harvested in 2010 and 2011 after Cycles 5. Half of the planted row at each of the two CO_2 levels was inoculated with a Fusarium pseudograminearum isolate spore suspension from Horsham. Whole plants with staple and soil were transferred into special pots in the control facilities in the CSIRO-Brisbane facilities in Australia after cycle 2. Special rooms that have a controlled CO_2 level were used to keep the same field treatments with both ambient (380ppm) and elevated CO_2 (700ppm) and with 25±2C° and 30% relative humidity. Seeds were harvested at the end of season and used to plant the crop for next season in order to mimic monoculture. Fusarium isolates were gained at the end of each season from the crown tissues.

Fungal isolation

Three 2 mm pieces of crown tissue from each stem were used to get fungal isolates. The pieces were surface sterilized in bleach (1% available chlorine) for 5 minutes, and washed twice in sterile water for 5 minutes. Then, the crown pieces were dried by placing them on sterile paper towel. Subsequently, crown tissue pieces were transferred onto quarter-strength potato dextrose agar (PDA) plates which contain 100 µg streptomycin sulphate and 10 µg tetracycline hydrochloride mL^{-1}. Plates were incubated at ambient temperature and placed under a combination of back light (F20 T9 BL-B 20W FL20S. SBL-B NIS, Japan) and standard white fluorescent light (35098 F18E/33 General Electric, USA) for 24 hours for 5 -7 days. Spore suspension was made by adding 3-4 drops of sterile distilled water on the fungal colonies that were grown around the plated wheat tissues using sterile flame-sterilized loop. This spore suspension was streaked onto 2% water agar media by using a flame-sterilized metal loop and plates were incubated under laboratory conditions for 24 h. A single germinated

macroconidium was transferred onto full-strength PDA media plate and incubated at ambient temperature according to Scott and Chakraborty (2010). All isolates were used for fungal identification to determine the relative frequency of Fusarium spp. and five isolates were randomly selected from each of the dominant Fusarium spp and used in bioassays for aggressiveness.

Fusarium populations and species identification

Fusarium species that examined in this study were collected by isolating monoconidial cultures from stored infected wheat crown tissues. Plants from (a) the initial 2007 FACE (b) second year 2008 FACE, and (c) after five cropping cycles in the CEF. Each population consisted of at least 200 monoconidial isolates, except FACE 2008 with 116 isolates due to the difficulties of isolation from the plant materials. For identifying Fusarium isolates, species-specific PCR primers were used which included Fusarium graminearum, F. pseudograminearum, F. acuminatum, F. culmorum, F. crookwellense, F. cerealis, F. equiseti, and F. oxysporum (Table 1). Over 1390 isolates were identified in this study from the four previously mentioned populations; this was conducted to determine if there was any shift in the ratio of each Fusarium spp under the two CO_2 treatments and wheat variety after one or more cropping cycles.

DNA extraction and PCR amplification

DNA was extracted from fungal isolates using Quick DNA Extraction Solution (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The fungal mycelia were swirled using tips around thoroughly inside the solution to ensure that the mycelium is deposited in the solution without grinding after adding 50 µL of QuickExtract Plant DNA Extraction Solution to 200-µL tubes or a well of a 96-well plate. Approximately 2 mg wet weight of mycelium from fungal culture were taken and immersed in the DNA Extraction Solution. The solution and the mycelia were incubated at 65^{o}C for 6 minutes and a further 2 minutes at 98^{o}C using PCR machine (GeneAmp® PCR System 9700 Base Module, Arlington, USA). PCR amplifications were performed in a total volume of 10 µL containing injection water 6.52 µL, PCR buffer (10x) 1.0 µL, $MgCl_2$ (25 mM) 1.0 µL, dNTPs (10 mM each dNTP) 0.2 µL, forward primer (10 µM) 0.12 µL, reverse primer (10 µM) 0.12 µL, and Taq polymerase (5 U/µl) 0.04 µL and 1 µL of the target DNA. The PCR master mix components were mixed gently many times by using pipette. After preparing the plates, they were placed in the thermocycler after setting temperature profile to conditions relevant to the primer reaction. The primers are shown in Table (1). Amplicons were separated by gel electrophoresis in 1% agarose gels in TAE (45 mM Trisacetic acid; 1 mM EDTA) that contain 0.1 ml^{-1} GelRed dye and visualised under UV light. Agarose gel electrophoresis 1% was prepared by adding 1 gm to 100 ml TAE (14). The solution was heated in the Microwave on High medium level for ~3 minutes and cooled down to lukewarm in water. Then, 10µL of Gel Red / 100mL agarose was added to the mix followed by pouring it into a levelled tank and inserting well templates. After that the samples were loaded by adding 5 µl of gel loading buffer per sample and 5µl of each sample were added to the tray holder using a multichannel pipette. Samples were loaded on the gel after covering it with TAE and ladder was added to the end wells (1µL in 5µL loading buffer). The gel was run with settings 100V, 100A, approximately~30 minutes and read after transferring it into the trans-illuminator to be visualised and taking photo figure (4, 5).

