Biochar as a carrier

Trichoderma harzianum on Biochar to promote disease suppression in Strawberry

Chris Blok[1*], Andrea Diaz[1], Nina Oud[1], Marta Streminska[1], Ming Huisman[1], Pham Khanh[1], Lydia Fryda[2] and Rianne Visser[2]

1. Wageningen University & Research, Glasshouse Horticulture, Violierenweg 1, 2665MV Bleiswijk, the Netherlands
2. Energy Research Centre of the Netherlands, (ECN), Part of TNO, P.O. Box 1, 1755 ZG Petten, The Netherlands
*Correspondence: Chris.Blok@WUR.nl; Tel.: (+31) 317 485679

Report WPR-893

WAGENINGEN
UNIVERSITY & RESEARCH

Referaat
In opdracht van het ministerie van economische zaken onderzoekt ECN (Energie Centrum Nederland, een onderdeel van TNO) de energieproductie uit hernieuwbare organische bronnen. Pyrolyse en gassificatie zijn technieken waarmee groen aardgas en platform chemicaliën gemaakt kunnen worden. Een ander optie is dat in co-productie bio-energie en biochar worden gemaakt. De economische haalbaarheid wordt sterk verbeterd als biochar als co-product een significante positieve waarde krijgt als onderdeel van potgronden in de glastuinbouw. Wageningen University & Research Glastuinbouw onderzoekt in dat kader of biochar gebruikt kan worden voor twee doeleinden. Het eerste doel is biochar als duurzamer veenalternatief in een potgrondmengsel voor de teelt van aardbeien. Het tweede doel is het gebruik van biochar als drager voor nuttige micro-organismen voor de teelt van aardbeien. In een teeltexperiment is gekeken naar groei-effecten bij het gebruik van een reeks verschillende biochar-veen mengsels. Daarbij is Trichoderma toegevoegd om de plant tegen een toediening van de ziekteverwekker Phytophthora te beschermen. De resultaten tonen dat 10, 20 of 30% v/v biochar in een veengrond geen effect heeft op vers en drooggewicht van de blad en stengel massa maar wel leidt tot een 5-10% hogere productie van vruchten. Het toedienen van Trichoderma met biochar leidde niet tot een betere overleving van Trichoderma. De toegediende Phytophthora leidde niet tot een verhoogde kans op ziekte in de aardbeiplanten maar de Phytophthora druk is lager als het percentage biochar toeneemt.

Abstract
For the ministry of economic affairs, ECN (Energy Centre Netherlands, part of TNO) develops energy production from renewable organic sources. Pyrolysis and gasification are technologies by which green bio gas and platform chemicals can be produced. Another option is to co-produce bio-energy and biochar. Biochar is the high-carbon low-solid product of the process. The economic feasibility of the latter processes dependents on the valorisation of biochar. A promising application is as (partial) peat replacement option in potting soil mixtures for greenhouse horticultural production. Wageningen University & Research Greenhouse Horticulture used biochar in dedicated research to accomplish two distinct goals. The first goal was to use biochar as an environmentally more friendly peat alternative in potting soil mixes for the production of strawberries. The second goal was to use biochar as carrier for beneficial micro–organisms for the production of strawberries. In a cultivation experiment the growth effects in a range of biochar–peat mixtures was studied. Trichoderma was added to protect the plant against a wilful addition of the disease Phytophthora. The results show 10, 20 and 30% v/v of biochar in a peat soil does not affect fresh or dry weight production of leaf and stem mass. The biochar addition does increase the fruit fresh weight production with about 5-10%. The addition of Trichoderma with biochar did not lead to an improved survival of the Trichoderma in the potting soil mixes. The added Phytophthora did not lead to a higher disease incidence, but the Phytophthora presence is lower in treatments with a higher dose of biochar.

Reportinfo

Report WPR-893
Projectnumber: 3742228900
DOI number: 10.18174/501687
Theme: Water and nutrients

Address

Wageningen University & Research, BU Greenhouse Horticulture
Violierenweg 1, 2665 MV Bleiswijk
P.O. Box 20, 2665 ZG Bleiswijk
The Netherlands
+31 (0) 317 - 48 56 06
glastuinbouw@wur.nl
www.wur@glastuinbouw.nl

Table of contents

Summary

The ministry of economic affairs stimulates research into energy production from renewable organic sources. Part of this research is provided by ECN (Energy Centre Netherlands, part of TNO). ECN develops, among others, pyrolysis and gasification technologies to produce green methane and platform chemicals for energy generation as well as replacements for fossil oil compounds in transport fuels, chemicals and materials. Another option is to co-produce biofuel and biochar. The burnable gas produced is combusted for energy generation by a conventional steam cycle, while the high-carbon / low-density solid product can be used as a material in a range of other applications. The economic feasibility of the latter process requires the valorisation of biochar.

One possible valorisation of biochar is as part of potting soil mixtures for greenhouse horticultural production provided the biochar is free of tars (PAH). This has the added advantage that the biochar can be used to partly replace peat. The replacement of peat can reduce the emission of carbon dioxide and methane usually associated with peat extraction.

Wageningen University & Research, Greenhouse Horticulture used biochar produced by ECN in dedicated research with strawberry on peat-biochar mixture with 10, 20 and 30% v/v of biochar. The first goal was to use biochar as peat alternative. The second goal was to use biochar as carrier for beneficial micro–organisms.

The results show 10, 20 and 30% v/v of biochar in a peat soil does not affect fresh or dry weight production of leaf and stem mass. The biochar addition does increase the fruit fresh weight production with about 5-10%. Possibly the growth was more generative because the biochar additions make a slightly drier rooting material, which is a known generative action. In conclusion biochar in quantities up to at least 30% v/v can be used as a viable peat alternative.

Biochar as carrier for the addition of Trichoderma did not lead to an improved survival of the Trichoderma in the potting soil mixes. In all treatments the Trichoderma counts went down two orders of magnitude within 3 weeks. In conclusion biochar does not help Trichoderma to survive in the system tested. Biochar as tested in this experimental set up is not a proper carrier of micro-organisms. There is some evidence from literature that biochar pre-treated with composts and or inorganic nutrients could act as carrier.

An unintended outcome was that the qPCR analyses used, showed unacceptably large variations in outcome. Differences of over two orders of magnitude between repetitions were no exception. Therefor a new protocol for the sample pre-treatment of rooting media samples for qPCR analysis was added to this report. The new protocol still needs to be validated though.

The addition of Phytophthora did not lead to a higher disease incidence. The Phytophthora presence –without diseasing the plants- was lower in treatments with a higher dose of biochar, possibly because the mixes with biochar are marginally drier in nature and Phytophthora thrives in wetter circumstances. In conclusion the Phytophthora was not pathogenic enough to allow conclusions on the interaction with Trichoderma/biochar combinations.

1 Introduction

1.1 Motivation and Background

In the Netherlands, the use of biochar in horticulture is still limited to the experimental phase. Nevertheless a proper quality of biochar could rapidly become relevant due to its role in the global reduction of carbon dioxide emissions in at least two ways:

- Contribution to the carbon sequestration by introducing the biochar that was used in horticultural cultivation into arable soils (Woods et al. 2009).
- The substitution of peat for container cultivation plants, as peat is not ecologically sustainable since peat is normally harvested from a relatively stable carbon store *(Verhagen et al. 2009*).

Furthermore biochar is known for improving water retention, plant protection/resilience against diseases (*Frenkel et al. 2017*) and increase of the biological control capacity of the growing media (*Debode et al. 2018*). Properties like porosity, pH and surface area are some characteristics of the biochar that might contribute to the establishment of beneficial microorganisms in the root-zone and therefore induce disease suppression. Since each biochar may differ in these properties, depending on its raw material and the pyrolysis procedure (*Fryda and Visser, 2015*), it is relevant to identify physical, chemical and biological properties of biochar.

ECN produces biochar specifically for horticultural purposes *(Fryda and Visser, 2015)*. That biochar has already been tested by WUR (*Blok et al. 2017*). The results indicate that ECN biochar improves water retention and the efficiency to buffer the pH. In order to understand the performance of microorganisms on the ECN biochar, it is also desirable to assess their establishment on growing media, as well as the subsequent development of the plants.

Trichoderma harzianum is a widely known as mycoparasite with direct biocontrol properties on soil-borne plant pathogens *(De Pascale et al. 2018; Debode et al. 2018).* Trichoderma has also indirect effects on plant resistance induction, as well as on the promotion of growth and development of the plant. Therefore, the survival study of this microorganism on a carrier material like biochar in growing media is of a great interest.

Phytophthora cactorum was first reported in glasshouse-grown strawberries in 1973, producing an internal red-brown discoloration of the upper crown, discoloration of leaves and wilting of the plant *(Harries and Stickels, 1981*). *Phytophthora cactorum* has been a problem and many producers had to deal with the consequent high economic and environmental impact. Some field experiments have shown that Trichoderma was able to reduce the incidence of *P. cactorum* in strawberry plants (*Porras et al. 2007*). Therefore, the opportunity to assess biochar as a carrier of *Trichoderma* in order to reduce the incidence of *P. cactorum* is of practical interest for strawberry producers.

1.2 Research objectives

1. Find if the addition of biochar to a peat mix affects the plant production on the mix in any way.
2. Identify to what extend beneficial microorganisms like *Trichoderma harzianum* are established on biochar, how this affects the survival in time of *Trichoderma harzianum* and how the presence of *Trichoderma harzianum* contributes to the performance of the plants.

In this report, the following questions will be answered:

- Is production of strawberries influenced by the addition of biochar to peat?
- Is *Trichoderma harzianum* able to survival on biochar?
- Is *Trichoderma harzianum* able to survive in a strawberry growing system when using biochar as carrier?
- Can *Trichoderma harzianum* contribute to the disease suppression of *Phytophthora cactorum* in a strawberry growing system with biochar as a carrier?

1.3 Approach

This project was realized in two experiments. The first experiment studied the survival of *Trichoderma harzianum* on the ECN biochar. The second experiment was focussed on determining if *T. harzianum* was able to survive in a strawberry growing system, and determining if *T. harzianum* contributes to the disease suppression of *Phytophthora cactorum* on strawberry plants by using ECN biochar as carrier. Prior to the establishment of the two experiments, the characterization and evaluation of physical, chemical and biological properties of the ECN biochar was completed

1.4 Organisation

This project was subsidised by the Dutch ministry of Economic Affairs via Rijksdienst voor Ondernemend Nederland (RVO). The project leader was the Dutch Energy Centre (ECN), which during the run time of the project became part of the TNO organisation. This report was developed and executed by Wageningen UR, Greenhouse Horticultural group.

Rian Visser from ECN provided the biochar that was especially produced for this project. Moreover, Rian executed and shared the results from the Scanning Electron Microscope (SEM) test to enrich the content of this report. At Wageningen UR, Andrea Diaz and Chris Blok designed, coordinated and executed the project with the support and advice of Marta Streminska as a specialist consultant on soil microbiology. Aat van Winkel, Nina Oud and Barbara Eveleens actively participated in the discussion and execution of the practical work of the project. The inoculation of microorganisms and DNA evaluation was performed by Khanh Pham and Huei Ming Huisman.

2 Materials and Methods

2.1 The rooting media used and treatments

A standard Baltic white peat was used as basic peat (Klasmann Deilmann, Schiedam the Netherlands). The biochar was a 2-4 mm coniferous chip product pyrolysed at 750 degrees Celsius. The test was performed on peat, biochar and mixtures of 10% v/v biochar-peat, 20% v/v biochar-peat and 30% v/v biochar-peat.

2.2 Evaluation of the Biochar

2.2.1 Water holding capacity

A water holding capacity test was carried out to determine the water retention of the used substrates CEN 13041, 2011. This test followed the method of the European Committee for Standardization (*CEN 13041, 2011*) by using a sand box set to test a range of different suction strengths. Samples were initially saturated and then used to fill double rings which were put to a suction head of -50 cm in order to compact the material. The complete filled and compacted lower ring was used for further tests. The water content of the samples was determined when the equilibrium was established between the water content and the suction force (usually after 48 hours). The suction force was defined as the height difference between the overflow of the water-tight sandbox (in which the samples stood on a bed of sand) and half the height of the sample rings in the sandbox *(Raviv et al. 2019)*.

2.2.2 Dry bulk density

The dry bulk density was determined by filling a cylinder with a known volume (1:1 height to diameter ratio cylinder of 20 litres) with the correspondent sample. In parallel with the water holding capacity test, the samples were saturated with water at a suction head of -10 cm. The mass was determined by weighting the content of the cylinder in fresh and dry state. The dry state is the weight after 24 hours at 105 degrees Celsius. Consequently, the density was calculated from the mass and the volume of the sample in kg/m^3 (*CEN 13041, 2011*). As part of this test, the combined percentage of water and air was also calculated as total pore space (%v/v) by subtracting the volume of mineral and organic solids from 100%-v/v. Thus, from the bulk density determination, the difference between the total volume and the volume fraction of the solid parts was calculated as the total pore space, which involves the volume occupied by water and air in percentage.

