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**Effect of elevated atmospheric CO2 and temperature on disease severity of *Fusarium oxysporum* f.sp. *lactucae* on lettuce plants**

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**1. Introduction**

Climate change will influence soil microbial communities both directly (warming) and indirectly (warming and increased CO2) via changes in quantity and quality of plant-mediated soil C inputs (Pritchard, 2011). Modifications mediated by industrial activity and new agricultural techniques induce complex responses of ecosystems to atmospheric CO2 (Zaehle et al., 2010). An increase of temperature and CO2 concentration in the atmosphere (up to 600 ppm) as predicted for the future (Lobell and Gourdji, 2012) could alter both substrate availability and microbial activity (Zheng et al., 2010). Numerous studies have investigated the effects of elevated CO2 on soil microbial composition and activity (Marilley et al., 1999; Rønn et al., 2002; Lipson et al., 2006) producing many conflicting results. Uncertainty still remains, largely due to the complexity of the interactions between plants, soil and soil microorganisms. To evaluate the effect of elevated CO2 on soil microbial composition and activity, soil extracellular enzyme activities have been used toexpressthe functioning of microbial communities, since they are sensitive and respond rapidly to environmental changes (Hanry, 2012). Microbial analysis alone may not be exhaustive to understand the shifts in microbial ecology of soil in response to climate change. Certain taxa may be influenced differently by CO2 conditions, and molecular methods such as polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) are optimized to monitor changes of microbial flora in soil undergoing changesin CO2 levels (Guenet et al., 2012). There is still a general lack of knowledge regarding the responses of different soil-borne microorganisms to elevated atmospheric CO2 conditions. It is also currently unclear how atmospheric CO2 affects fungal diseases, as studies are limited on this topic. Several studies address the effect of climate change on increasing/decreasing disease incidence (Scherm and Coakley, 2003; Garrett et al., 2006; Chakraborty et al., 2008). However, to our knowledge, none have investigated the effect of different atmospheric CO2 levels on the interaction between *Fusarium oxysporum* f.sp. *lactucae* (FOL) and its host plant. *Fusarium oxysporum* f. sp. *lactucae* (FOL) is a pathogen causing lettuce wilt worldwide (Matheron and Gullino, 2012). The pathogen was first reported in Japan as the causal agent of lettuce root rot (*Lactuca* *sativa* L.) (Matuo and Motohashi, 1967). The same pathogen has been reported in the United States (Hubbard and Gerik, 1993), Taiwan (Huang and Lo, 1998), Brazil (Ventura and Costa, 2008), Italy (Garibaldi et al., 2002) and Arizona (Matheron and Koike, 2003).

The aim of this work was to study the effect of *Fusarium oxysporum* f.sp. *lactucae*, artificially infested in growing substrate, on lettuce plants grown under simulated climate change conditions by altering levels of CO2 and temperature in phytotron plant growth chambers. Various temperature and CO2 concentrations were chosen to represent the present ambient condition under which the disease develops and its possible forecast for the future. To evaluate the effect of climate change, the disease incidence, microbial cultivable abundance, enzymatic assays, and shifts in fungal communities were analysed.

**2. Materials and methods**

*2.1. Microorganism and inoculum preparation*

*F. oxysporum* f. sp. *lactucae* (FOL) strain MYA3040 (Agroinnova, Torino) was cultured in Erlenmeyer flasks containing potato dextrose broth (PDB, Difco) plus 25 mg l-1 streptomycin (AppliChem) for 10 days at 25 °C. Chlamydospores were recovered by centrifugation for 20 min at 6000 x *g* at 15 °C, followed by removal of mycelia fragments by sieving through cheesecloth. Chlamydospore suspensions were dried and mixed with sterile talc (Locke and Colhoun, 1974) and stored at room temperature for further use.

