Root-Associated Microorganisms Prevent Caffeine Accumulation in Shoots of *Salvia officinalis* L

Margot Schulz[1,*], Mona Knop[1], Carmen Muellenborn[2], Ulrike Steiner[3]

[1]IMBIO (Institut für Molekulare Physiologie und Biotechnologie der Pflanzen), Universität Bonn, Bonn, 53115, Germany
[2]IBG-2, Plant Sciences, Forschungszentrum Jülich GmbH, Jülich, Germany
[3]INRES (Institut für Nutzpflanzenwissenschaften und Ressourcenschutz), Universität Bonn, Bonn, 53115, Germany

Abstract *Salvia officinalis,* previously used as an intercrop in coffee plantations, absorbed caffeine from incubation media. Caffeine was mainly found in the roots, together with traces of theobromine, while solely a small amount of caffeine was found in the shoots. In sterile plants, the caffeine accumulation was similar in both the roots and the shoots. The addition of antibiotics to the incubation medium of non-sterile sage revealed an involvement of plant-associated microorganisms in the caffeine degradation. Three most active microorganisms were isolated from non-sterile sage roots and were identified by molecular and microscopic studies as *Trichoderma hamatum* (fungus)*, Pseudomonas putida* (bacterium) and *Rhodotorula glutinis* (fungus). Whereas *P. putida* and *R. glutinis* were associated with the rhizosphere, *T. hamatum* existed as an endophyte inside the roots, as ascertained by colonization and re-isolation studies. The study demonstrates that the degradation of caffeine is initiated by the ability of the microorganisms to perform demethylations, whereas the xanthine degradation may be attributed to either the plant or the microorganisms. Plants, which do not contain caffeine as a secondary product, have not yet been investigated for degradation of the compound. *Trichoderma hamatum* was not known as a caffeine degrading species and as an endophyte of *Salvia officinalis*. We propose a novel eco-biochemical interaction between endophytic *T. hamatum* and sage plants in caffeine degradation. If aromatic plants are used as intercrops in coffee plantations, the occurrence of caffeine degradation in those plants is of great importance.

Keywords Allelopathy, Endophyte, Caffeine Degradation, Plant-fungus Interaction, *Pseudomonas Putida, Rhodotorula Glutinis, Salvia Officinalis* L., *Trichoderma Hamatum*

1. Introduction

In recent decades, caffeine has been identified as a compound with allelopathic potential that causes the autotoxicity of coffee plants in older plantations since this purine alkaloid, mainly released by the fruits and leaves, accumulates in the soil over time. Caffeine accumulation is proposed to be one of the factors responsible for the observed worldwide premature decay phenomenon in unshaded older coffee monocultures[1, 2, 3]. However, several investigators have argued that soil microorganisms, such as *Pseudomonas putida, Serratia marcescens,* species of the genera *Alcaligenes, Rhodococcus*, and *Klebsiella* and several fungi mainly belonging to the *Penicillium* and *Aspergillus* genera and the yeast *Trichosporon asahii*, are able to degrade caffeine[4, 5]; some of these investigators, therefore, doubt the contribution of caffeine to the allelopathy of coffee[4]. Caffeine is often used as a specific marker for the detection of ground and surface water

contamination with waste water, and several species of caffeine-degrading bacteria, such as *Pseudomonas putida, Alcaligenes* species and species of *Klebsiella* and *Rhodococcus*, have been isolated from domestic waste water or surface water[6]. Therefore, caffeine degradation may occur rather slowly in environments with a low density of suitable microorganisms. Moreover, caffeine is toxic to most soil microorganisms. Recently, a photo-Fenton process for caffeine degradation was developed as an alternative method to microbial degradation[7].