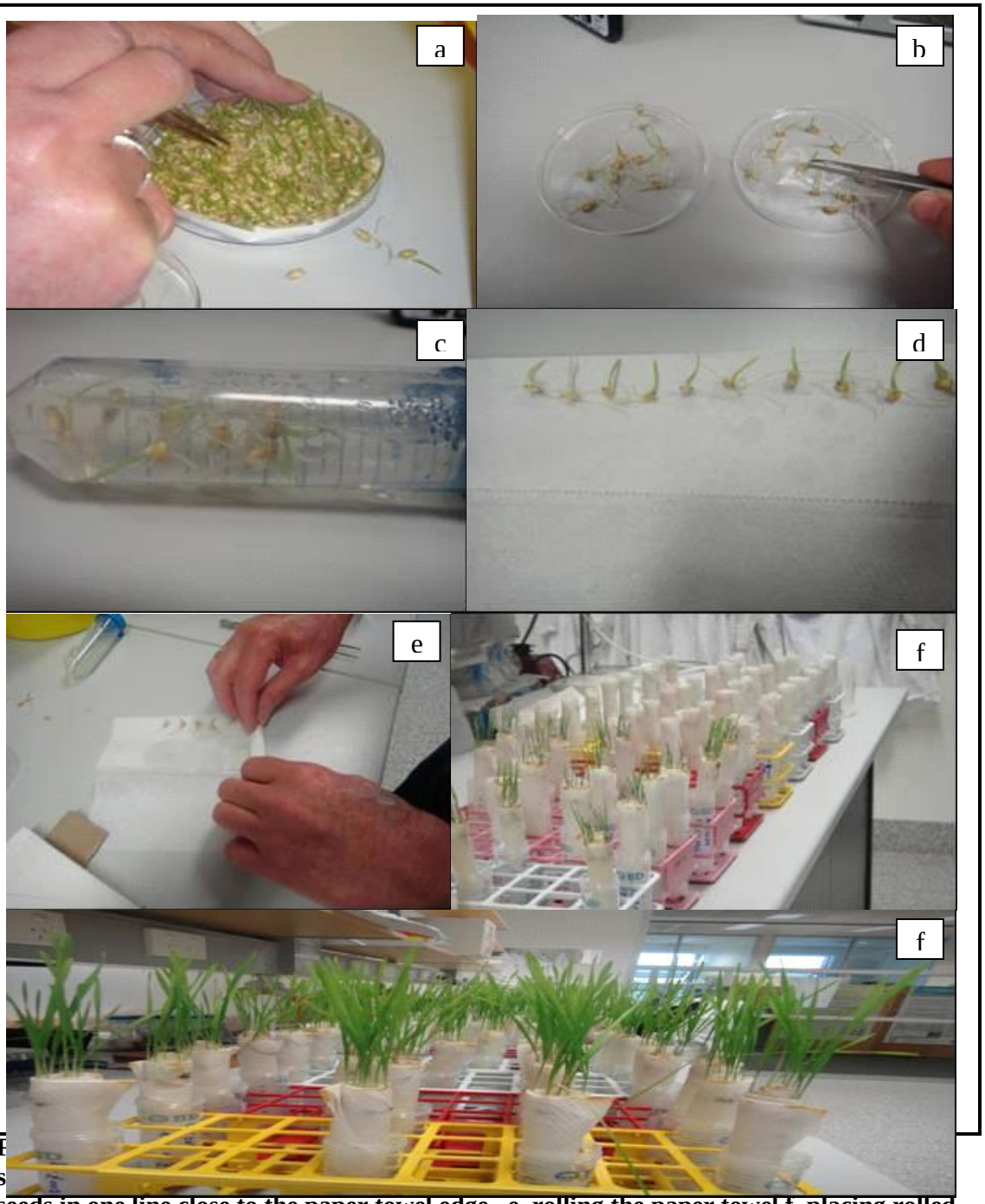
Table1: Fusarium species- specific primers, and their sequence and annealing temperature that used in this study