*2.2. Plant growth and experimental design*

Plastic tanks containing 100 l of a mixture of peat-perlite substrate (1:3 v/v) (Tecno2, Turco Silvestro sphagnum peat moss, Albenga, Italy; pH 6, salinity 0.56 mS cm-1, porosity 90%, density 150 kg m-3) were artificially infested with FOL to reach a final concentration of 1×104 CFU g-1 of substrate. A non-infested tank was used as control. Subsequently, 32 pots (2 l each) were prepared from the infested tank and 32 pots were prepared from the non-infested tank (control). Lettuce plants were then transplanted (2 plants/pot), and 8 pots of both infested and non-infested substrate were laid in 4 different phytotrons (32 plants/phytotron; PGC 9.2, TECNO.EL, Italy). Each replicate consisted of 2 pots with 4 plants.

Plants of *L*. *sativa* cv Crispilla Bianca were sown in plug trays and kept at 20-26 °C, 70% relative humidity (RH) and natural light conditions. After 15 days, when the first seedling-leaves had developed, lettuce plants were transferred to phytotron conditions for 7 days before transplanting in the infested and non-infested substrate. Each of the 4 phytotrons were operated under different combinations of temperature and CO2 as follows: phytotron 1: 800 ppm CO2, 22-26 °C; phytotron 2: 800 ppm CO2, 18-22 °C; phytotron 3: 400 ppm CO2, 22-26 °C; phytotron 4: 400 ppm CO2, 18-22 °C. Other relevant parameters such as light and humidity were regulated in the same way for all phytotrons in order to simulate natural conditions. Seven, 14, 21, and 28 days after transplanting, soil samples were taken from each phytotron for further analysis. The experiment was carried out three times.

*2.3. Disease incidence*

The plants were checked weekly for FOL disease development by monitoring the severity of symptoms with the use of a disease index rating from 0 to 100; 0 corresponding to no infection; 25, initial symptoms of leaf chlorosis, one or two yellow leaves; 50, severe leaf chlorosis, growth reduction and initial wilting; 75, incipient symptoms of leaf chlorosis and growth inhibition and severe wilting; 100, leaves totally yellow, wilting of plants followed by death, as previously reported (Garibaldi et al., 2004).

The final disease index was determined at the end of each 8 pot experiment per phytotron (16 plants from infested samples and 16 plants from control samples). Following harvest of each replicate, stem sections of wilted plants were cut and placed on Komada medium (Komada, 1975) to confirm the presence of the wilt pathogen. At the last assessment, the fresh weight (FW) of the survey plants was also measured.

*2.4. Microbial analysis*

Substrate samples (25 g) were taken (at 1-30 cm depth) from 4 pots per sample (infested and control) in sterilized polyethylene bags using sterilized spatula, and stored at 4 ºC until examination. Samples were passed through a sieve (1.7 mm mesh) to remove debris and vegetation, and mixed at room temperature for 30 min with 225 ml of quarter strength Ringer’s solution (Merck, Germany) in sterilized polyethylene bags. Decimal dilutions in quarter strength Ringer’s solution were prepared and 1 ml aliquots of the appropriate dilutions were poured in triplicate on to the following selective media: plate count agar (PCA, Oxoid, Milan) incubated at 28 °C for 42 h for total bacterial counts (TBC) of mesophilic bacteria, and Komada medium (Komada, 1975) kept at room temperature for 7 days for enumeration of *Fusarium* spp. Results were calculated as the mean of Log counts for three replicates.

*2.5. DNA extraction from substrates inoculated with FOL and PCR-DGGE analysis*

For DNA extraction, infested substrate samples (250 mg) were taken from 4 pots/phytotron at different times (7, 14, 21, and 28 days after transplanting) and extracted using the NucleoSpin® Soil kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. The DNA solution was incubated at 37 °C for 30 min with 5 µl of RNAse (Macherey-Nagel), and then stored at -20 °C. DNA was quantified by using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and was standardized at 50 ng μl-1. For ascomycete communities, a portion of the fungal 18S-ITS gene was amplified using a modified NS1 forward primer (Danon et al., 2010) and the specific reverse primer ITS4A (Larena et al., 1999). These PCR products were then used as templates in nested PCR with the primer set ITS1/ITS2 (Gardes and Bruns, 1993; Anderson et al., 2003) to generate products for DGGE analysis. A GC clamp was added to the ITS2 primer according to Muyzer et al. (1993). Amplification was carried out in a T-Gradient thermal cycler (Biometra, Goettingen, Germany) as previously described (Danon et al., 2010). PCR products of approximately 300 bp were analyzed by DGGE (Bio-Rad Dcode apparatus). Samples were applied to 6% (wt/vol) polyacrylamide gels in 1 X TAE buffer. Parallel electrophoresis experiments were performed at 60 °C using gels containing a 20 to 60% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (wt/vol) formamide). The gels were run for 16 h at 75 V, stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Milano) for 30 minutes, visualized under UV-transillumination and photographed by Bio-Rad Gel Doc system (Bio-Rad, Milano, Italy).