While the biosynthesis of caffeine and related purine alkaloids has been extensively studied in *Coffea arabica* and *Camellia ptilophylla* and to some degree in *Theobroma* species, insights into the catabolism of caffeine *in planta* have been obtained solely by tracer experiments using ^{14}C-labeled purine alkaloids[4]. Based on these data, a major catabolic pathway was constructed that starts with the demethylation of caffeine to produce theophylline and xanthine, which is subsequently degraded via the purine catabolic pathway. However, according to the MetaCyc database of metabolic pathways, the plant enzymes and genes responsible for the demethylation of the purine alkaloids, have not been identified and characterized[8]. In bacteria, demethylating and oxidative catabolic pathways

are involved in the degradation of caffeine[9, 10,11]. In fungi, theophylline was described as an intermediate in the degradation pathway[12]. Roussos et al.[13] collected 272 strains of filamentous fungi collected from the soil and the coffee leaves and fruits in coffee-growing areas. Only 5 strains of *Aspergillus* species and 2 strains of *Penicillium* species, exclusively, were able to degrade the caffeine present in the culture medium. In another study, 131 epiphytic and endophytic fungi from coffee leaves were determined[13].Vega et al.[14] isolated hundreds of endophytes with an unknown role from coffee plants collected from different geographic sites. Thus, coffee plants harbor a high diversity of microorganisms which may be important for the prosperity of the particular ecosystem coffee plant-microorganisms. However, whether endophytes may drive caffeine degradation *in planta*, was not directed. The traditional coffee plantations in Coatepec (Mexico) have ground cover vegetation that is dominated by Poaceae and Asteraceae for sunny plots and *Commelina* species in the shade, sometimes in combination with trees[1, 2]. It may be speculated that in this ecosystem weeds and trees are able to reduce the amount of coffee allelochemicals in the soil by absorption of the compounds, but the contribution of the ground-cover plant species, intercropped species and their associated microorganisms in the reduction of caffeine in the soil is unknown.

Aromatic plants, such as sage (*Salvia officinalis*), oregano (*Origanum vulgare*), mint (*Mentha piperita*) and basil (*Ocimum basilicum*), have been used as intercrops in coffee plantations. These aromatic plants stimulated the plagiotropic growth of the coffee plants, whereas their own growth was negatively influenced. In young plantations, the negative effect was reduced in basil and sage plants compared to other aromatic species[15, 16]. As found by uptake experiments performed in the IMBIO lab, University of in Bonn, all of the species were able to absorb caffeine, which accumulated in the roots. When basil was transferred to a caffeine-free medium, nearly all of the absorbed caffeine was released from the plant into the medium, whereas with sage, only a small portion could be detected. This result prompted us to elucidate the fate of the caffeine that is absorbed by sage, a species lacking purine alkaloids as natural constituents. *In planta* degradation of caffeine is of great importance when herbs like sage will be used as intercrops in coffee plantations. Presently no study is available that directs caffeine degradation in other plants than those belonging to the genera *Coffea, Camellia* and *Theobroma*.

2. Materials and Methods

Plant Material and Caffeine Incubations

Six-week-old *Salvia officinalis* plants (accession No. 23581), cultivated under greenhouse conditions, were incubated with 5 mM caffeine for 24 to 120 hours (h) in sterile tap water. In some experiments, the incubation

medium was mixed with both Rifampicin (40 mg/mL) and Cefotaxime (250 mg/mL) (Duchefa Biochemie, The Netherlands) to suppress bacterial growth. Aliquots (100 µL each) of the incubation medium were stored at -20°C until analysis; the caffeine and degradation products were analyzed immediately after the thawing of the aliquots. For the determination of caffeine absorption, the roots and shoots of sage plants from all incubations were harvested separately and homogenized with quartz sand and 5 volumes (w/v) of 50% methanol. The homogenates were centrifuged, and the supernatants were analyzed for caffeine, theophylline and theobromine by HPLC using the method described in[14]. Caffeine and the degradation products, theobromine and theophylline, were identified by co-chromatography with synthetic compounds. For the experiments with sterile *Salvia officinalis*, the seeds were surface-sterilized with 80% ethanol for 1 min, followed by a 20 min treatment with 2% hypochlorite, supplemented with 0.1% Tween 20. After washing three times with sterile H_2O, the seeds were transferred either to pots with sterilized soil (50% sand, 50% sieved compost; Hawita Kompost and sand, Kissener´s Gartenmarkt, Bonn, Germany) or cultivated in sterile glass jars with ½ strength Murashige and Skoog (MS) salts, plus vitamins and additionally containing 0.8% plant agar, 30 g/L sucrose and 0.5 g/L at a pH of 5.8. The plant cultivation was performed under sterile conditions in a Persival-Scientific culture chamber under conditions of 20^0C, 12 h of light (PAR: 100 microeinstein m^{-2} s^{-1}), and 55% relative humidity. The plants were used for experiments after the development of 5 to 7 leaves. All experiments were repeated at least 3 times.