Annealing Temperature C°	Primer sequence(3-5)	Direction	primers	Species
57	CGGGGTAGTTTCACATTTCCG GAGAATGTGATGACGACAATA	Forward Reverse	Fp1-1 Fp1-2	F. pseudograminearum
60	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	Forward Reverse	FcOIF FcOIR	F. culmorum
60	CTCAGTGTCCACCGCGTTGCGTAG CTCAGTGTCCCAATCAAATAGTCC	Forward Reverse	CRO-AF CRO-AR	F. crookwellense
57	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	Forward Reverse	Fg16NF Fg16NR	F. graminearum
58	CATACCTATACGTTGCCTCG TTACCAGTAACGAGGTGTATG	Forward Reversed	FEF-1 FER-1	F. equiseti
69	TAGCCCACCGTGTAATACCCTGGG CGGGGGATAAAGGCGG	Forward Reverse	PFO2 PFO3	F. oxysporum
56	GGGATATCGGGCCTCA GGGATATCGGCAAGATCG	Forward Reverse	FAC-F FAC-R	F. acuminatum

Bioassay for aggressiveness

Pathogenicity and aggressiveness of selected isolates from the different Fusarium spp were tested on one wheat variety (Kennedy) using a paper towel assay as previously described by Ma et al. (11). Five isolates of the dominant Fusarium spp. from each wheat variety, cycle and CO_2 treatment (high CO_2, low CO_2, Tamaroi, and 2-49) were selected to investigate if there were any differences between treatments and cycles in terms of Fusarium spp. pathogenic ability. A total of 254 isolates were used. Fifty seeds from the susceptible wheat variety Kennedy were placed in Petri dishes containing 2-3 sterile filter-papers. Sterile distilled water was added to the filter papers to enhance seed germination. Seeds were germinated for 3 days prior to inoculation with Fusarium spp. A spore suspension for each Fusarium isolate was prepared by adding 5ml of sterile water to Petri dish; then, by using a sterile metal scraper, fungal mycelia were scraped and the solution was poured into a 50ml Falcon tube after filtering the solution through sterile Miracloth. The spore concentration was determined using haemocytometer and concentration adjusted to 1×10^5conidia/mL. Ten germinated seeds were placed into 50ml of the spore suspension in a Falcon tube and soaked and swirled gently for 3-5 minutes. These seeds were then placed onto a paper towel which had previously been folded with one third of the towel doubled up against the central third. The seeds were placed with the coleoptile facing the double thick edge, in order for the seedling to grow out of the paper at the top about 2cm from the edge. Then, carefully the paper was rolled up enclosing the seeds (Figure 1).

The rolled towel was held using adhesive tape. Thirty droplets of inoculum were added to the outside of the rolled towel. The rolls were separately placed into 50 mL Falcon tubes containing 10 ml sterile distilled water. The tubes were incubated at ambient light and temperature for 12 days and watered daily with 10 mL SDW. After incubation, rolls were removed from the 50ml Falcon tubes and unrolled or sometimes rolls were cut under the seeds. For each set of ten seeds, the seedling length and the length of browning were measured (excluding the seeds). Seeds that failed to produce a noticeable shoot or completely rotted were rated as, seedling length = 0, and browning length =0. Aggressiveness was measured as the proportion of seedling length affected by browning due to Fusarium infection.