2.2.3 Organic and mineral matter content

The same samples of the water holding capacity test were dried at 105°C for 12h in a tared container. After drying, the samples were weighted and then ignited at 600°C during 3h. The organic matter content was determined as the loss on ignition. The mineral fraction was determined as the weight of the cool ashes (*CEN, 2007*).

2.2.4 Oxygen uptake rate and stability

This method determines the degradation of the organic material by microorganisms under ideal conditions, measuring the oxygen consumption of the microorganisms in a sealed vessel for a certain period of time.

Samples were mixed with a full nutrient solution to ensure that microbial activity was not limited by nutrients or moisture. A nitrification inhibitor was added to avoid false readings by nitrogen gas emission and the pH of the substrate-nutrient mixtures was adjusted to a pH between 5.5 and 6.5. Then, the samples were put in closed vessels and placed on a horizontal shaker (120 rpm) for five days at 30°C. The pressure in the vessels was continuously measured over the course of incubation period. The CO_2 was captured from the gas phase by a solution of KOH in a small vessel included in the sample vessel. Therefore, the decrease in pressure could only be attributed to the O_2 consumption. The maximum O_2 consumption rate was analyzed from the correlation of O_2 concentration and time. Results are expressed as oxygen consumption per unit of time (dO_2/dt in mmol/min) and oxygen consumption per unit of mass of dry organic matter (mmol/kg/h).

2.2.5 Nutrient content

A nutrient analysis was executed for the prepared substrates of the second experiment (pot experiment), which include peat (with and without *T. harzianum*), and mixtures (10% v/v biochar-peat, 20% v/v biochar-peat and 30% v/v biochar-peat). Rooting media samples were collected at planting and during the growing season of the strawberry plants. The 1:1,5 extraction method was followed to estimate the composition of the substrate solution by adding fresh water to the substrate until it reaches approximately a water content of -32cm of water head (*Raviv et al. 2019*). Then, a sub sample of 60 ml of the substrate was placed in a beaker with 90 ml of water, forming a suspension that was shaken for 30 min with a magnetic agitator. Later, the suspension was filtered by using filtering paper in order to clear the water suspension. The collected samples were analysed by an external laboratory (Groen Agro Control).

2.2.6 Phytotoxicity test

Biochars may contain certain phytotoxic compounds with effects on plant growth. Therefore, Phytotoxicity was tested on the ECN biochar -and peat. Seventeen grams of dry substrate with 43 ml of standard cucumber nutrient solution was prepared (Table 2.1). Thereafter, the pH and EC values were adjusted to pH 5.5 using concentrated nitric acid, and adding extra nutrients or demi water to reach an EC of 2.0 mS/cm.

Table 2.1
Standard nutrient solution for the phytotoxicity test.

Parameter	Unit	Value	Parameter	Unit	Value
pH	-log[H^+]	5.5	NH4	mmol/l	0.6
EC	mS/cm	2.2	K	mmol/l	11.5
			Na	mmol/l	0.5
			Ca	mmol/l	2.5
			Mg	mmol/l	1.1
Fe	µmol/l	14.4	Si	mmol/l	< 0.1
Mn	µmol/l	7.7	NO3	mmol/l	15.1
Zn	µmol/l	9.3	Cl	mmol/l	0.2
B	µmol/l	49	SO_4	mmol/l	1.1
Cu	µmol/l	1.4	HCO_3	mmol/l	0.1
Mo	µmol/l	0.53	P	mmol/l	2.1

The bioassays were carried out using three different species: *Sorghum saccharatum* (sorghum), *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard). The seeds were placed on filter paper on granulate rock wool, and then put into closed transparent plates. For each treatment, 4 plates with 10 seedlings were prepared, in total 40 seedlings per treatment. After 3 days of incubation at 20°C, in darkness, photos of the plates were taken, the number of germinated seedlings were counted and the root- and shoot length of seedlings were measured to express toxicity as percentage of growth inhibition when compared with the control (Figure 2.1).

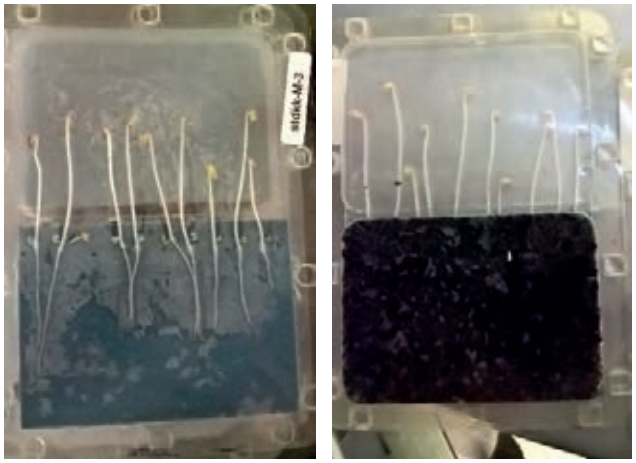


Figure 2.1 *Transparent test plates with the germinated seeds (sorghum, garden cress and mustard) after 3 days of incubation for peat (left) and biochar (right).*

2.2.7 pH buffering capacity

The acid-neutralizing capacity of the biochar was determined by titration using an automatic titration unit. A step-wise titration procedure was used in which concentrated acid (HCl 0.2M) was dosed for five minutes followed by an equilibration period of 45 minutes without acid dosing. During dosing, the HCl was applied until a pH of five was reached. During equilibration, the pH slowly increases again due to buffering of the substrate. This procedure was repeated ten times. The titration therefore took nine hours. The total amount of acid dosed during the titration was set as equal to the acid-neutralising capacity of the substrate.

2.3 Experiment 1: Survival of *Trichoderma harzianum*

This experiment consists of four treatments represented as substrates (peat and biochar) with and without inoculation of *Trichoderma harzianum*. Each treatment consists of four replicates that were assessed during three sessions in order to quantify the DNA at different times after the inoculation of *T. harzianum*. Therefore, a total of 48 transparent plastic pots, which contained the corresponding treatment, were distribute as described in Table 2.2:

Table 2.2
Description of the treatments for the experiment 1.

Treatment	Inoculation	Growing medium preparation	DNA analysis	Repetition per session
Peat+Th	*Trichoderma harzianum*	100ml peat + 10ml inoculum + 35ml nutrient solution	7, 16 & 25 (dai)*	4
Biochar+Th		100 ml biochar + 10 ml inoculum +35 ml nutrient solution		4
Peat	non-inoculation	100 ml peat + 45 ml nutrient solution	7, 16 & 25 (dai)*	4
Biochar		100 ml biochar + 45 ml nutrient solution		4

* Days after inoculation

The initial pH of the ECN biochar was 6, and the results from the titration test indicate that the biochar had a pH buffering capacity of 48.5 mmol of acid per kg of dry matter. Since the pH target value for the inoculation was also 6, the ECN biochar was not neutralized for this experiment.

The setup of the experiment followed the next steps: **a)** Preparation and inoculation of the *T. harzianum* inoculum; **b)** incubation; **c)** isolation of *T. harzianum* for DNA analysis.

2.3.1 Preparation and inoculation of *Trichoderma harzianum*

The preparation of the *Trichoderma harzianum* (Th) inoculum was made from the commercially available Trianum®, produced by Koppert (Dutch company). To grow the *T. harzianum* spores, 5 grams of Trianum were mixed with 40 ml of sterile demi water in a 50ml tube. Trianum is not soluble in water, so before placing the inoculum on a plate the tube was well shaken. Then, 100 µL were placed on 60 PDA (Potato Dextrose Agar) plates with the addition of antibiotics (streptomycin sulphate, salt and oxytetracycline hydrochloride to prevent bacterial growth).
The inoculum was allowed to grow for one week and then, a layer of tap water was applied to the plates with the fully grown *T. harzianum*. With a Drigalski spatula, the Trichoderma was scraped off the plate and collected in a bottle. The yield of 50 plates was 1.1 litre approximately, from which the Trichoderma was filtered in order to be counted under the microscope. In this case, 9.7×10^6 spores per mL were harvested (Figure 2.2).

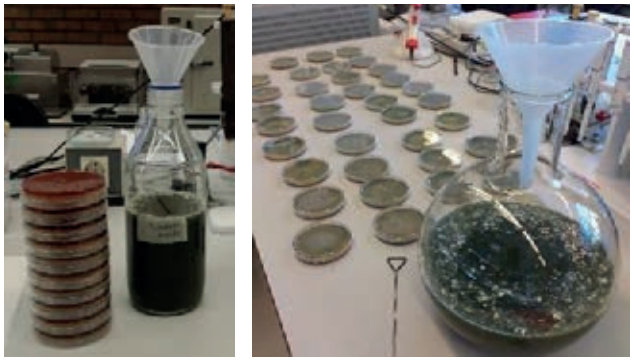


Figure 2.2 *Harvest of Trichoderma harzianum from 50 cultivation plates with Drigalski spatula.*

The treatments were inoculated with 10^5 spores of *T. harzianum* per ml of growing medium where the added suspension was 10 mL per pot (i.e. 10^6 spores/mL). Although only 24 pots were required for inoculation, the dilution was made based on 25 pots in order to have extra inoculum as buffer. To get the correct dilution of the Trichoderma concentration, the calculation was made as follows:

- 25 pots x 100 mL = 2500 mL substrate.
- 10^5 sp/mL × 2500 mL = 2.5×10^8 spores that were required.
- 10 mL volume of inoculum x 25 pots = 250 mL volume of inoculum.
- 2.5×10^8 spores (required) / 9.7×10^6 sp/mL (harvested) = 26 mL concentrated inoculum.
- 26 mL concentrate inoculum + 224 mL tap water = 250 mL diluted inoculum.
- 2.5×10^8 spores (required)/ 250 mL diluted inoculum = 10^6 spores/mL diluted inoculum.

To check whether the dilution was correct, the counting of spores was repeated under a microscope using a counting chamber. Afterwards, 10 mL were taken with a pipette from the diluted inoculum of Trichoderma and poured on the 100 mL substrate pots (10 * 10^6 / 100, which is 10^5 spores per ml substrate). The substrate and the inoculum were mixed with a spoon through the whole volume of the substrate (100 mL) on each pot.

2.3.2 Incubation

Right after the inoculation with *Trichoderma harzianum*, all the treatments were incubated in a climate chamber with 70% relative humidity and 20°C in darkness. The incubation period lasted for 25 days.

2.3.3 Isolation of *Trichoderma harzianum* for DNA analysis

In order to quantify the *Trichoderma harzianum* inoculum survival, samples of 10 mL of substrate were collected from the correspondent pots and then placed in plastic tubes of 15 mL. The samples were collected at 7, 16 & 25 days after the inoculation of the growing media.

On each of the three sessions 16 samples were collected for DNA extraction and qPCR analysis with a total of 48 samples to be analysed in this experiment. In addition to the 48 samples, 2 samples of *Trichoderma* inoculum from the 10^6 spores/mL inoculum solution were also collected for DNA/qPCR analysis for reference purposes. The protocol for the DNA extraction and qPCR are described in the Annex 1 and Annex 2 respectively.

2.4 Experiment 2: Disease suppression by *Trichoderma harzianum* on Strawberry plants using Biochar as carrier

This experiment used a strawberry pot cultivation system (variety Elsanta) to identify if the addition of biochar to the substrate would promote plant growth and/or disease suppression. More specific, the establishment of biochar as carrier of beneficial microorganisms like *Trichoderma harzianum* might contribute to the strawberry plant resilience against *Phytophthora cactorum* (Pc) infection.

This experiment consisted of ten treatments that compared substrates (4 growing media; peat and the three mixes of biochar with peat at different ratios), all with *Trichoderma harzianum* (added/not added) and these treatments repeated with addition of *Phytophthora cactorum* (added/not added). As the treatments with *Phytophthora* could infect nearby containers with the disease via the drain water, the treatments with *Phytophthora* were put in a separate greenhouse compartment with an independent drain water collection system. The plants with only *Trichoderma* were in another greenhouse compartment. Each compartment had three $4m^2$ ebb and flood tables. Control pots without *P. cactorum inoculum* were added to the greenhouse compartment with *Phytophthora* treatments in order to check for cross contamination (Table 2.3).

Each treatment consisted of six replicates with seven pots as sampling units with a total of 42 pots per treatment. Therefore, a total of 420 pots were distribute as described in Table 2.3 and Annex 3.