Isolation of the root-associated microorganisms

Pieces of the sage roots were placed onto sterile Czapek or malt agar plates and incubated in the dark until the most predominant microorganisms were observed. Clean colonies of these plates and 200 ml of the *Salvia officinalis* incubation medium were used as the inoculum for cultures in sterilized, liquid Czapek or malt medium with and without the antibiotics, Rifampicin (200 µl of a 40 mg/mL stock solution) and Cefotaxime (200 µl of a 250 mg/mL stock solution). The inoculation flasks were incubated on a rotary shaker (100 rpm) at room temperature. After 3 days, the liquid culture was supplemented with caffeine (0.5 mM), and 200 µl from each flask was stored for the caffeine determination at the start of the incubation (day 0). The degradation of caffeine was monitored by HPLC over a period of 5 days by collecting and analyzing 200 µl samples of the media. At the end of the incubation period, the three microbial suspensions that were able to degrade caffeine were filtered with filter paper, and the supernatants were discarded after collecting 200 µl aliquots. Two fungi and one bacterial isolate capable of degrading caffeine (isolates ThSoW29508, RhSoW01 and PsSoW01) were placed on Czapek or malt agar and subcultured as described above. The isolates were each incubated in liquid medium (100 mL) with 5 mM caffeine, directly after the isolation and 8 weeks after the isolation.

Identification of the microorganisms – barcoding PCR, cloning and sequencing

To characterize the isolate ThSoW29508 on a molecular level, mycelia grown on Czapek medium were scraped off the plates and ground in liquid nitrogen. DNA was extracted using the DNeasy plant mini kit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. Three primer combinations were chosen for the barcoding of the isolate ThSoW29508. For the PCR amplification of the Internal Transcribed Spacer Region (ITS), primers ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′), according to[17] were used. Additionally, a partial sequence of the large exon of tef1 was amplified with the primers tef1afw (5′-GTGAGCGTGGTATCACCATCG-3′) and tef1arew (5′-GCCATCCTTG GAGACCAGC-3′), and the primerpairChit42-1a (5′-GCTYTCCATCGGTGGCTG GAC-3`) and Chit42-2a (5`-GGAGTTGGGGTAGCTC AGC-3`) was used to amplify a fragment of the gene encoding endochitinase 42 (ech42)[18]. A second fungal isolate, RhSoWo01, cultivated on Sabouraud dextrose agar, was used for in-cell PCR with the primers ITS1F (5′-TCCGTAGGTGAACCT- GCGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC- -3′), according to[17]. The identification of the bacterial isolate, PsSoW01, cultivated on *Pseudomonas* agar F/P, was also accomplished by in-cell PCR with the primers P13P (5′-AGGCCCGGGAACGTATTCAC-3′) and P11P (5′-GAGGAAGGTGGGATGACGT-3′) for amplification of the 16S ribosomal RNA gene[19]. For all PCR reactions, either 20 ng of genomic DNA or cells were mixed with 5 μL Taq-buffer (10x), 5 μL MgCl$_2$ (25 mM), 0.4 μL dNTPs (10 mM), 0.2 μL Taq-Polymerase (2.5 U, Fermentas) and 2.5 μL of each primer (10 pmol/μL) in a 50 μL PCR reaction. The parameters for the PCR reactions were as follows: 95$^°$C for 5 min for the initial denaturation; 35 cycles each of 95$^°$C for 30 seconds (sec), 58$^°$C (primer pair ITS), 64$^°$C (primer pair tef1), 63$^°$C (primer pair Chit42) or 55$^°$C (primer pair P13P/P11P) for 30 sec, respectively, 72$^°$C for 1 min 30 sec; and a final extension at 72$^°$C for 7 min. The PCR products were separated and visualized on a 1% agarose gel stained with ethidium bromide. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and used directly for sequencing. The PCR products were sequenced with the same primers used for the barcoding PCR using the Quick Start Kit (Beckman-Coulter, München, Germany). A magnetic bead purification (Agencourt Clean Seq, Beckman-Coulter, München, Germany) of the sequencing fragments was accomplished, and the fluorescent-labelled fragments were separated and detected at 6.0 kV for 60 min by means of capillary gel electrophoresis, equipped with a laser-induced fluorescent detector (CEQ 8800, Beckman-Coulter, München, Germany). The evaluation of the data and the alignments and the analysis of sequences were performed with Beckman-Coulter software, and the sequences were analysed by a Blast search in NCBI for the identification of the species.