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seeds in one line close to the paper towel edge. e. rolling the paper towel f. placing rolled towels in 50 ml racks separately. g. Grown wheat seedlings after 12 days.

Statistical analysis

The frequency and aggressiveness data were analysed using a complete factorial design using generalised linear model procedure in SAS. Variety, species, CO_2, Cycles, and their interaction were included in the models that the analysis investigated their effects and differences. Significant differences were considered at ($p<0.05$).

RESULTS AND DISCUSSION

Data were grouped according to pathogenic and saprophytic species, and for each species separately were analysed. There was a significant (P=0001)

differences between Cycle 1 (FACE 2007) and Cycle 5 (CEF 2010) in terms of species frequency. The mean of species frequency in Cycle 5 was significantly higher than Cycle 1 at 0.146 while it was at 0.080 in Cycle 1. This indicates that the incidence of Fusarium spp increased noticeably in Cycle 5 in comparisons with species occurrence in Cycle 1 expressed in the seven primers that were used in order to speciate Fusarium isolates from the cycles 1 and 5 (Figure 2).

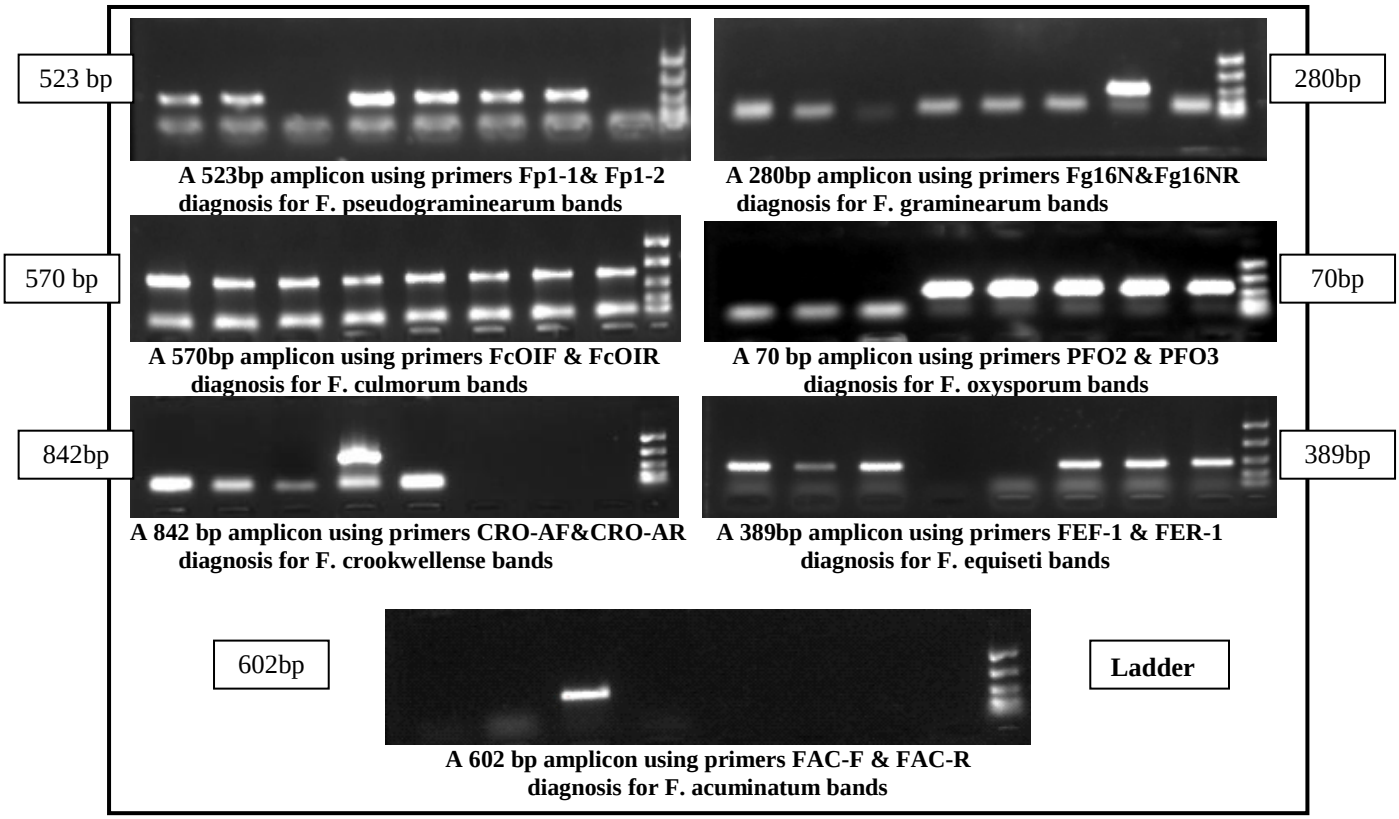

Figure2: Amplicons from the used seven primers with different sizes depending on Fusarium species.

In both CO_2 treatments Cycle 5, had the highest effect on selection of Fusarium spp. F. pseudograminearum had the highest frequency rate among other Fusarium species followed by F. equiseti then F. culmorum in Cycle 5 (Table 2). The effect of CO_2 level in the elevated CO_2 treatment was significant on Fusarium spp in analysed data from Cycle 5 (P = 0001).

Table 2: The overall Fusarium species frequency after 5 cycles (CEF 2010).

Fusarium species	Mean of frequency
F. pseudograminearum	0.18897 A
F. equiseti	0.17181A
F. culmorum	0.13107 B
F. sp	0.12500 B
F. oxysporum	0.07596 B

Different letters in Ryan-Einot-Gabriel-Welsch Multiple Range Test column indicate significant difference.

Aggressiveness of dominant Fusarium species

The effect of CO_2 level (ambient and elevated) on the aggressiveness of Fusarium spp was significant in both analysed cycles 1 and 5 ($p<0.05$). The mean of aggressiveness of Fusarium species at ambient CO_2 treatment was significantly higher at 0.158 in comparison with elevated once at 0.135 (Table 3).