Table 2.3

Description of the treatments for the experiment 2.

Treat. Code	Microorganism	Growing media	DNA analysis
P nTh nPc	*Trichoderma harzianum*	Peat	15 & 27 days after Trichoderma inoculation
P yTh nPc		Peat+ *T. harzianum*	
B10 yTh nPc		10/90% v/v biochar/peat + *T. harzianum*	
B20 yTh nPc		20/80% v/v biochar/peat + *T. harzianum*	
B30 yTh nPc		30/70% v/v biochar/peat + *T. harzianum*	
P nTh yPc	*Trichoderma harzianum* and *Phytophthora cactorum*	Peat + *P. cactorum*	
P yTh yPc		Peat+ *T. harzianum* + *P. cactorum*	
B10 yTh yPc		10/90% v/v biochar/peat + *T. harzianum* + *P. cactorum*	
B20 yTh yPc		20/80% v/v biochar/peat + *T. harzianum* + *P. cactorum*	
B30 yTh yPc		30/70% v/v biochar/peat + *T. harzianum* + *P. cactorum*	
Cont. yTh nPc		P and B10 growing media + *T. harzianum*	

* 5 (type of growing media) x 2 (yes/no disease infection) x 6 (replicates) x 7 (sampling units) = 420 pots + 7 control pots

** Although the two series of DNA analysis were performed after the *T. harzianum* inoculation, the first analysis was performed before the *P. cactorum* infection while the second analysis was later.

In order to avoid cross contamination, the treatments with and without disease infection were separated in two greenhouses following a randomized distribution (see Annex 3). Strawberry plants were planted on pots of 2 litres after a cooling period and then distributed on 6 tables with a total area of 35m^2. Then, they were allowed to grow for 3 weeks before induce the disease infection with *P. cactorum*. The total growing period of the strawberry cultivar was from the 18th February until the third week of May, where the flowering and first harvest was expected.

2.4.1 Preparation of the rooting media

The rooting media for this second experiment were mixed from biochar (produced by ECN from 100% coniferous wood) and white peat (non-fertilized nor limed). As same as in the first experiment, the biochar for this experiment was not neutralized as the results from the titration test showed a pH buffering capacity of 48.5 mmol of acid per kg of dry matter with an initial value of pH 6.0.

Four mixtures of growing media were prepared for this experiment. On each mixture, the pH, EC and the total volume of peat and/or biochar were taken into account in order to have the same values after the liming of the mixtures with a target value of pH 5.5. Table 2.4 shows the calculations for the peat and biochar volumes, as well as the total amount of $CaCO_3$ and water that was applied for each treatment.

Table 2.4

Calculation for the preparations of the mixtures of growing media.

Mixtures of growing media	No. pots	Volume/substrate (litre)			substrate (Kg)		Initial pH	Initial EC (µS/cm)	$CaCO_3$ (Kg)	water (lit)	water/ peat (% v/v)
		Peat	Biochar	Total	Peat	Biochar					
Peat	168.0	336.0		336.0	73.4		4.2	110	1.2	113.3	33.7
10/90% v/v biochar/peat	84.0	151.2	16.8	168.0	33.0	3.7	4.4	88	0.5	42.0	25.0
20/80% v/v biochar/peat	84.0	134.4	33.6	168.0	29.4	7.4	4.6	67.2	0.4	33.7	20.0
30/70% v/v biochar/peat	84.0	117.6	50.4	168.0	25.7	11.1	4.6	59.5	0.4	23.5	14.0
Total	420.0	739.2	100.8	840.0	161.5	22.2					
* Density of peat		0.2185	kg/L								
**Density of biochar		0.2201	kg/L								

The pH and EC were measured in a 1:1.5 solution extraction. To calculate the amount of kilograms of substrate to be used for each mixture, the bulk density of the peat and the biochar were measured. The addition of $CaCO_3$ was calculated following the ratio of 3.5 gr/L.

Figure 2.3 *Preparations of the growing media mixtures.*

A big scale and big bins were used for the weight and preparation of the mixtures. Subsequently, each mixture was mixed using a concrete mixer and was placed in a big container to allowed an homogenous distribution and mix of its components (peat, biochar, $CaCO_3$ and water) (Figure 2.3).

When all the mixtures were prepared, pots of 2 litres were filled according to the correspondent treatment on weight base. Meanwhile, one plant of strawberry (variety Elsanta) was planted on each pot and thereafter distributed in the greenhouse according to the planned setup (Figure 2.4).

Figure 2.4 *Planting and distribution of the treatments in the greenhouses.*

The planting date was on the 26th of March and 2 days later the inoculation of *Trichoderma harzianum* was done. The infection of *Phytophthora cactorum* was carried out 3 weeks after planting.

2.4.2 Preparation and inoculation *Trichoderma harzianum* and *Phytophthora cactorum*

The preparation and inoculation of *Trichoderma harzianum* followed the same concentration (10^5 spores/mL of growing medium), method and procedure of the experiment 1, but with a difference in the total amount of substrate to be inoculated and the volume of diluted inoculum per pot. While in experiment 1 a total of 2.5 litres of substrate were inoculated with 10 ml of diluted inoculum per pot, in the experiment 2 the inoculation was used for a surplus of substrate later used to fill 336 pots with 2.0 L per container, calculated as:

- The harvest of *T. harzianum* was $9.5*10^7$ spores/mL undiluted inoculum.
- In total $8.4*10^{10}$ spores were needed for 21 L diluted inoculum, because 420 pots (surplus) * 2000 mL = 840 000 mL substrate, for which in total $8.4*10^{10}$ spores (840 000 mL* 10^5 spores/mL) were required in 21 L (420 pots * 50 mL diluted inoculum = 21 L).
- The undiluted inoculum contained $9.5*10^7$ spores/mL (harvest), so $8.4*10^{10}$ / $9.5*10^7$ = 884 mL of undiluted inoculum was needed, which was supplemented with tap water to 21 L in total to create the diluted inoculum of $4*10^6$ spores/mL.
- Each pot received 50 mL of diluted inoculum, this means $50*4*10^6$ spores per 2.0 L, which is about $1*10^8$ spores per litre growing medium or $1*10^5$ spores per mL growing medium.

The preparation of *Phytophthora cactorum* was made from a dormant strain that WUR (Business unit of Greenhouse Horticulture) keeps stored. In order to activate and grow the *P. cactorum*, the initial colony was place on 480 V8 (tomato agar without antibiotics) plates during 2 weeks at 25°C. Then, the content of the of the plates were harvested with a Drigalski spatula and mixed with a blender during 10 seconds (Figure 2.5).

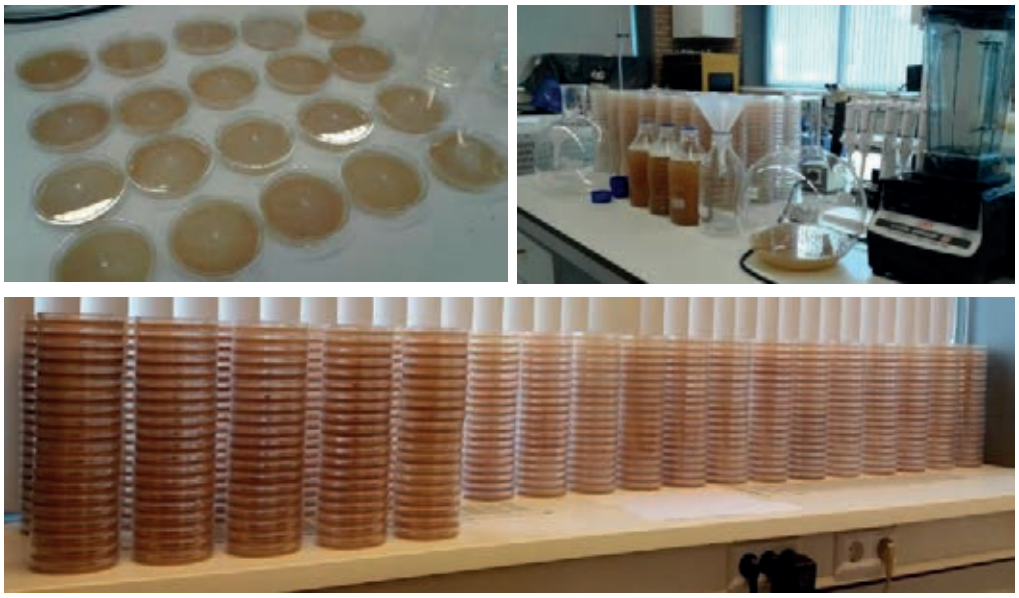


Figure 2.5 *Activation and harvest of Phytophthora cactorum from 500 cultivation plates.*

The suspension was placed in the fridge at 4-6 °C during 1.5 hours and then, the spores were counted with a counting chamber (Fuchs-Rosenthal). Based on the number of the spores, a dilution was made with tap water in order to achieve the target concentration (10^5 spores/mL of substrate of *P. cactorum*). The yield was 2.4 x 10^8 spores per mL. To get the correct dilution of the *P. cactorum*, a calculation was made as follows:

- 250 pots (more than required) x 2000 mL = 500,000 mL substrate.
- 10^5 sp/mL × 500,000 mL = 5 × 10^{10} spores that were required.
- 50 mL volume of inoculum x 250 pots = 12,500 mL --> 12,5 L volume of inoculum.
- 5 × 10^{10} spores (required) / (12.5 L) = 4x10^9 spores per litr inoculum.
- It was decided to transport batches of 5 L: i.e. 5 * 4 x10^9 = 2 x 10^{10} spores per 5L.
- 2 × 10^{10} sp / 2,4 x 10^8 spores/mL (harvested) = 85 mL concentrated inoculum for a 5L batch.
- 85 mL concentrate inoculum + 4915 mL tap water = 5000 mL diluted inoculum.
- The diluted inoculum contains 2 x 10^{10} / 5000 ml = 4 x 10^6 spores/mL.
- 50mL of this dilution per 2L container means (4x10^6 x50)/2 = 10^8 spores per L i.e. 10^5 spores per mL growing medium.

At the end, each pot of strawberry received 50 mL of *P. cactorum* inoculum over the crown of the plant.

2.4.3 Water management

After the sowing of the strawberry plants, the EC and nutrient management was the same for all the treatments following the standard nutrient solution explained. The selected nutrient solution was for an open drain system of strawberry pot plants (Table 2.5).

Table 2.5
Strawberry nutrient solution for an open drain system.

EC	pH	NH4	K	Ca	Mg	NO_3	SO_4	H_2PO_4	Fe	Mn	Zn	B	Cu	Mo
dS/cm	-lgH+	mmol/l							µmol/l					
1.5	5.0	1.2	7.4	4.36	1.68	15.3	2.0	1.33	20	10	7	12	0.75	0.5

Taking into account that each treatment has a different need of irrigation due to the effect of biochar on the water retention, 15 pots (5 mixtures x 3 tables) were selected for the recording of water uptake. Every week this measurement was recorded on the greenhouse where the infection of *P. cactorum* was not executed in order to avoid cross contamination between plants.

The water uptake was calculated from the difference of the pot weight (before and after an irrigation cycle), and the drip collection container of each greenhouse. The fertigation volume was adapted to maintain around 40% -v/v of water in the pots by quantifying the water supply per irrigation cycle on each week. The differences of water retention between treatments were corrected with extra addition of water using a hand sprinkler.

The nutrients were monitored every two weeks by the 1:1.5 nutrient extraction analysis for each of the prepared mixtures (peat, peat+ Th, 10/90% -v/v, 10/90% -v/v, and 10/90% -v/v biochar/peat).

2.4.4 Disease incidence determination and plant growth

After the Phytophthora inoculation the incidence of the disease was recorded 2 times per week by visual observation of wilting and/or stunting of the plants. Whenever a plant showed those symptoms, a cut in the crown of the plant was done in order to confirm the presence of Phytophthora (Figure 2.6).

Figure 2.6 *Disease incidence determination. An example of a wilted plant and a completely damaged crown.*

After two months of cultivation 2 of the 7 plants per row were taken out in order to create more space. Of these 5 plants (new sampling unit) all fruits were harvested weekly. At the end of the growing period, 3 plants in the middle of the row per replicate were selected, their root crown was scored for Phytophthora presence and the fresh and dry biomass mass of the above ground tissues were collected.