Fungal colonization of the plant tissue

Sterile plants were used for the colonization studies; plants were cultivated as described above. Inoculation of the plants was done by the application of a spore suspension (5 x 10^5 spores/mL) into the soil (50 mL/pot). The spores were harvested from fungal colonies grown on potato dextrose agar (PDA) for 14 days (d). After inoculation, seedlings were grown for 21 d in a controlled-environment chamber (16-h light/8-h dark photoperiod) at 25°C. For the re-isolation of *Trichoderma hamatum*, plants were harvested 21 days after inoculation by cutting the roots and stems into sections of 0.5 cm in length and up to a height of 5 cm above the soil line. Roots and stems were taken from 20 different plants. The plant samples were either non-sterilized or surface sterilized using NaOCl (1.3% available chlorine) for 1 min, followed by three washes in sterile, distilled water, and placed into Petri dishes on water agar containing antibiotics (50 mg/L penicillin, 50 mg/L chlortetracycline and 50 mg/L streptomycin). The plates were incubated at 24°C in the dark for 3 days and then transferred to the light (photoperiod of 12 h) for an additional 4 days. For the detection of fungal colonization, the samples were examined microscopically using a Leica MZ 16 F stereomicroscope (Leica, Bensheim, Germany) with 10-fold magnification. The numbers of samples with *Trichoderma* colonies on each Petri dish were recorded after 7 days, and the data were expressed as % isolation rate.

Microscopic studies

Investigations of fungal structures by light and fluorescence microscopy were carried out with a Leitz DMR photomicroscope (Leica, Bensheim, Germany). The fungal structures were stained in the dark with 0.05% Uvitex 2B (Syngenta, Basel, Switzerland) in 0.1 M Tris/HCl buffer, pH 8.0. The microscope was equipped with a digital camera (KYF 75 Sony) and Diskus software (TB Hilgers, Königswinter, Germany) for image processing.

Statistical analyses

Values were presented as means ± standard deviations from at least three independent treatments. These data were subjected to ANOVA analysis of variance (Bartlett´s test for homogeneity of variance, Tukey´s student range test and Dunn t test, P < 0.05) using SAS version 8.1.