Table 3: species frequency under both ambient and elevated CO_2 after five cropping cycles

Grouping	Mean	N	CO_2
A	0.158150	0.135689	Ambient
B	756	621	Elevated

Means with the same letter are not significantly different.

Table (4) shows that most of the treatments in the experiment had significant effects on aggressiveness ($p<0.05$) comparing ambient CO_2 with elevated CO_2. CO_2, variety, cycles and their interaction affected Fusarium spp aggressiveness significantly (P=0001).

Table 4: Aggressiveness data analysis from cycles 1 and 5 for all Fusarium species comparing CO_2, variety, cycles, species and their interaction; ($p<0.05$) was the significant standard

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CO_2	1	0.135	0.135	4.99	0.0257
variety	1	0.146	0.146	5.40	0.0203
cycle	1	0.916	0.916	33.76	<.0001
species	4	13.48	3.372	124.18	<.0001
CO_2+cycle	1	0.410	0.410	15.11	0.0001
Variety+cycle	1	0.035	0.035	1.30	0.2548
CO_2+variety	1	0.024	0.024	0.90	0.3433
CO_2+variety+cycle	1	0.016	0.016	0.59	0.4409
CO_2+species	3	0.622	0.207	7.65	<.0001
Variety+species	3	0.178	0.059	2.19	0.0872
Cycle+species	3	1.273	0.424	15.64	<.0001
CO_2+vari+cycle+speci	11	1.142	0.103	3.83	<.0001

F.culmorum had the highest aggressiveness ability among the other Fusarium spp in both cycles (Table 5); however, its ability was noticeably higher at ambient CO_2 treatment in both cycles. F. pseudograminearum had the second highest aggressiveness ability among the rest Fusarium species; while other Fusarium species such as F. equiseti, F. oxysporum and unidentified Fusarium spp did not have any significant differences between their aggressiveness ability in both cycles and CO_2 treatments.

Table 5: Aggressiveness data analysis for all Fusarium species together from cycles 5 assessed via comparing the proportions of browning tissue to seedlings length

Mean of aggressiveness	species
0.29148 A	F. culmorum
0.16687 B	F. pseudograminearum
0.04856 C	F. oxysporum
0.03241 C	F. equiseti
0.02690 C	F. sp

Different letters in Ryan-Einot-Gabriel-Welsch Multiple Range Test column indicate significant difference.