2.4.5 Isolation of *Trichoderma harzianum* for DNA analysis

The isolation of *T. harzianum* for DNA and qPCR was done in two sessions for the second experiment. The samples for the first session were collected 2 weeks after the inoculation of *T. harzianum*, and for the second session the samples were collected 1 week after the disease infection with *P. cactorum* (or 4 weeks after the inoculation of *T. harzianum*). The samples for DNA and qPCR analysis were collected for 6 treatments and 4 replicates, with a total of 24 samples per session (48 samples in total for this experiment). The replicates corresponded to the tables 1- 2 (greenhouse without disease infection), and 4-5 (greenhouse with disease infection) of the experimental setup (Annex 3). The selected treatments for DNA analysis were: Peat; Peat+Th; Mix (20/80%-v/v) Biochar/Peat+Th; Peat+Pc; Peat+Th+Pc; Mix (20/80%-v/v) Biochar/Peat+Th+Pc. Each sample was collected from 3 pots (randomly selected from each line of 7 sampling units) in a tube of 50 mL. The surrounded rhizosphere and substrate were collected from the middle height of each pot with a spoon. After the collection of the sample, the 50 mL tubes were labelled and then processed for DNA extraction. The protocol for the DNA extraction and qPCR are described in the Annex 1 and Annex 2 respectively.

3 Results

3.1 Biochar evaluation

Table 3.1
Overview of basic parameters for the treatments.

	units	mix 10 % biochar	mix 20 % biochar	mix 30 % biochar	100 % biochar
		Mix 10% biochar	Mix 20% Biochar	Mix 30% Biochar	Mix 100% Biochar
WHC	% v/v	87	87	86	45
DBD	kg/m^3	118	124	114	138
OM	% w/w	95	96	96	99
MM	% w/w	5	4	4	1
OUR	mmol/kg/h				1.15**
Phytotox					n.s.d *
PH-buffer	mmol H^+/kg				48.5

* no significant difference detected compared to a standard

** more stable than the peat reference at 2.7 mmol/kg/h

The data in Table 3.1 show that biochar has a water holding capacity which is almost half that of regular peat (data not shown, 80-90%-v/v). However, this property does not influence the water holding capacity of mixtures with biochar up to 30%-v/v. The reason is that pure biochar has a lot of macro pores in between the material which reduce the water holding capacity. Once in a mix with finer peat material, the macro pores are filled with peat and the water holding capacity is very much like peat. A slight reduction in water holding capacity is visible in the 30%-v/v biochar mix. Of course when using a finer or coarser biochar, these properties might be different. In parallel the density is decreasing with increasing biochar content. Organic matter and mineral matter are as expected. The degradability as measured with the OUR method (*CEN 16087-1, 2011*) shows the material is slightly more stable than most peats, which are about 1.5 - 2.

3.2 Biochar nutrient analysis

Table 3.2
Overview of biochar nutrient analysis.

		Mix 10% biochar	Mix 20% Biochar	Mix 30% Biochar	Reference100% Peat
pH		5.4	5.7	5.9	5.5
EC	$dS.m^{-1}$	0.49	0.27	0.30	0.37
NH4	$mmol.L^{-1}$	0.5	0.3	0.3	0.5
K	$mmol.L^{-1}$	1.3	0.9	1.2	0.6
Na	$mmol.L^{-1}$	0.4	0.3	0.3	0.3
Ca	$mmol.L^{-1}$	0.5	0.2	0.2	0.4
Mg	$mmol.L^{-1}$	0.6	0.2	0.2	0.5
Si	$mmol.L^{-1}$	0.1	0.1	0.1	0.1
NO3	$mmol.L^{-1}$	1.8	0.5	0.6	1.4
Cl	$mmol.L^{-1}$	0.3	0.2	0.2	0.3
SO4	$mmol.L^{-1}$	0.6	0.5	0.5	0.3
HCO3	$mmol.L^{-1}$	0.2	0.1	0.3	0.3
P	$mmol.L^{-1}$	0.25	0.2	0.25	0.2
Fe	$\mu mol.L^{-1}$	4.8	5.1	4.9	3.9
Mn	$\mu mol.L^{-1}$	0.5	0.2	0.2	0.6
Zn	$\mu mol.L^{-1}$	0.7	0.5	1	0.5
B	$\mu mol.L^{-1}$	4	4	4	4
Cu	$\mu mol.L^{-1}$	0.1	0.2	0.1	0.1
Mo	$\mu mol.L^{-1}$	0.1	0.1	0.1	0.1

Table 3.2 shows the pH is increased by the added biochar but not beyond acceptable limits. The other elements are not influenced by the biochar addition.

3.3 Yield data

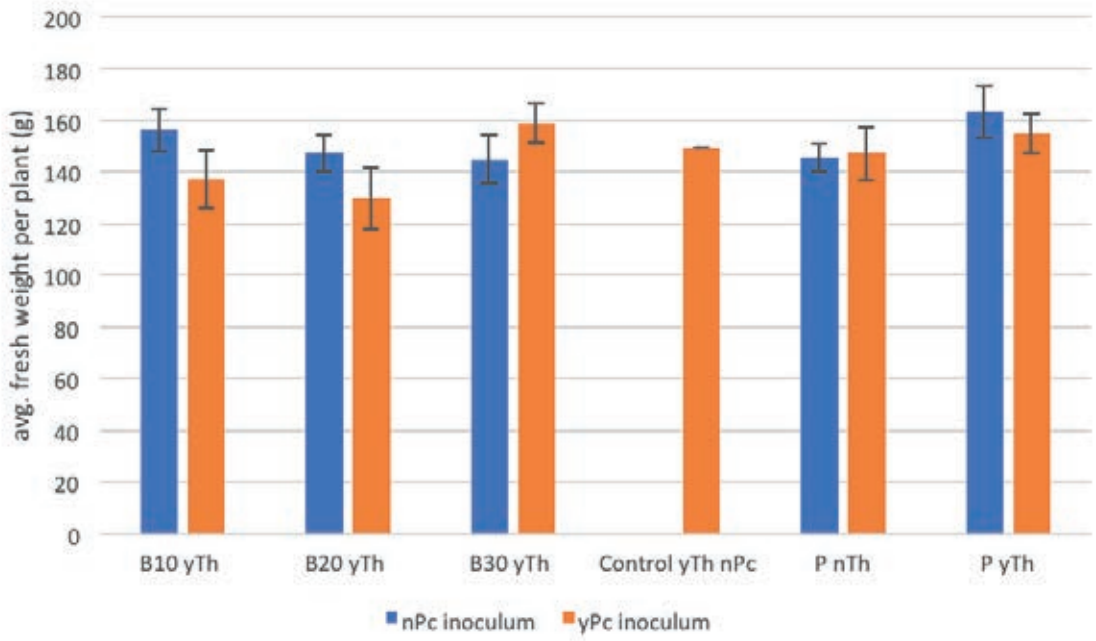


Figure 3.1 *Overview of yield in gram of fresh weight per plant for the treatments with biochar levels, and simultaneous application of Trichoderma harzianum or Phytophthora. P = 100% peat. B20 = 20% biochar/80% peat. nTh = no Trichoderma harzianum inoculum. yTh = yes Trichoderma harzianum inoculum. nPc = no Phytophthora cactorum inoculum. yPc = yes Phytophthora cactorum inoculum*

Figure 3.1 shows no remarkable effects of biochar addition, Trichoderma harzianum or Phytophthora on fresh weight.

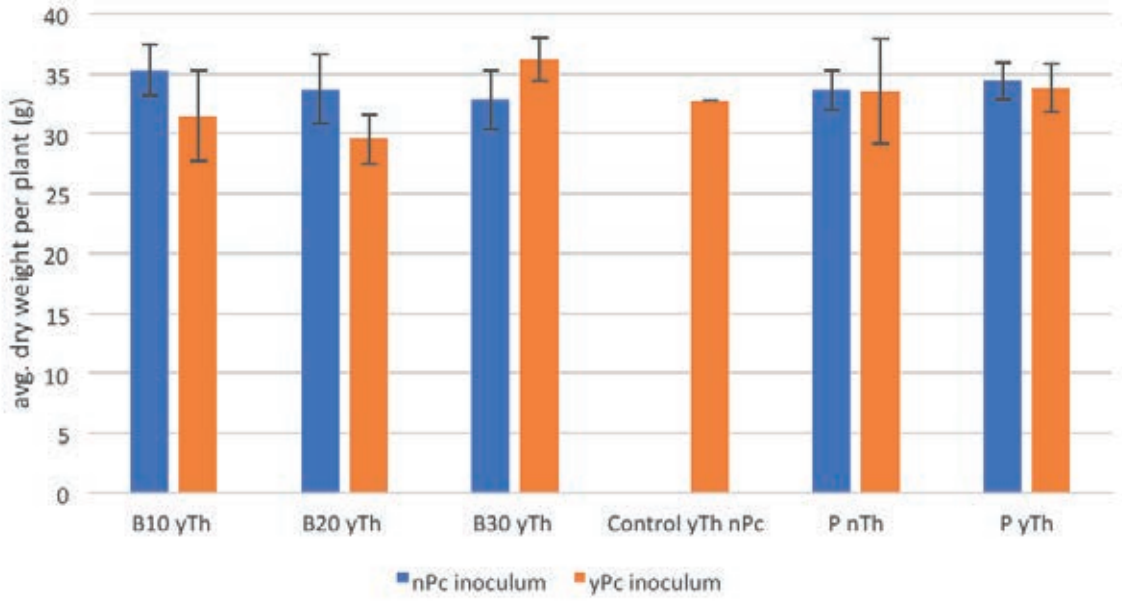


Figure 3.2 *Overview of yield in gram of dry weight per plant for the treatments with biochar levels, and simultaneous application of Trichoderma harzianum or Phytophthora. P = 100% peat. B20 = 20% biochar/80% peat. nTh = no Trichoderma harzianum inoculum. yTh = yes Trichoderma harzianum inoculum. nPc = no Phytophthora cactorum inoculum. yPc = yes Phytophthora cactorum inoculum*

Figure 3.2 shows no remarkable effects of biochar addition, Trichoderma harzianum or Phytophthora on dry weight.

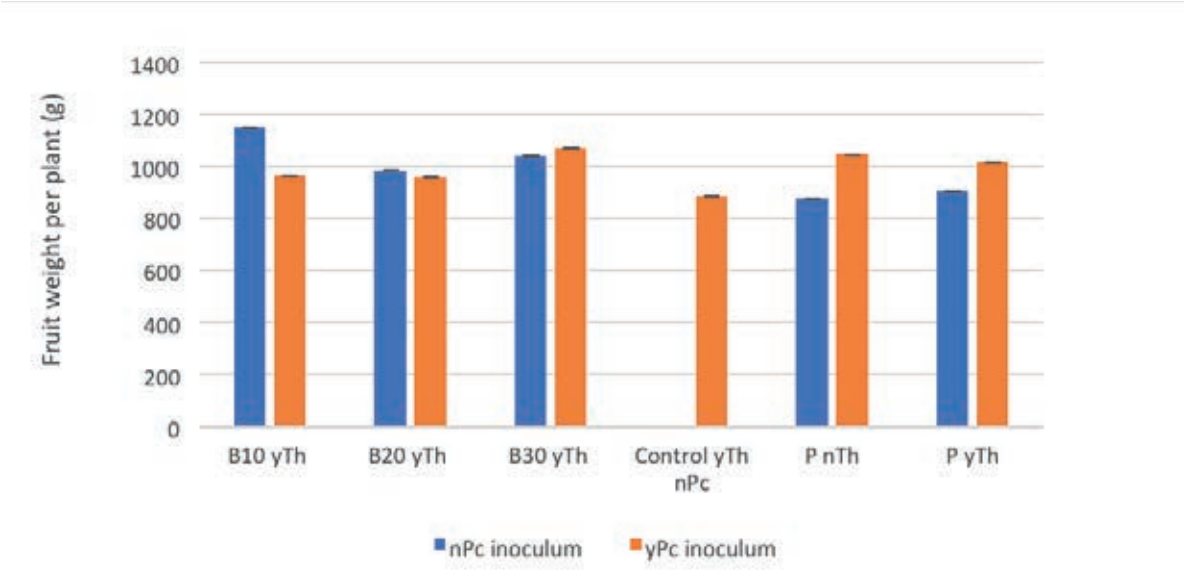


Figure 3.3 *Overview of yield in gram of fresh fruit per plant for the treatments with biochar levels, and simultaneous application of Trichoderma harzianum or Phytophthora. P = 100% peat. B20 = 20% biochar/80% peat. nTh = no Trichoderma harzianum inoculum. yTh = yes Trichoderma harzianum inoculum. nPc = no Phytophthora cactorum inoculum. yPc = yes Phytophthora cactorum inoculum*

Figure 3.3 shows a small positive effect of biochar on fruit yield no effect of Trichoderma harzianum and a small positive effect of Phytophthora cactorum on fruit yield.