3. Results

Decrease of caffeine in the incubation medium

In the incubation medium of non-sterile sage plants, the level of caffeine started to decrease to 80 to 90% after three days of incubation and dropped to 10 to 20% at day five (Fig. 1). Traces of theobromine could be found at days three and four. No other catabolite was detected. As for the sterile plants, the caffeine amount applied remained almost constant over five days. When the incubation medium of non-sterile plants was supplemented with the antibiotics Rifampicine and Cefotaxime, 75% of the caffeine was still present after five days.
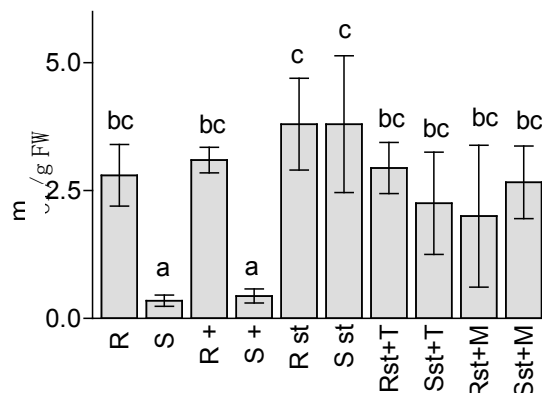
of 17 to 26% after six days.
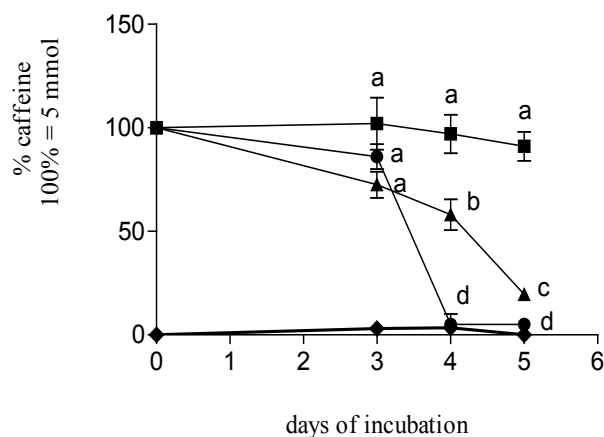
Figure 1. Decrease of caffeine (μM) in the incubation media of sage plants. Data are means of ± SE of the three replicates and bars with different letters indicate significant differences at P<0.05 (ANOVA). Same letters are not significantly different. ■: sterile sage plants; ▲: non-sterile sage plants; ●: sterile sage plants inoculated with Pseudomonas putida, Trichoderma hamatum and Rhodotorula glutinis; ♦: theobromine in the medium of non-sterile plants

Figure 2. Caffeine accumulation in roots and shoots of sage. R: roots; S: shoots; R+, S+: accumulation in the shoots and roots of sage plants incubated in the presence of antibiotics; R st, S st: roots, shoots from sterile plants; +T: inoculated with the Trichoderma species; +M: inoculated with the three microorganisms Rhodotorula glutinis, Trichoderma hamatum; Pseudomonas putida. Data are means of ± SE of the three replicates and bars with different letters indicate significant differences at P<0.05 (ANOVA). Same letters are not significantly different

Caffeine accumulation in sterile and non-sterile Salvia officinalis plants

After three days of incubation, the caffeine concentration in the non-sterile and sterile plants was determined. The root and shoots were analysed separately. The roots of the non-sterile plants contained 2 to 3 μmol caffeine/g fresh weight, and the caffeine accumulation in the roots of the sterile plants was of a similar range. In the root extracts of the non-sterile plants, traces of theobromine, but no theophylline, were detected. The addition of antibiotics did not influence the caffeine accumulation pattern in the roots and shoots of the non-sterile plants. The most striking difference between the non-sterile and sterile plants was approximately a 10-fold, significantly higher accumulation of caffeine in the shoots of the sterile plants, whereas the concentration in the roots was similar to that of the non-sterile plants. In the sterile plants, no theobromine was detected (Fig. 2). Thus, it is clear that plant-associated microorganisms were of a high importance for the observed caffeine degradation, while the sage plants seemed to be unable to degrade the purine alkaloid on their own. These results were decisive for the subsequent isolation of three most prominent microorganisms involved in the caffeine degradation.