*2.6. Sequencing of DGGE fragments*

Selected DGGE bands were excised from the gel with sterile pipette tips and purified in water according to Ampe et al. (1999). One l of the eluted DNA was used for reamplification by using the primers and the conditions described above, and the PCR products were checked by DGGE; The original PCR product was run on the gel as the control. Products that migrated as a single band and at the same position as the control were purified by using a QIAEX PCR purification kit (Qiagen, Milano, Italy) according to the manufacturer’s instructions and then were sequenced. To determine the closest known relatives of the partial 18S-ITS gene sequence obtained, searches were performed in public datalibraries (GenBank) with the Blast search program (http://www.ncbi.nlm.nih.gov/blast/).

*2.7. Microbial activity*

The substrate samples collected were tested for soil biological parameters such as soil β-Glucosidase activity (βGA) and fluorescein diacetate hydrolysis (FDA). βGA was determined by colorimetric measurement of the reduction of 4-nitrophenyl-β-D-glucopiranoside to p-nitrophenyl according to the method of Nannipieri et al. (1979). FDA was measured using 3,6-diacetyl fluorescein, and measuring the absorbance of released fluorescein at 490 nm according to the method of Schnurer and Rosswall (1982). Enzyme activities were expressed as μg per g of substrate after one hour of incubation. The measures were done in triplicate. Assays without a substrate were performed at the same time as a control.

*2.8. Statistical analysis*

Data obtained in the three replicates were analyzed by using one-way analysis of variance (ANOVA) for each individual date, with treatments being the main factor. ANOVA analyses were performed with an SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA). The Duncan HSD test was applied when ANOVA revealed significant differences (P<0.05).

**3. Results**

*3.1. Disease assessment*

The disease index (DI, 0-100) in the three replicates was influenced significantly by temperature (Table 1). At 22-26 °C (phytotrons 1 and 3), DI increased more than 50%, when compared with the DI observed at the lower temperature. Change in carbon dioxide level showed no significant effects when temperature ranged between 18-22 °C (phytotrons 2 and 4) and 22-26 °C (phytotrons 1 and 3). No disease was observed in lettuce plants grown in non-infested pots (Table 1). DI was high in all conditions, reaching more than 50% of wilted plants in phytotron 1 and 3 as a consequence of temperature (22-26 °C), while fresh weight (FW) data did not change significantly between the different phytotrons (Table 1). Diseased plants produced less than 80% biomass compared to not inoculated plants, with the exception at 18-22 °C (phytotron 2 and 4), in which the FW of diseased lettuce plants was slightly higher when compared with plants at 22-26 °C. Finally, ANOVA analyses on the four conditions of inoculated plants showed no statistical differences between the three trialscarried out.

*3.2. Microbial analysis and enzymatic activity of substrate samples*

The results of the fungal and bacterial plate counts from three replicates on specific media are shown in Tables 2 and 3. Plate counts of *Fusarium* spp. (Table 2) from infested substrate samples were not affected by different CO2 concentrations, nor by combinations of CO2 and temperature conditions, but showed significant differences (P<0.05) due to temperature conditions alone. Samples at 22-26 °C in phytotrons 1 and 3 showed an increase from 4.20 to 4.49 and from 4.19 to 4.63 Log CFU g-1, respectively. However, samples at 18-22 °C did not show a significant development. Different concentrations of CO2 did not affect the growth of *F. oxysporum*. No *Fusarium* spp. was detected in non infested substrate samples.