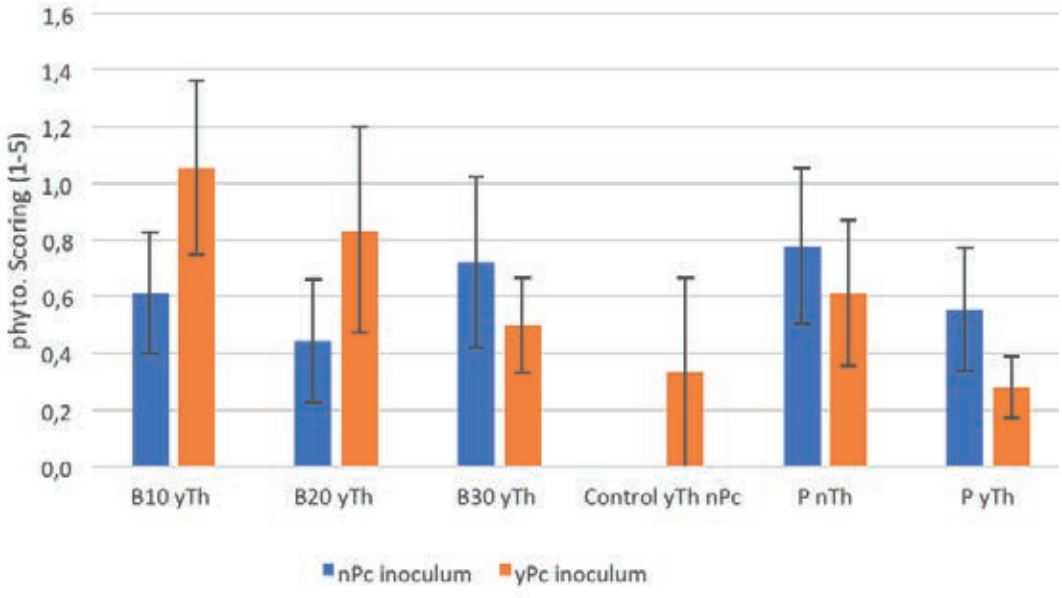


Figure 3.4 *Overview of Phytophthora severity score per plant for the treatments with biochar levels, and simultaneous application of Trichoderma harzianum or Phytophthora. P = 100% peat. B20 = 20% biochar/80% peat. nTh = no Trichoderma harzianum inoculum. yTh = yes Trichoderma harzianum inoculum. nPc = no Phytophthora cactorum inoculum. yPc = yes Phytophthora cactorum inoculum*

Figure 3.4 shows a decrease of Phytophthora with an increase in Trichoderma treated biochar. As the variation in outcomes is really big, these data are not statistically different. The score in Figure 3.4 is based on the score system in Table 3.3. This system is explained here rather than in the "Methods" section as the score was developed on the plant material in this experiment.

Table 3.3

Scoring table of Phytophthora cactorum presence with a 0-5 range.

0	1	2	3	4	5
White/yellow inside crown	1 spot or multiple small spots visible (d=1mm) inside the crown	Clearly damaged spot, but not further expanded, d < 4 mm	Clearly damaged, larger spot, clearly red, top of the crown is still clearly white/yellow. Could be partly cork/wood	Almost completely damaged crown, red. Partly cork/wood. Top of the crown still a small edge white/yellow	Completely damaged inside crown, clearly cork/wood. Completely red, or if white is present then enclosed by red cork/wood

The qPCR data proved very variable and comparisons between treatments were difficult. Therefore the results were expressed in a percentage of the average for the total of one experiment. This means the averages used for experiment 1 and 2 are different and the percentages may not be used to compare experiment 1 and 2! Experiment 1, without plants, shows that the level of Trichoderma incidence is the same for peat with (PyThnPc) and without addition of Trichoderma (PnThnPc), i.e. the natural level of Trichoderma was as high as the addition of Trichoderma. The addition of biochar seems to reduce the incidence of Trichoderma. In all cases the level of Trichoderma decreases over time i.e. the Trichoderma is present in the substrate prior to growing but dies off during storage (moisture content = xx; T= xx; oxygen supply is ample).

Table 3.4

qPCR (results: one value (average total all treatments per experiment) for calculation %) experiment 1.

Date	P nTh nPc	P yTh nPc	B20 nTh nPc	B20 yTh nPc
12-mrt	475%	127%	65%	2%
26-mrt	23%	281%	105%	1%
1-apr	9%	112%	0%	0%

P	100% peat
B20	20% biochar/80% peat
nTh	no Trichoderma harzianum inoculum
yTh	yes Trichoderma harzianum inoculum
nPc	no Phytophthora cactorum inoculum
yPc	yes Phytophthora cactorum inoculum

In experiment 2, with plants, the addition of Trichoderma slightly increases the level of Trichoderma but again in all treatments the Trichoderma dies off. The presence of Phytophthora does not seem to interact with Trichoderma.

Table 3.5

Experiment 2, in greenhouse qPCR (results: one value (average total all treatments per experiment) for calculation %).

Date	P nTh nPc	P nTh yPc	P yTh nPc	P yTh yPc	B20 yTh nPc	B20 yTh yPc
12-mrt	1.4%	0.8%	11.3%	1150.3%	24.2%	4.5%
26-mrt	0.0%	0.1%	1.2%	2.2%	2.8%	1.4%

P	100% peat
B20	20% biochar/80% peat
nTh	no Trichoderma harzianum inoculum
yTh	yes Trichoderma harzianum inoculum
nPc	no Phytophthora cactorum inoculum
yPc	yes Phytophthora cactorum inoculum

When samples were sent to the laboratory based on the visual scores as presented in Table 3.5, it became clear that the addition of Phytophthora did correlate with the symptoms found (Table 3.6).

Table 3.6

DNA check for presence of Phytophthora ssp. (Ph spp.), Pythium spp. (Py spp.), Cylindrocarpon destructans (Cd), Rhizoctonia solani (Rs), Colletotrichum spp. (C spp.), Verticillium alboatrum (Va) and Verticillium dahiae (Vd) on a selection of samples based on their visual Phytophthora cactorum scoring (Pc scoring). Treatment: no (n), yes (y), Trichoderma Harzianum inoculation(Th), Phytophthora cactorum inoculation (Pc), substrate of 100% peat (P) or mix % biochar/peat: 10/90 (B10), 20/80 (B20), 30/70 (B30). DNA check: close to zero (-), weak (-/+), moderate (+).

Treatment	Pc Scoring	Ph spp.	Py spp.	Cd	Rs	C spp.	Va	Vd
Control yTh nPc	0	-	-	+/-	-	-	-	-
P nTh yPc	0	-	-	+/-	-	-	-	-
B30 yTh yPc	0	-	-	-	+/-	-	-	-
Control yTh nPc	1	-	-	-	-	-	-	-
P nTh nPc	1	-	-	+/-	-	-	-	-
P nTh nPc	1	-	-	+/-	-	-	-	-
B30 yTh yPc	1	-	-	+/-	-	-	-	-
B10 yTh nPc	2	-	-	+	-	-	-	-
B30 yTh yPc	2	+	-	-	+/-	-	-	-
B10 yTh yPc	3	+	+/-	+/-	+	-	-	-
B20 yTh yPc	3	+	-	+/-	+/-	-	-	-
B10 yTh yPc	4	+	-	+	+	-	-	-
P nTh yPc	4	+	+/-	+	-	-	-	-
B20 yTh yPc	5	+	-	-	+/-	-	-	-

P	100% peat
B20	20% biochar/80% peat
nTh	no Trichoderma harzianum inoculum
yTh	yes Trichoderma harzianum inoculum
nPc	no Phytophthora cactorum inoculum
yPc	yes Phytophthora cactorum inoculum

4 Discussion and conclusions

The biochar low bulk density and high water holding capacity ensured that differences with the peat used were small. As, on the one hand, the pure biochar is drier in nature than the peat used, but on the other hand the peat fills many of the larger pores of the biochar, the mixes were marginally drier in nature. The biochar used was not phytotoxic at all. The biochar was very stable according to the OUR method (*CEN 16086-2, 2011*), slightly more so than peat. This means at least in principle, biochar is perhaps not able to support more micro-organisms than peat. It therefore may be wise to add both, mineral and organic feed for the microorganisms to the biochar, possibly well before mixing with other potting soil constituents. An additional indication biochar is to stable to support much microbial activity is the observation by ECN that in peroxide only 10%-w/w biochar is lost while peat is totally destructed by the peroxide.

The buffer capacity of the biochar is such that it can neutralise peat in a quantity of 50% to 65% of its own volume based on the figures alone. This worked well although the pH-buffer method, used to measure the acid neutralisation by biochar, somewhat underestimated the amount of alkalinity in biochar. For practical purposes this means that the highest dose for this combination with peat was at least 30% biochar. To safely use biochar, it must be either neutralised or (as we did) mixed with a moderately acid peat. As some acidity was remaining in the mixes, especially when less biochar was used, the remaining acidity was neutralised by adding lime.

The fresh weight and dry weight of above ground parts excluding fruits of strawberries (i.e. leaves and stems) did not show any differences between the treatments, i.e. weight was not influenced by the choice of substrate, addition of Trichoderma or addition of Phytophthora.

The fresh weight of fruits harvested did show a small positive effect of the addition of biochar to the mixes. This could very well be the result of the slightly drier nature of the mixes with biochar. Drier materials tend to be a generative impulse to many plants. Furthermore, from strawberry practise it is known that coir or coir peat mixes are also drier and exercise a generative impulse on strawberry. In this case the drier nature was an advantage but in practise a grower might grow on pure peat by anticipating the more vegetative growth with an adapted irrigation or fertigation schedules.

The Trichoderma did not react to any part of the treatment as far as we noticed. Furthermore, Trichoderma levels for both, added and resident Trichoderma, dropped down quite rapidly with two orders of magnitude. This indicates that Trichoderma is present in the initial potting soil but is not able to compete with the developing microbiome in moistened, fertilised and or cultivated potting soil.

There was no influence of biochar on the establishment of Trichoderma. Biochar did therefore not act as carrier for Trichoderma under the conditions of this experiment. Several sources suggested Trichoderma could be carried on biochar and could survive at higher levels for more than a few weeks (*De Tender et al. 2016; Debode et al. 2018*). It is also suggested to pre-treat biochar with either nutrients, composts or both (*Hagemann et al. 2017; Joseph et al. 2018*). It may therefore be too soon to discard biochar as carrier all together. Better approaches could be to load the biochar with inorganic and organic nutrients for the micro-organisms to be carried and to soak or infiltrate the biochar with the target organisms rather than add organisms to the mix as we did.

The Phytophthora at no point became a threat to the vitality of the strawberry plants. The Phytophthora was however present in the DNA check. This could mean that the plants for some reason or another could withstand the disease or that the Phytophthora strain used was not pathogenic to these strawberry plants. One surprising pattern was the apparent decrease in Phytophthora score with an increase in biochar from 10 to 20 to 30% v/v. The variation was however quite large. A possible explanation is the effect of a drier medium. It is well known Phytophthora, which uses swim spores, is less active in drier media like has been described for Pythium (*Termorshuizen et al. 2006; Van der Gaag and Wever, 2005*).

The variation in quantities measured for Trichoderma and Phytophthora was unacceptable. A preliminary assessment of the methods used by microbiologist so far showed several possibilities to improve the methods to reduce variation:

- To increase the sample volume to a volume larger than the spatial variation in numbers.
- To homogenize the larger sample to allow taking a very small sub sample for the qPCR.
- To define the quantity of reactants necessary on both volume base and weight base to avoid artefacts because of the 10-20 times lower bulk weights of substrate samples.
- To run a reference sample with a known concentration per volume field sample to check on the integrity of the procedures and to avoid or compensate artefacts.

These improvements have been discussed with Pham Khanh, Marta Streminska and Yaite Cuesta Arena and resulted in a preliminary improved method (Annex 3). This method remains to be validated before we can conclude the problems with quantification via qPCR methods are solved.

In conclusion:

- The experiments showed the biochar used can be mixed with peat to create safe and convincing potting soil mixes, suitable for the growth of strawberries at equal green mass growth. Biochar can be used in quantities of at least 30%v/v.
- In the experiments performed, the potting soils mixtures with biochar resulted in fruit production of strawberry at a level equal to or slightly higher than that for peat without biochar.
- The addition of Trichoderma did not increase the Trichoderma levels with more than one order of magnitude and in all cases the levels dropped off two orders of magnitude in a matter of weeks. It seems Trichoderma is not able to compete with the species resulting from fertigating the potting soils (with or without plants). It is quite possible Trichoderma is very sensitive to the water content (or air content) of the mixes as potting soils are stored at moisture contents of 20-30%v/v and used at moisture contents of 50-70%v/v.
- Biochar did not function as carrier for the Trichoderma but there is enough evidence in literature and practise not to abandon this path right away.
- The Phytophthora added proved to be present but not active. In this case the chances are the rooting media were not wet enough to allow Phytophthora to become pathogenic. If that was the case, the moisture level for pathogenicity would be over 70%v/v.
- The qPCR measurements showed large variations believed to be manageable by applying an improved protocol (Annex 3). It is advised to validate the improved protocol as soon as possible.