When the incubation medium of the sterile sage plants was inoculated with the three freshly isolated microorganisms, a rapid decrease of caffeine in the medium was noted after a lag phase of three days. During the following day, 90% of the caffeine was degraded (Fig. 1). The addition of only one of the microorganisms led to a decrease of caffeine, between 25 to 33%, within three days. The inoculation of the medium with the microorganisms reduced the content of caffeine in the shoots and roots, but the amounts of caffeine that were determined in the plant material varied drastically (Fig. 2). Inoculation of the medium with one of the species of microorganisms resulted in a decrease of the level of caffeine

Identification of microorganisms associated with Salvia officinalis

The most prominent fungi and bacteria emerging from the root segments were cultivated in media supplemented with caffeine to select only those species that were able to degrade the caffeine. The three most active microorganisms, two fungi and one bacterium, were isolated and identified. The first isolate formed spores (3.8 x 3.0 μm, l/w), which were ellipsoidal and green with a smooth surface. The morphological features of the freshly isolated and plated fungus (isolate ThSoW29508) are shown in Fig. 3. Using the ITS primers ITS1F and ITS4R, a single, specific band was amplified with a fragment size of 646 bp (GenBank ID: HM488365). For the other two primer pairs, a major fragment of 254 bp for tef1 (GenBank ID: HM488367) and a major fragment of 900 bp for echit42 (GenBank ID: HM488366) were sequenced for further species identification. The sequence analysis revealed a high homology to diverse Trichoderma hamatum isolates for the ITS region (e-value: 0.0, max. identity: 99%) and for the chit42 (E-value: 0.0, max. identity: 99%) and tef1 (E-value: 3e-123, max. identity: 98%) genes. According to these results, this fungus belongs to the Trichoderma hamatum complex. The morphological features of the second fungal isolate indicated the genus Rhodotorula. The sequence analysis of the obtained fragment (GenBank ID: HM488368) revealed a high homology to the sequences of Rhodotorula glutinis isolates (E-value: 0.0, max. identity: 100%). Due to growth of the bacterium isolate on Pseudomonas isolation agar and according to the sequence analysis of the obtained fragment (GenBank ID: HM488364) with a high homology to the 16S ribosomal gene of Pseudomonas putida (E-value: 2e-83, max. identity: 99%), the bacterium was grouped as a member of the Pseudomonas putida complex[20]. Additional

PCR product information is presented in Table 1. In the following text; the isolated species are named Trichoderma hamatum, Rhodotorula glutinis and Pseudomonas putida.

Table 1. PCR product used for the molecular identification of microorganisms isolated from roots of Salvia officinalis

| Isolate	GenBank ID Accession No.	Sequence length(bp)	Literature source
ITS region			
ThSoW29508	HM488365	628	[17]
translation elongation factor 1 (tef1)			
ThSoW29508	HM488367	254	[18]
endochitinase 42 gene(echit42)			
ThSoW29508	HM488366	814	[18]
ITS region			
RhSoW01	HM488368	654	[17]
16 S ribosomal RNA gene			
PsSoW01	HM488364	175	[20]
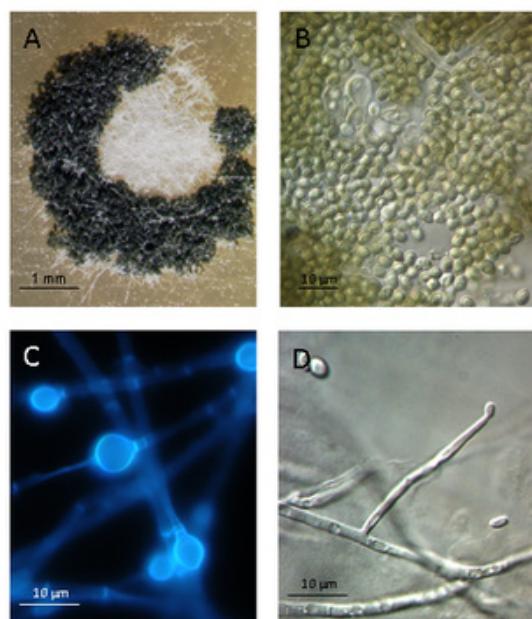
Figure 3. Morphological features of endophytic Trichoderma hamatum in Salvia officinalis. A: a sporulating colony on PDA after three weeks of incubation; B: the green globose spores; C: the chlamydospores formed terminal and intercalary; D: a synanamorph forming sparingly in the aerial mycelium conidiophores