For mesophilic bacteria (Table 3), total bacterial counts (TBC) from infested substrate samples increased with time, only at the standard CO2 concentration (400 ppm CO2). Infested substrate samples measured after 7 and 28 days at 400 ppm CO2 increased from 6.82 to 7.60 Log CFU g-1 at 18-22 °C, and from 7.12 to 7.86 Log CFU g-1at 22-26 °C. By contrast, the TBC statistical values of substrate samples at 800 ppm CO2 remained constant throughout the whole experiment. The same behaviour was observed in the control sample.

Results relating to enzymatic activity are shown in Tables 4 and 5. The FDA activity (Table 4) was only affected by CO2 concentration and was generally lower at 800 ppm CO2 than at 400 ppm CO2. In particular, the FDA in infested substrate samples measured after 28 days at 400 ppm CO2 increased significantly (P<0.05) from 13.59 to 56.20 µg FDA h-1 g-1 at 18-22 °C, and from 10.67 to 56.03 µg FDA h-1 g-1 at 22-26 °C. No significant changes were observed in the control. On the other hand, βGA concentrations (Table 5) in all samples and under all CO2 and temperature conditions decreased significantly after 28 days. Elevated CO2 levels had no significant effect on the enzyme measurement. For both enzymes assayed, the presence of FOL had no significant effect (P<0.05) on enzyme activity profiles.

*3.3. Identification of microbial species*

PCR-DGGE fingerprints of the ascomycete community obtained from DNA directly extracted from infested samples in all the conditions adopted are presented in Fig.1 (see Fig. S1 for the two other replicate fingerprints); the results of the band sequencing are shown in Table 6. Fragments arising from different fingerprints were sequenced and those migrating the same distance gave the same results in terms of closest relative species and percent identity. Repeated DNA extraction and PCR-DGGE analysis confirmed the fingerprinting obtained. Fungal profiles did not show significant changes in relation to CO2 concentration and temperature conditions (Fig.1). Sample profiles were similar and showed bands identified as *Fusarium oxysporum, Pseudallescheria fimeti* and *Hypocreales* sp. (bands 3, 5 and 8, respectively) (Table 6) in all the fingerprints. The occurrence of other fungi such as *Trichoderma asperellum,* *Preussia* sp*., Myriococcum thermophilum* and *Peziza ostracoderma* (bands 1, 2, 4 and 6) was observed (Fig. 1, Table 6).

**4. Discussion**

The aim of this work was to study the effect of simulated climate change on the severity of *Fusarium oxysporum* f.sp. *lactucae* on lettuce plants. Changes to the natural environment were simulated in phytotron growth chambers using different levels of CO2 and temperatures. Phytotrons have been used to provide controlled and reproducible conditions for several types of studies in plant physiology (Gullino et al., 2011). Experiments carried out in these chambers can be compared to field data, not only in a qualitative but also in a quantitative way (Moot et al., 1996; Hsiao and Xu, 2000;). The ability to reproduce different climatic conditions for experimental purposes can be very useful in simulating climatic scenarios and monitoring plants, pathogens and plant-pathogen interactions. The results of this study were interpreted in terms of disease incidence, cultivable abundance, enzymatic assays, and variability of fungal profiles by PCR-DGGE. Other studies have shown that the response of soil microorganisms to elevated CO2 conditions is highly variable and strongly depends upon the plant–soil system (Moscatelli et al., 2005; Drigo et al., 2008). Different levels of CO2 used in several long term studies have shown to decrease (Ebersberger et al., 2003), increase (Hungate et al., 1999; Williams et al., 2000; Cheng et al., 2011) or not induce changes (Nussbaumer et al., 1997; Zak et al., 2000) in microbial diversity and activities. Temperature increase of few °C may have very little effect on the development of soil microbial flora (Scharpenseel et al., 1990). A temperature increase in the range predicted for future climate change, under ambient and elevated CO2 concentrations, did not show consistent effects on soil microorganism development (Kandeler et al., 1998).