References

Blok, C., Salm, C.v.d., Hofland-Zijlstra, J., Streminska, M., Eveleens, B., Regelink, I., Fryda, L., and Visser, R., 2017.

Biochar for Horticultural Rooting Media Improvement: Evaluation of Biochar from Gasification and Slow Pyrolysis. Agronomy 7.

CEN 13037, 2011.

Soil Improvers and Growing Media—Determination of pH.

CEN 13038, 2011.

Soil Improvers and Growing Media—Determination of Electrical Conductivity.

CEN 13039, 2011.

Soil Improvers and Growing Media—Determination of Organic Matter Content and Ash.

CEN 13040, 2007.

Soil Improvers and Growing Media—Sample Preparation for Chemical and Physical Tests, Determination of Dry Matter Content, Moisture Content and Laboratory Compacted Bulk Density.

CEN 13041, 2011.

Soil Improvers and Growing Media—Determination of Physical Properties—Dry Bulk Density, Air Volume, Water Volume, Shrinkage Value and Total Pore Space.

CEN 13652, 2001.

Soil Improvers and Growing Media—Extraction of Water Soluble Nutrients and Elements.

CEN 16086-2, 2011.

Soil Improvers and Growing Media—Determination of Plant Response—Part 2: Petri Dish Test Using Cress.

CEN 16087-1, 2011.

Soil Improvers and Growing Media—Determination of the Aerobic Biological Activity—Part 1: Oxygen Uptake Rate (OUR).

De Pascale, S., Rouphael, Y., and Colla, G., 2018.

Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. Eur.J.Hortic.Sci. 82:277-285.

Debode, J., De Tender, C., Cremelie, P., Lee, A.S., Kyndt, T., Muylle, H., De Swaef, T., and Vandecasteele, B., 2018.

Trichoderma-Inoculated Miscanthus Straw Can Replace Peat in Strawberry Cultivation, with Beneficial Effects on Disease Control. Frontiers in Plant Science 9.

De Tender, C.A., Debode, J., Vandecasteele, B., D'Hose, T., Cremelie, P., Haegeman, A., Ruttink, T., Dawyndt, P., and Maes, M. (2016).

Biological, physicochemical and plant health responses in lettuce and strawberry in soil or peat amended with biochar. Applied Soil Ecology 107, 1-12.

Fryda, L. and Visser, R., 2015.

Biochar for Soil Improvement: Evaluation of Biochar from Gasification and Slow Pyrolysis. Agriculture 5:1076-1115.

Frenkel, O., Jaiswal, A.K., Elad, Y., Lew, B., Kammann, C., and Grabrer, E.R. 2017.

The effect of biochar on plnat diseased: What should we learn while designing biochar substrates?. Journal of Environmental engineering and landscape management. 25:105-113.

Hagemann, N., Joseph, S., Schmidt, H.-P., Kammann, C.I., Harter, J., Borch, T., Young, R.B., Varga, K., Taherymoosavi, S., Elliott, K.W., et al. (2017).

Organic coating on biochar explains its nutrient retention and stimulation of soil fertility. Nature Communications 8, 1089.retention and release. The Science of the total environment 618, 1210-1223.

Harries, D.C. and Stickels, J.E., 1981.

Crown rot (Phytophthora cactorum) in glasshouse-grown strawberries at East Malling Research Station. Pl. Path. 30: 205-212.

Joseph, S., Kammann, C.I., Shepherd, J.G., Conte, P., Schmidt, H.P., Hagemann, N., Rich, A.M., Marjo, C.E., Allen, J., Munroe, P., et al. (2018).

Microstructural and associated chemical changes during the composting of a high temperature biochar: Mechanisms for nitrate, phosphate and other nutrient

Porras, M., Barrau, C., Arroyo, F.T., Santos, B., Blanco, C. and Romero, F., 2007.
Reduction of Phytophthora cactorum in Strawberry fields by Trichoderma spp. and soli solarization. Plant Disease. 91:142-146.
Raviv, M., Lieth, J.H., and Bar-Tal, A., 2019.
Soilless Culture: Theory and Practice. Second Edition., Amsterdam, the Netherlands.
Termorshuizen, A.J., van Rijn, E., van der Gaag, D.J., Alabouvette, C., Chen, Y., Lagerlöf, J., Malandrakis, A.A., Paplomatas, E.J., Rämert, B., Ryckeboer, J., Steinberg, C., and Zmora-Nahum, S., 2006.
Suppressiveness of 18 composts against 7 pathosystems: Variability in pathogen response. Soil Biology and Biochemistry 38:2461-2477.
Van der Gaag, D.J. and Wever, G., 2005.
Conductiveness of different soilless growing media to Phytium root and crown rot of cucumbers under near-commercial conditions. Eur. J. Plant Pathology 112:31-41.
Verhagen, J., van den Akker, J., Blok, C., Diemont, H., Joosten, H., Schouten, M., Schrijver, R., Verweij, P., and Wösten, H. 2009.
Climate Change. Scientific Assessment and Policy Analysis. WAB 500102 027. Peatlands and carbon flows. Outlook and importance for the Netherlands. PRI, Wageningen, the Netherlands.
Woods, W.I., Teixeira, W.G., Lehmann, J., Steiner, C., WinklerPrins, A., Rebellato, L., Hofwegen, G., Kuyper, T.W., Hoffland, E., Broek, J.A., and Becx, G.A., 2009.
Opening the Black Box: Deciphering Carbon and Nutrient Flows in Terra Preta, p. 393-409, Amazonian Dark Earths: Wim Sombroek's Vision. Springer Netherlands.European Committee for Standardization, 2011. Soil improvers and growing media - Determination of physical properties - Dry bulk density, air volume, water volume, shrinkage value and total pore space. CEN-EN 13041.
https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1365-3059.1981.tb01258.x?purchase_referrer=onlinelibrary.wiley.com&tracking_action=preview_click&r3_referer=wol&show_checkout=1

Annex 1 DNA extraction with DNeasy® PowerSoil® Kit

The DNA extraction is realised on the test samples. Reference is a series of factor 10 dilutions starting from the initial concentration of the inoculum to a 10^9 dilution. The kit is designed for a certain soil volume and consists of a series of steps in which 6 different solutions are added (Figure 1). First the sample is ground to a pulp, then cells are opened up (lysis). Consequently some interfering substances are separated (inhibitor removal) before all DNA is loaded onto a column and rinsed. Finally the DNA is washed off the column again and ready for a measurement.

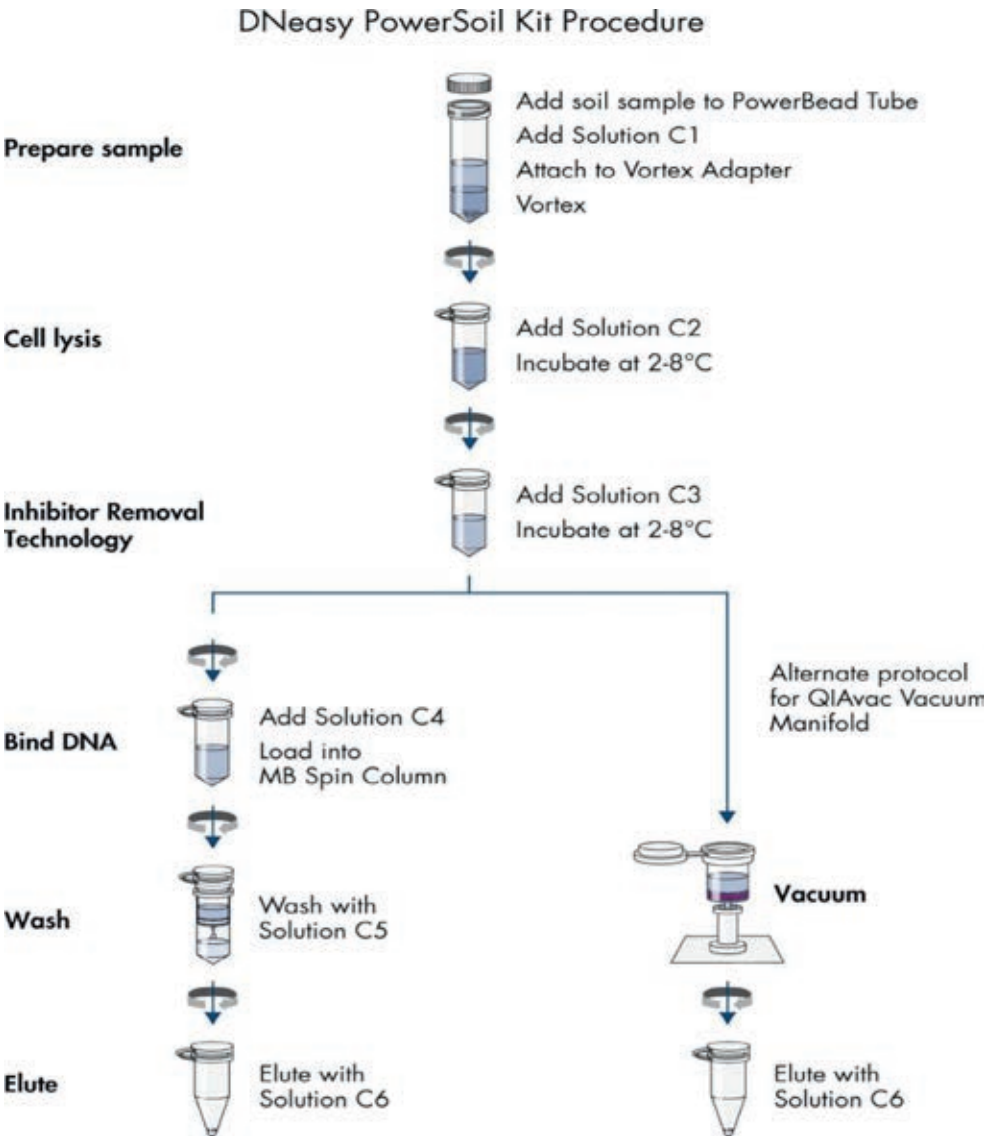


Figure 1 *Overview of the steps to add solutions and harvest all DNA in the sample.*

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.

1. Add 0.25 g of sample to the PowerBead Tube provided, in which there is 700 microL lysibuffer and some dozens of beads to mash up the material inserted Figure 2).
   a. Gently vortex to mix.

Figure 1 *The initial PowerBead Tube with 700 microL of lysibuffer and the beads which will smash up the sample. This only works when the sample volume is smaller than the volume of the lysibuffer.*

2. Add 60 µl of Solution C1 and
   a. Invert several times or vortex briefly.
      Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder.
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x g for 30 s.
6. Transfer the supernatant to a clean 2 ml collection tube.
   Note: Expect between 400–500 µl of supernatant, which may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step.
8. Centrifuge the tubes for 1 min at 10,000 x g.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml collection tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through. Centrifuge again for 1 min at 10,000 x g.
18. Carefully place the MB Spin Column into a clean 2 ml collection tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no. 17000–10).
20. Centrifuge at room temperature for 30 s at 10,000 x g. Discard the MB Spin Column. The DNA is now ready for downstream applications.
    Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Comments by Chris Blok 2019 05 01: the amount of sample is specified by weight (0.25 g). The quantities of additives, especially the solution in the PowerTube and C1 (700 + 60 microL), are related to the volume expected in that particular weight of soil. 0.25 g of soil is about 250 microL so therefore emerged in the solution.
In a typical rooting medium 0.25 g is 2500 microL with a pore space of 2300 microL which is clearly more than the 760 microL added. As the material is not only adsorbent but also absorbent, the C1 will be caught in the internal pores but may remain insufficient to harvest all DNA.
This can be proven by running a series with increasing amount of lysibuffer + C1 up to submerging the sample.

From our Belgian colleagues (Jane Debode):
Voor Trichoderma in substraat gebruiken we meestal nog de klassieke uitplating op TSM medium en voor Phytophthora een loktoets (zie artikel Vercauteren in bijlage).

We gebruiken de DNA kit wel voor algemene microbioom analyses. Voor grond en compost werkt deze kit prima maar voor witveen substraat hebben we gelijkaardige moeilijkheden ondervonden. Dan hebben we meestal minder dan 0.25g genomen (0.15 tot 0.20) en met een steriel tipje het witveen 'ondergeduwd' in de lysisbuffer. We hebben dit nog maar gedaan op een heel beperkt aantal stalen, aangezien we met witveen meestal hebben gewerkt met rhizosfeer ipv bulk en bij rhizosfeer doet het probleem zich niet voor.