This study manifested that there was a significant change in aggressiveness ability among Fusarium spp under CO_2 treatment (ambient and elevated). There was a significant increase in the ability of pathogenic Fusarium spp to colonize wheat tissues especially under elevated CO_2. Results of analyses from Cycle 1 and 5 revealed a significant increase in Fusarium spp in terms of aggressiveness ability. F. pseudograminearum and F. culmorum showed higher level of aggressiveness rate under elevated CO_2 in comparison with ambient especially after five cycles of monoculture of wheat. F. pseudograminearum expressed a higher aggressiveness rate under ambient CO_2 than elevated suggesting that high CO_2 level can increase aggressiveness ability among pathogenic Fusarium spp. or

fasting their colonization rate after cycle 5. F. culmorum revealed same trend as F. pseudograminearum with higher aggressiveness rate at elevated CO_2 than ambient once. Found a similar trend from F. culmorum aggressiveness rate in durum wheat in Tunisia under ambient conditions, scoring the highest colonization rate followed by F. pseudograminearum. Dyer et al., (4) also reported that F. culmorum is the most aggressive Fusarium sp followed by F. pseudograminearum in terms of causing CR in wheat in Canada. Moreover, monoculture wheat cycles revealed a significant impact on Fusarium spp ability to colonize wheat tissue. From the data analysis, Fusarium species at Cycle 5 were higher in terms of their aggressiveness ability than species from cycle 1 which indicate that cycles' number can increase aggressiveness ability in Fusarium spp under monoculture wheat cropping system. Lamprecht et al. (9) points out that monoculture wheat cropping system increases the disease CR especially increasing F. pseudograminearum severity. The interaction between cycles, species, variety and CO_2 was also significant, indicating that all these factors can influence Fusarium species pathogenicity rate even though under field conditions, increasing the disease CR aggressiveness rate. The greater the number of cycles appears to increase disease severity significantly and have major effects on Fusarium species selection under monoculture wheat cropping system. Cycle 5 scored the highest significant influence on Fusarium spp shifting and disease severity especially F. Pseudograminearum. The interaction between cycles, CO_2, variety and species has significant effects on Fusarium spp selections and aggressiveness. Future studies should focus on expanding this experiment with more isolations under more than seven cycles. Increasing the number of varieties in the future studies in order to have better indication about variety influences on Fusarium spp. selection. Increasing the number of isolates can be tested in the aggressiveness experiment.

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تأثير زيادة تركيز ثاني اوكسيد لكاربون في قابلية الشراسة لأنواع الفيوزاريوم في زراعة الحنطة المنفردة

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الملخص

تؤثر زيادة تركيز ثاني اوكسيد الكربون في الغلاف الجوي فسلجياً وفي الكتلة الحيوية للنبات وتفاعله مع المسببات المرضية. يعد مرضاً تعفن السنابل وتعفن التاج الفيوزارمي في الحنطة من بين أهم أمراض الحبوب المسببة لخسائر كبيرة في الإنتاج لمحصولي الحنطة والشعير في العديد من بلدان العالم بضمنها استراليا. تم جمع **1390** عزلة من أربعة انواع من فطر فيوزاريوم من منطقة التاج لصنفين من الحنطة من حقل لمعاملة التعرض الحر لثاني اوكسيد الكاربون في عامي **2007** و**2008** ومن معاملة غرف التربية المسيطر عليها في سي اس اي ار او – استراليا. تم اختيار خمس عزلات من كل نوع من أنواع الفيوزاريوم الأكثر تكراراً في العزل لمعاملة فحص شدة الشراسة .اظهرت النتائج وجود زيادة معنوية في قابلية الشراسة في أنواع الجنس **Fusarium** بزيادة تركيز ثاني اوكسيد الكاربون. كانت هناك زيادة معنوية ملحوظة في شراسة الفطرين **F. Culorum** و **F. Pseudograminearum** بزيادة تركيز CO_2 بعد خمس دورات زراعية منفردة .

وزارة العلوم والتكنولوجيا–بغداد – العراق.

سي اس اي ار او الصناعة النباتية، كوينزلاند– استراليا.