In our study, the disease incidence of FOL and plate counts of *Fusarium* spp. in infested substrate samples were not affected by variations in CO2 concentration, but showed significant differences (P<0.05) as a function of temperature. As previously reported higher temperatures generally corresponded to greater severity of Fusarium wilt in lettuce (Scott and Gordon, 2010). This behaviour is well correlated with disease incidence. Total bacterial counts in infested substrate samples showed a greater increase at the standard CO2 concentration than under higher CO2 conditions. In the conditions adopted in this study, the results suggested that the forecast increase in CO2 concentration due to climate change would have a limited effect on bacterial development. Results of this study also showed that the microbial enzyme activity involved in the carbon cycle was not affected by elevated CO2 concentration. Recent reports (Dorodnikov et al., 2009; Guenet et al., 2012) have shown similar results, while other studies reported conflicting results about the enzymatic activity (Freeman et al., 2004). This variability of results can originate from differences in several factors such as the composition of the substrates used and the availability of plant nutrients. The variability of fungal profiles was evaluated by PCR-DGGE, a technique that has also been employed with good results in other studies to assess the soil microbial population under different CO2 levels (Guenet et al., 2012). In this study the PCR-DGGE analysis of DNA extracted from substrate samples showed that the fungal species diversity at different levels of CO2 did not change significantly. The results of this study are in agreement with recent studies showing that species diversity is not influenced by elevated CO2 levels (Lipson et al., 2006; Guenet et al., 2012). PCR-DGGE analyses showed that temperature does not affect the species diversity in the substrate samples analyzed.

**5. Conclusions**

The global warming predicted for the future could induce an increase in the incidence of diseases caused by plant pathogenic *Fusarium* species. Previous studies have shown that exposure to high atmospheric CO2 levels and high temperatures can cause a low, but significant modification in plant growth and in the development of FOL in lettuce. We cannot make specific predictions on what would happen in the field, but results from the simulation experiment, performed in phytotron growth chambers, showed that temperatures of 22-26 °C induced an increase in disease incidence compared with standard conditions, although no effect was observed on TBC or on the stimulation of the monitored extra-cellular enzymes. No significant effects were detected as a consequence of elevated CO2 concentration. However, the potential impacts of elevated atmospheric CO2 on soil microbial composition and activity deserve further studies.

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**Figure 1 -** PCR-DGGE profiles of the 18S-ITS amplicons from microbial DNA directly extracted from infested substrate samples after 28 days growth in phytotrons; subscript numbers indicate the number of days after transplantation when the sample was taken. Single band numbers indicate the sequenced fragments reported in Table 6.

**7 14 21 28 7 14 21 28 7 14 21 28 7 14 21 28**

**2**

**(800ppm CO2 18-22°C)**

**1**

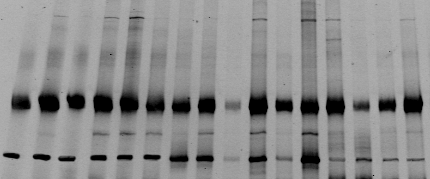
**(800ppm CO2 22-26°C)**

**4**

**(400ppm CO2  18-22°C)**

**3**

**(400ppm CO2  22-26°C)**



**7 14 21 28 7 14 21 28 7 14 21 28 7 14 21 28**

**2**

**(800ppm CO2 18-22°C)**

**1**

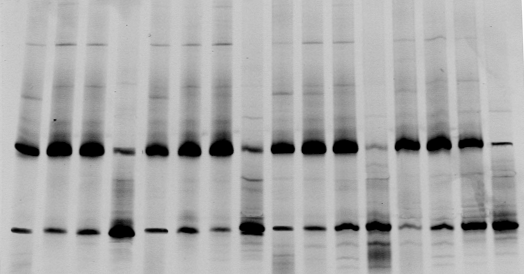
**(800ppm CO2 22-26°C)**

**4**

**(400ppm CO2  18-22°C)**

**3**

**(400ppm CO2  22-26°C)**



|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | Phytotron\* | | | |
|  | Parameters | 1 800 ppm CO2  22-26°C | 2 800 ppm CO2  18-22°C | 3 400 ppm CO2  22-26°C | 4 400 ppm CO2  18-22°C |
| Infested samples | Disease Index (0-100) | 58.3c | 29.7b | 54.7c | 31.3b |
| FW (g plants-1) | 4.2a | 5.8a | 4.1a | 5.6a |
| Control | Disease Index (0-100) | 0.00a | 0.00a | 0.00a | 0.00a |
| FW (g plants-1) | 15.4b | 15.9b | 16.2b | 15.5b |