Annex 2 qPCR analysis with GoTaq® qPCR Master Mix

The total nucleic acid concentration of the inoculum is determined by spectrometry at 260nm. The DNA is prepared for the qPCR with the GoTaq® qPCR Master Mix which include a nuclease free solution and specific primers for the reproduction of the DNA. The PCR is run according to the method described in Joshi & al. (2006), The solution is incubated at 95°C for 3 min, following 40 cycles of PCR at 95°C for 30 s, 55°C for 30s and 72°C for 15s.

Use of the GoTaq® qPCR Master Mix

The protocol for a 50µl reaction is outlined below. Component volumes may be scaled as appropriate. This protocol assumes that 20% of the reaction volume is DNA template (e.g., 10µl of DNA template added to 40µl of reaction mix). If the volume of DNA template is more or less than 10µl, adjust the volume of Nuclease-Free Water accordingly so that the final reaction volume is 50µl.

Preparing the DNA Template

1. Prepare standards and experimental template dilutions in nuclease-free water.
2. Add 10µl of DNA (or water for no-template control reactions) to the appropriate wells of the reaction plate.

Preparing the qPCR Reaction Mix

Prepare 50 microL reaction mix by combining Sample, GoTaq® qPCR Master Mix, Nuclease-Free Water and primers in the order listed. See Notes 1 and 2.

Component	Volume per 50µl Reaction	Final Concentration
GoTaq® qPCR Master Mix, 2X	25µl	1X
Nuclease-Free Water	to a final volume of 40µl	
upstream and downstream PCR primers	_____µl	0.2µM or 0.05–0.9µM each

***Figure** 1 The preparation of 50 microL runs as follows: 10 microL of sample (not shown in figure) then 25 microL of GoTaq qPCR MasterMix, then 5 microL nuclease free water then some PCR primers of unknown volume.*

Notes

1. See the GoTaq® qPCR Master Mix Technical Manual #TM318 for a list of instruments that require addition of CXR Reference Dye.
2. Some instruments such as the BioRad instruments require addition of a normalization dye (e.g., fluorescein).
3. Carefully pipet 40µl of reaction mix into each reaction well.
   Seal the plate, and centrifuge at low speed for 1 minute.
4. Program the thermal cycler with the desired thermal cycling conditions as per the manufacturer's instructions.
5. Place the plate in thermal cycler, and press "Start". When the run is complete, analyse the data using your usual procedures.

Comment by ChrisBlok 2019 05 02.
What does the procedure finally reports: the absolute quantity of DNA in the well or the concentration in the well as read by the machine?

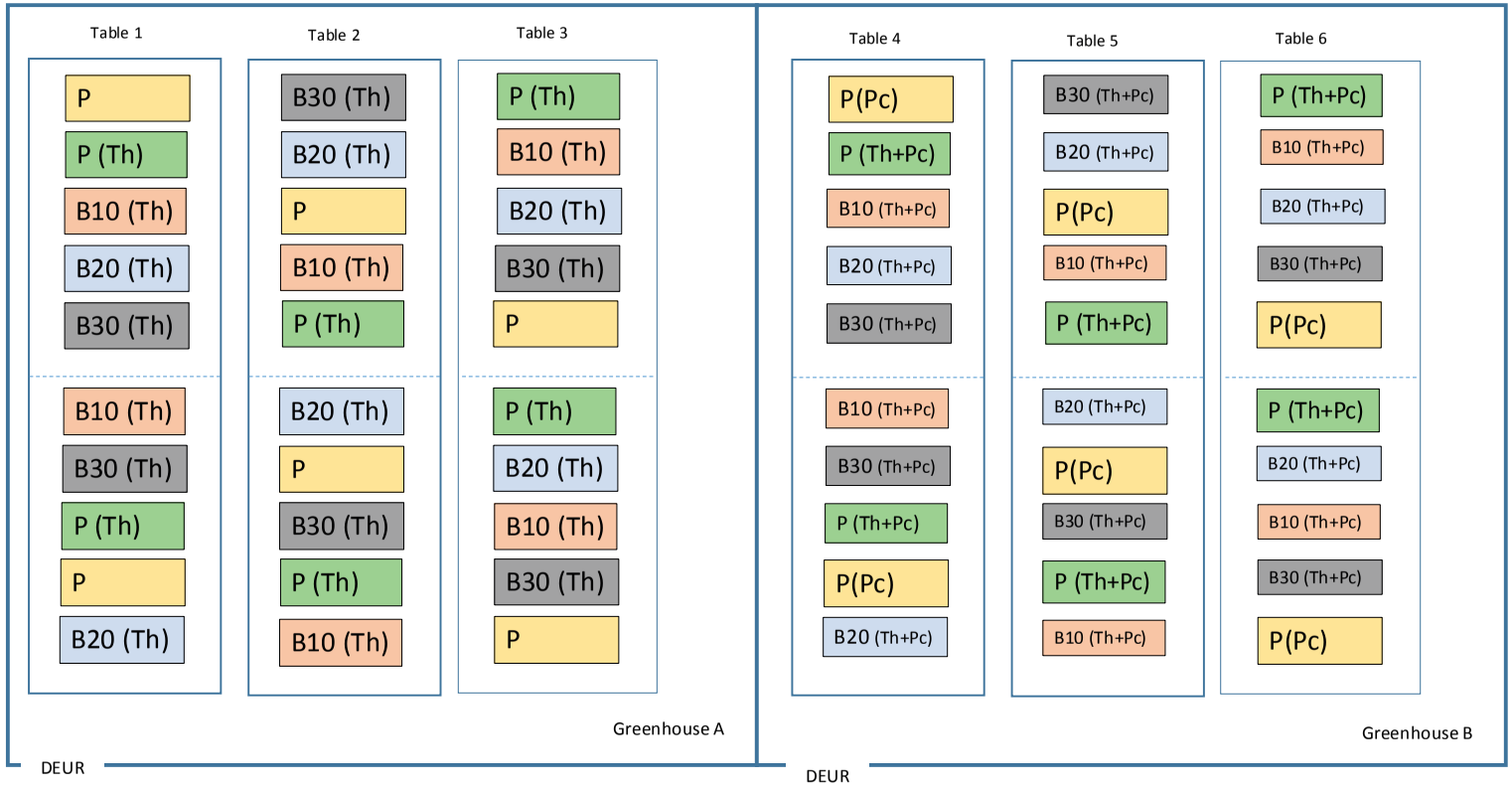
- If absolute quantity, how is this calculated back to quantity per mL substrate?
- If concentration, how is this calculated back to quantity per mL substrate?

As an example suppose the outcome is 20 ng DNA/microL:

- Then there is 50*20 = 100ng in the well (a well is 50 microL).
- In the well there was 10 microL sample taken from 100 microL monster (yield of the DNA extraction).
- So 100/10*100ng= 1000 ng from a 0.25 g sample.
- 0.25 g rooting medium was about 2.5 mL
- This means there was 1000ng/2.5 mL is 400 ng/mL rooting medium

The quantity per volume rooting medium is the answer of importance for the final report and the horticultural application.

Annex 3 Setup of the experiment in the Greenhouse



INFORMATION:

Greenhouse: 2.205 and 2.206
Period: 18 Feb- 20 May
Cultivar: Strawberry var. Elsanta

Plants/m2= 12 → Sampling unit = 7
Plants/treatment= 42
Plants/table= 70
Total plants = 420

TREATMENTS:

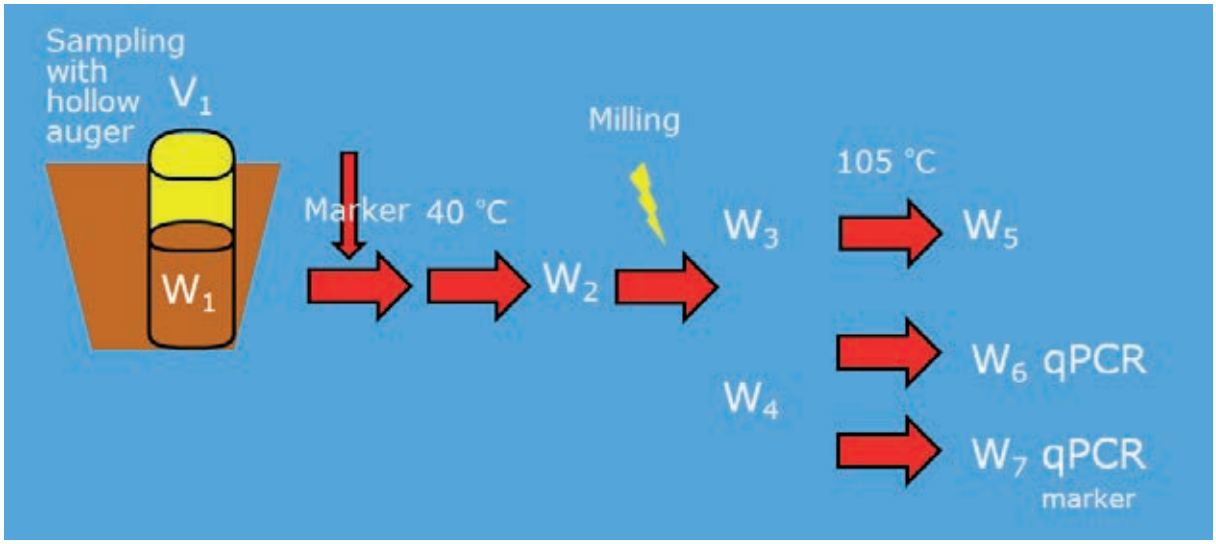
Greenhouse A
P → Peat
P (Th) → Peat + Trichoderma harzianum
B10 (Th) → Biochar 10% v/v + Th
B20 (Th) → Biochar 20% v/v + Th
B30 (Th) → Biochar 30% v/v + Th

Greenhouse B
P → Peat + Phytophthora cactorum
P (Th) → Peat + Trichoderma harzianum + Pc
B10 (Th) → Biochar 10% v/v + Th + Pc
B20 (Th) → Biochar 20% v/v + Th + Pc
B30 (Th) → Biochar 30% v/v + Th + Pc

Annex 4 Protocol qPCR pre-treatments

Rooting Media sample preparation for qPCR related techniques.

We are going to take a sample (A) from one container with an hollow auger with a razor sharp edge. To get a razor sharp edge, always sharpen the edge before using, removing any unevenness on the edge.



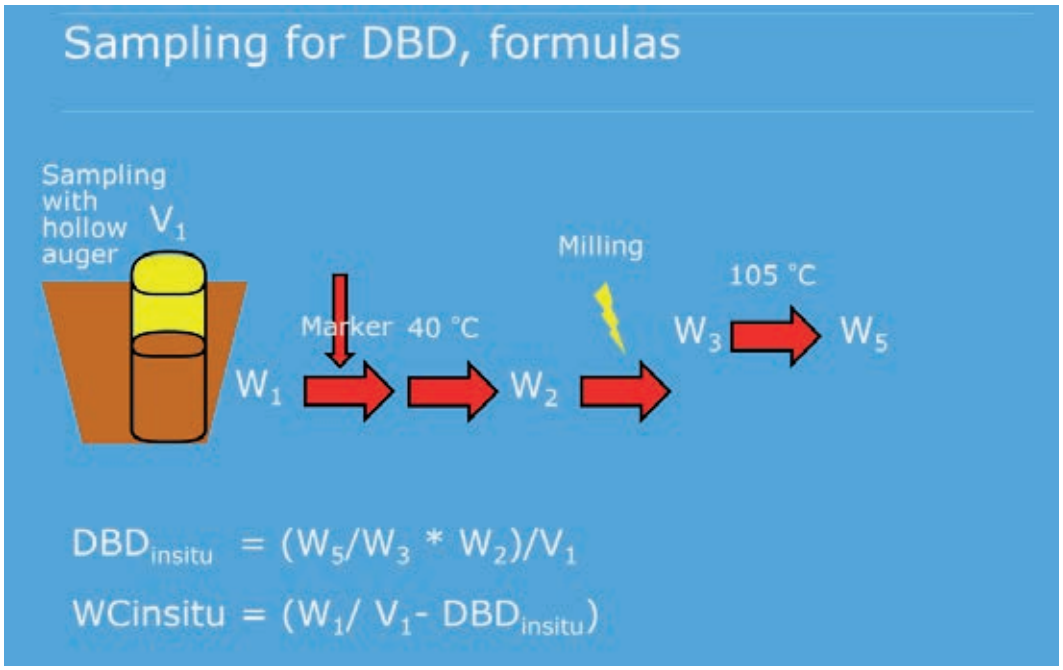
1. The volume of the sample is recorded as the volume of the sample in situ e.g. without compaction. To arrive at this volume measure the height of the rooting medium before applying the hollow auger. Use this height to calculate the sample volume in mL as V_1=H*PI*R^2, in which:
   a. H is the height of the rooting medium on the container in cm.
   b. PI is the constant value 3.14.
   c. R is the radius of the auger in cm.
2. Take a sub sample with an hollow auger of
   a. 10% of the container volume for container volumes up to 1000 mL.
   b. 100 mL up to 4000 ml containers.
   c. 250 mL up to 50 L containers.
3. Weigh the sample directly after taking the sample as W_1 in mg.
4. Add a marker of 1 mL of solution with 10^10 spores of plasmid DNA or an equivalent concentration in ng DNA and distribute this evenly by spraying onto the spread out sample. Homogenize by mixing. NB this 1 mL does not influence the water content and DBD calculations as it does not influence W_1 and W_2. In general: add X_z (ng) of the marker organism and express this quantity over the sample volume V_1 as $Y_z = X_z/V_1$ in ng/mL.
5. Dry the sample for 24 h at 40 °C. The low temperature is to preserve DNA. Make sure the sample is spread thinly. The oven should be ventilated.
6. Weigh the sample directly after taking the sample from the oven as W_2 in mg. Store all samples in fully filled air tight containers.
7. Split the sample in two samples B, and C taking care not to create any separation of the main sample into graded parts.
8. Weigh the samples directly after taking the sample as W_3 and W_4 in mg.
9. Dry the sample W_3 for 24 h at 105 °C in order to in the true DBD. Make sure the sample is spread thinly. The oven should be ventilated.
10. Weigh the sample directly after taking the sample from the oven as W_5 in mg. Store he sample in a fully filled air tight container.
11. Calculate dry bulk density DBD_{insitu} as $(W_5/W_3*W_2)/V_1$ (mg/mL = g/L)
12. Calculate water content (%) in situ as $WC_{insitu} = (W_1/ V_1 - DBD_{insitu})*100\%$.