**Table 1**

Disease index (0-100) and total plant fresh weight of plants at the end of the replicates (FW)

\* The Disease index (0-100) and total plant fresh weight (FW) were expressed as a mean of the three replicates. Values with different superscripts in the same row differ significantly (P<0.05)

**Table 2**

Plate counts of *Fusarium* spp. from infested substrate samples following incubation in phytotrons for 28 days

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | \* Log CFU g-1 ± SD | | | |
| Sampling day | 1  (800 ppm CO2 22-26°C) | 2  (800 ppm CO2 18-22°C) | 3  (400 ppm CO2  22-26°C) | 4  (400 ppm CO2  18-22°C) |
| 7 | 4.20 ± 0.22abA | 4.21 ± 0.32aA | 4.19 ± 0.32aA | 4.38 ± 0.33 bA |
| 14 | 3.88 ± 0.66 aA | 4.22 ± 0.61aA | 3.89 ± 0.67 aA | 4.35 ± 0.36 bA |
| 21 | 4.41 ± 0.36bA | 4.25 ± 0.17aA | 4.26 ± 0.30 abA | 4.19 ± 0.19 abA |
| 28 | 4.49 ± 0.15bBC | 4.28 ± 0.51aAB | 4.63 ± 0.30 bC | 3.88 ± 0.58 aA |

\* Results were calculated as the meanLog count for the three replicates. The plate counts were expressed as the mean of the three trials. Values with different superscripts (lowercase letters) in the same column differ significantly (P<0.05).Values with different superscripts (uppercase letters) in the same row differ significantly (P<0.05).

**Table 3**

Total bacterial counts (TBC) of mesophilic bacteria from infested and control substrate samples following incubation in phytotrons for 28 days.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | \* Log CFU g-1 ± SD | | | |
|  | Sampling day | 1  (800 ppm CO2 22-26°C) | 2  (800 ppm CO2 18-22°C) | 3  (400 ppm CO2  22-26°C) | 4  (400 ppm CO2  18-22°C) |
| Infested samples | 7 | 6.84 ± 0.58 aA | 6.94 ± 0.33 aA | 7.12 ± 0.27aA | 6.82 ± 0.19 aA |
| 14 | 7.15 ± 0.50 abA | 7.49 ± 0.34 bB | 7.21 ± 0.37 aAB | 7.44 ± 0.32bB |
| 21 | 7.44 ± 0.73 bAB | 7.07 ± 0.47aA | 7.12 ± 0.66 aA | 7.64 ± 0.64 bB |
| 28 | 7.20 ± 0.43 abA | 7.19 ± 0.33abA | 7.86 ± 0.31bB | 7.60 ± 0.06 bB |
|  |  |  |  |  |
| Control | 7 | 6.93 ± 0.47 aAB | 6.81 ±0.27 aA | 7.27±0.13 aC | 7.20±0.36 aBC |
| 14 | 7.36 ± 0.60 aA | 7.48 ±0.37 bA | 7.36±0.53 abA | 7.39±0.27 aA |
| 21 | 7.47 ± 0.77aA | 7.49 ±0.90 bA | 7.58±0.59 abA | 7.40±0.45 aA |
| 28 | 7.55 ± 0.72 aA | 7.59±0.74 bA | 7.81±0.46 bA | 7.48±0.47 aA |

\* Results were calculated as the mean Log count for the three replicates. The plate counts were expressed as the mean of the three replicates. Values with different superscripts (lowercase letters) in the same column differ significantly (P<0.05).Values with different superscripts (uppercase letters) in the same row differ significantly (P<0.05).

**Table 4**

Total microbial activity (FDA) in infested and control substrate samples following incubation in phytotrons for 28 days.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | bµg FDA h-1 g-1 | | | |
|  | Sampling day | 1  (800 ppm CO2 22-26°C) | 2  (800 ppm CO2 18-22°C) | 3  (400 ppm CO2  22-26°C) | 4  (400 ppm CO2  18-22°C) | |
| Infested samples | 7 | 9.31 ± 3.44 aA | 7.42 ± 0.00a A | 10.67 ± 1.23aA | 13.59± 0.67 aB | |
| 14 | 4.13 ± 0.56 aA | 13.79 ± 0.78aAB | 25.72 ± 0.06bB | 26.75 ± 3.28 bB | |
| 21 | 5.12 ± 7.56 aA | 22.53 ± 0.56bAB | 34.10 ± 4.56 bB | 17.70 ± 0.78 aAB | |
| 28 | 6.14 ± 3.17 aA | 20.87 ± 4.39 bAB | 56.03 ± 0.44cC | 56.20 ± 5.00 cC | |
|  |  |  |  |  |  | |
| Control | 7 | 12.44 ± 0.13 bA | 14.89 ± 0.00 abA | 15.13 ± 2.65 aA | 23.13 ± 0.22 aAB | |
| 14 | 3.27 ± 7.83 aA | 23.46 ± 0.61 bB | 18.65 ± 4.11 aAB | 23.74 ± 0.17 aB | |
| 21 | 4.11 ± 4.56 aA | 6.64 ± 2.83 aA | 18.45 ± 4.11 aAB | 24.45 ± 6.44 aB | |
| 28 | 1.39 ± 3.06 aA | 6.93 ± 0.28 aAB | 19.33 ± 10.11aC | 16.49 ± 0.78 aC | |

bResults were calculated as the mean Log count for three replicates. Values with different superscripts (lowercase letters) in the same column differ significantly (P<0.05).Values with different superscripts (uppercase letters) in the same row differ significantly (P<0.05).

**Table 5**

β-Glucosidaseactivity (βGA) in infested and control substrate samples following incubation in phytotrons for 28 days.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | cµg p-nitrophenol h-1 g-1 | | | |
|  | Days of sampling | 1  (800 ppm CO2 22-26°C) | 2  (800 ppm CO2 18-22°C) | 3  (400 ppm CO2  22-26°C) | 4  (400 ppm CO2  18-22°C) |
| Infested samples | 7 | 32.10±3.77 abA | 30.58±12.68 abA | 45.89±5.01 bAB | 50.98±6.30bAB |
| 14 | 53.67±3.01 cA | 55.21±0.63 cA | 100.28±0.09 cC | 87.21±5.89 cAB |
| 21 | 31.99±9.11 abA | 43.80±3.40 bcA | 43.95±8.83bA | 47.27±8.84 bA |
| 28 | 5.18±1.93 aA | 13.11±0.13 aB | 1.77±1.04 aA | 11.04±0.32 aB |
|  |  |  |  |  |  |
| Control | 7 | 39.80±5.37 bcA | 58.57±16.69 cAB | 43.99±17.69 bAB | 65.33±4.47 cB |
| 14 | 47.57±0.38 cA | 48.65±3.71 cA | 68.25±4.18 cB | 60.14±2.34 cAB |
| 21 | 34.40±4.26 bB | 14.16±2.53 bA | 28.21±1.04 abB | 10.82±5.09 bA |
| 28 | 3.92±0.54 aA | 2.72±1.36 aA | 18.85±0.38 aB | 6.67±0.16 aA |

bResults were calculated as the mean Log count for three replicates. Values with different superscripts (lowercase letters) in the same column differ significantly (P<0.05). Values with different superscripts (uppercase letters) in the same row differ significantly (P<0.05).

**Table 6** – Fungal species identification following sequencing of 18S-ITS genes purified from PCR-DGGE profiles of infested soil samples.

|  |  |  |  |
| --- | --- | --- | --- |
| Banda | Closest relative | Identity (%) | Closest relative Accession No. |
| 1 | *Trichoderma asperellum* | 98 | FJ797513 |
| 2 | *Preussia* sp*.* | 99 | FJ210518 |
| 3 | *Fusarium oxysporum* | 98 | JF439472 |
| 4 | *Myriococcum thermophilum* | 99 | JF412008 |
| 5 | *Pseudallescheria fimeti* | 97 | AY879799 |
| 6 | *Peziza ostracoderma* | 97 | U40472 |
| 7 | Uncultured fungus | 100 | HQ215860 |
| 8 | *Hypocreales* sp. | 99 | EF060712 |

a bands 1 to 8 are indicated in Figure 1.