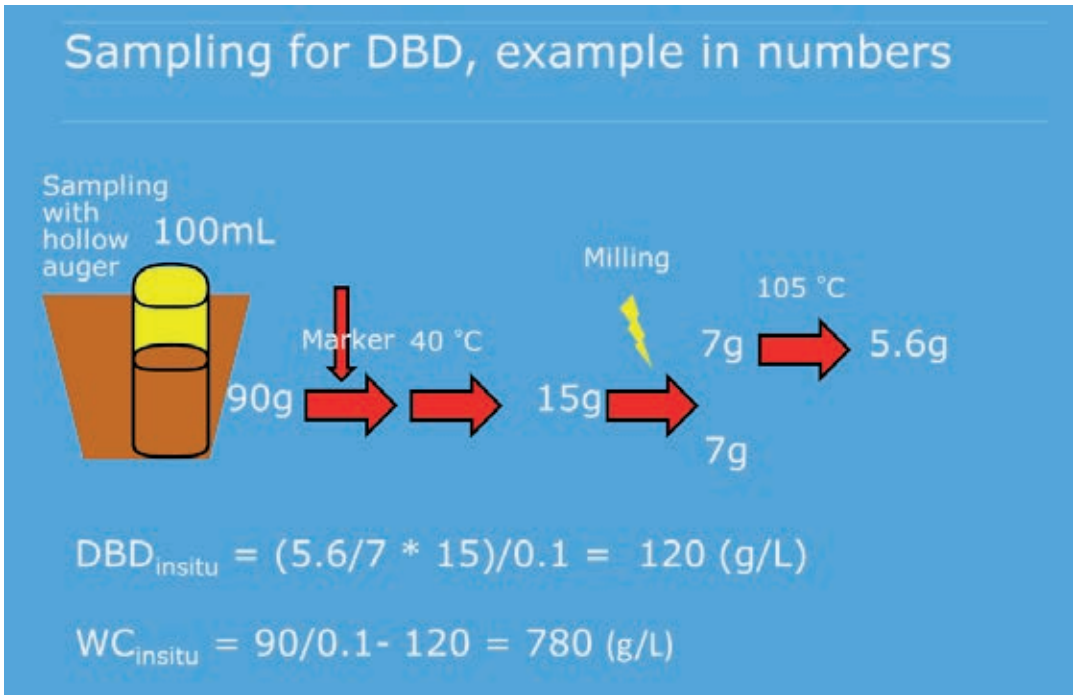
13. Put the sample is a disc mill and powder the sample for 10 minutes at 140 movements per minute with 10 discs of xx g [this needs to be further specified by the operator].
    NB Clean the mill after each use observing the following:
    - Remove all dust with a brush
    - Use compressed air to further clean
    - Use a clean damp cloth to wipe all parts.
14. From sample C (W_4) Take sub samples D and E as prescribed by the relevant qPCR tests and report the weight (mg) as W_6 and W_7. Sample D is used for the marker concentration and sample E for the concentration of the target organism.
15. Report the readings of the qPCR for the marker in subsample D as $[X_m]$ in ng/g dry matter.
    a. Calculate and report the content of marker $[Y_m]$ per unit value of original sample by using $[Y_m] = [X_m]*DBD_{insitu.}$
16. Check on the losses due to processing by comparing $[Y_m]$ and $[Y_z]$ and reporting $[Y_m] / [Y_z]$. If this value is 80-100% the method is true. If the value is below 80% the processing affects the outcome.
17. Report the readings of the qPCR for the target organism in subsample E as $[X_t]$ in ng/g dry matter.
    a. Calculate and report the content of the target organism $[Y_t]$ per unit value of original sample by using $[Y_t] = [Y_m] / [Y_z] * [X_t] * DBD_{insitu}$

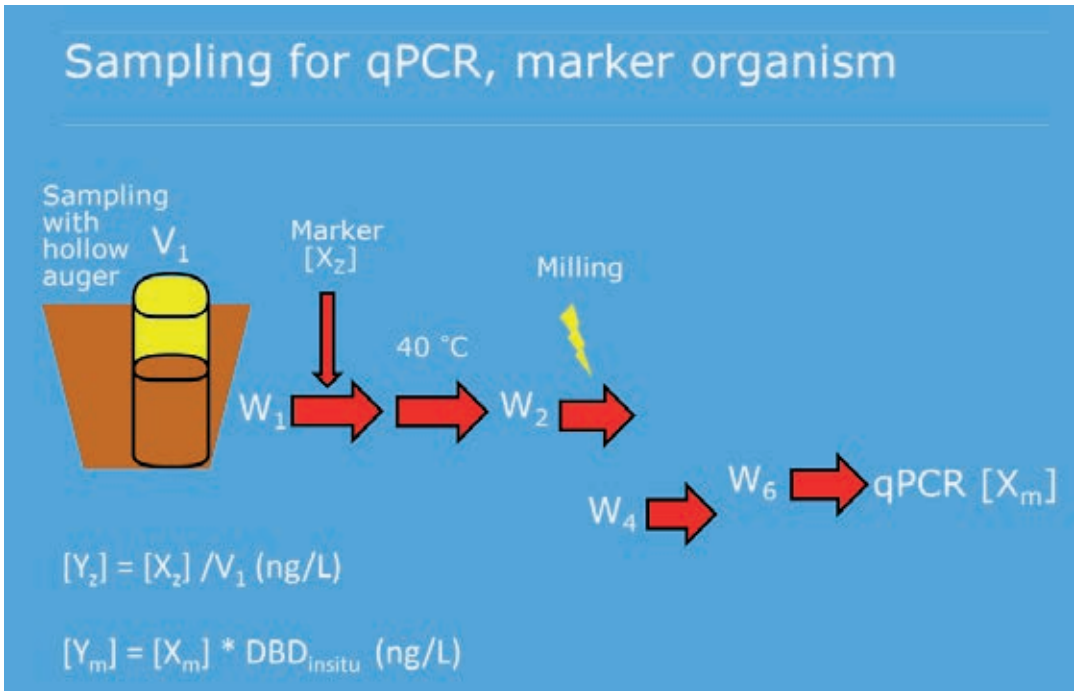
Adapted method for samples with >40% v/v of biochar

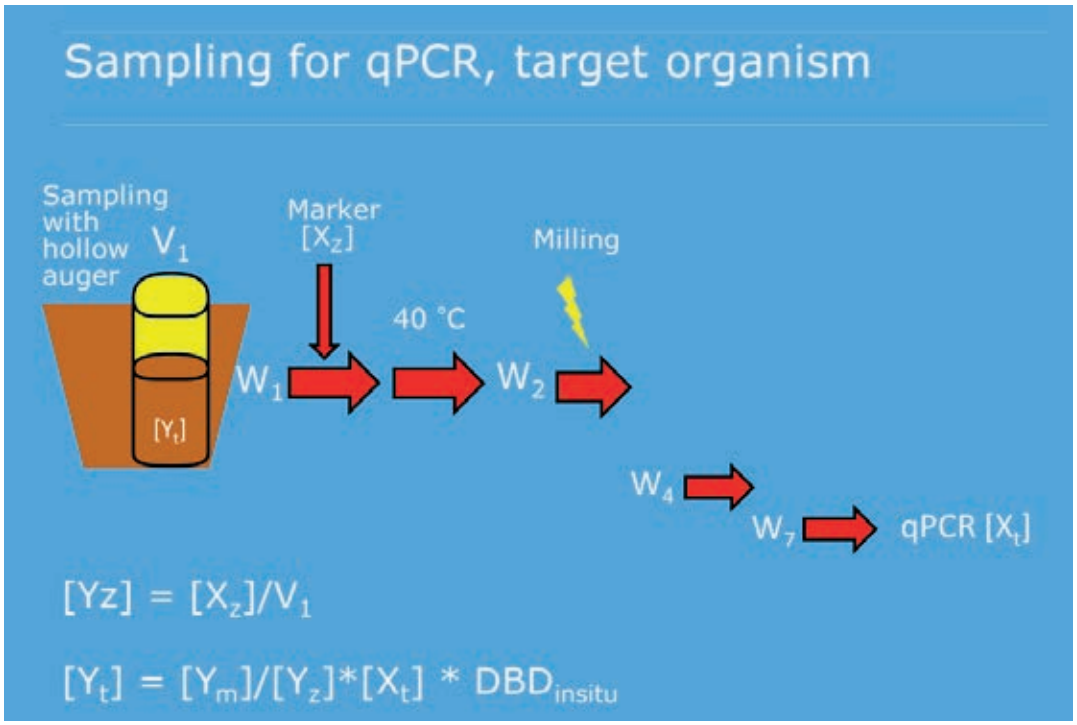
1. As biochar allegedly interferes with DNA by preferential adsorption of DNA on the surface, care must be taken not to break the particles into smaller units. Therefore the homogenisation by milling step is replaced by a homogenisation step by:
    b. Spreading the dry sample on a sheet in a layer of about 1 mm, separating coagulated parts into the smallest possible units without crushing the biochar.
    c. Hand mixing the particles several times.
    d. Spreading the dry sample homogeneously on a sheet in a layer of about 1 mm, dividing the sample into the required number of sub samples, before weighing and storing.
2. As biochar allegedly interferes with DNA by preferential adsorption of DNA on the surface, a reference sample without biochar must be included. The reading of the marker found back in the reference compared to the biochar containing sample will give an indication of the possible loss of DNA by adsorption to the biochar.
3. The sub samples used for qPCR or related methods now run the risk of not being representative of the sample they will be taken from (which is other than with the milled powdered mass used in the original method). Therefore the dry sample is spread homogeneously on a sheet in a layer of about 1 mm, drawn in squares (a grid) and the required weight is taken from one or two squares. This is to prevent taking graded samples straight from the storage.

Further graphic representations:






Sampling for qPCR, marker organism
Sampling with hollow auger
V1
Marker [Xz]
Milling
40 °C
W1
W2
W4
W6
qPCR [Xm]
[Yz] = [Xz] /V1 (ng/L)
[Ym] = [Xm] * DBDinsitu (ng/L)


Sampling for qPCR, target organism
Sampling with hollow auger
V1
Marker [Xz]
Milling
40 °C
W1
W2
W4
W7
qPCR [Xt]
[Yt]
[Yz] = [Xz]/V1
[Yt] = [Ym]/[Yz]*[Xt] * DBDinsitu

To explore the potential of nature to improve the quality of life

Wageningen University & Research,
BU Greenhouse Horticulture
P.O. Box 20
2665 ZG Bleiswijk
Violierenweg 1
2665 MV Bleiswijk
The Netherlands
T +31 (0)317 48 56 06
www.wur.nl/glastuinbouw

Report WPR-893

The mission of Wageningen University and Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 5,000 employees and 10,000 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.