Colonization of plant tissue

After incubation of the sage plants cultivated in soil inoculated with spores of Trichoderma hamatum, the fungus was re-isolated from the roots and stem pieces, which were either surface sterilized or not sterilized. The re-isolation rate of the non-sterilized roots was, on average, approximately 30 % higher than that of the surface-sterilized roots. The stems showed a lower level of colonization compared to the level in the roots, which depended on the distance from the soil line but was the highest in the lower part of the stem (Fig. 4). Because the re-isolation rate from the surface-sterilized stems was only 30 % less compared to the non-sterilized ones, an endophytic existence of the fungi within the roots

and lower parts of the stem is likely.
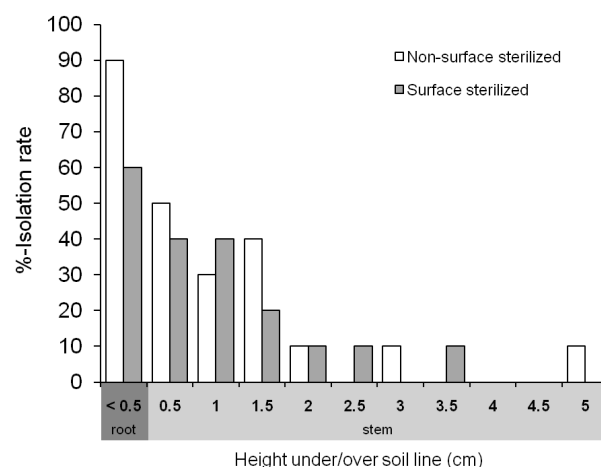
Figure 4. Re-isolation of Trichoderma hamatum from surface-sterilized and non-sterilized roots and stem pieces of sterile-grown sage plants inoculated with Trichoderma spores

4. Discussion

While the demethylation reactions of caffeine were performed by the microorganisms, both the microorganisms and the sage plants could have been responsible for any further degradation. Because we could not detect any xanthine in the incubation medium, it might have been metabolized rapidly. *Pseudomonas putida,* a widespread saprotrophic soil bacterium, colonizes the roots of different plants. Strains of *Pseudomonas* species from the soil of coffee plantations, which showed positive chemotaxis toward caffeine, exhibit higher rates of caffeine degradation than other strains of this bacterial species[21]. Yu et al.[22] suggested that *P. putida* possesses multiple N-demethylases that are involved in caffeine and methylxanthine demethylation. *R. glutinis* was not yet known to degrade caffeine. To our knowledge this is a first report of *Trichoderma hamatum* being associated as an endophyte with *Salvia officinalis* and, furthermore, being involved in degrading caffeine in a *Trichoderma* -sage-interaction. The ability to degrade caffeine is of high importance when sage plants will be used as intercrops in coffee plantations.

Trichoderma species, often found in the rhizosphere of plant roots, are widely used for the biocontrol of plant diseases and have been shown to alter plant metabolism by interacting with plant roots. These fungi can act as opportunistic, avirulent plant symbionts and induce systemic resistance against pathogens[23, 24, 25, 26]. However, cooperation with plants in degrading xenobiotics has not been described to date[27]. It has recently been demonstrated that *Trichoderma hamatum* exists as a beneficial endophyte in *Theobroma cacao*[28], and *T. stromaticum* is already known to exist endophytically in the vascular system of cocoa[29]. Numerous fungal endophytes have been isolated from coffee plants. Although not yet investigated[14], these plant-associated fungi may contribute to the degradation of

caffeine *in planta* and, perhaps more generally, to the degradation of xenobiotic compounds, including allelochemicals. Here, we propose the existence of a beneficial biochemical interaction between endophytic *Trichoderma hamatum* and sage in caffeine degradation inside the root. It is likely that root-associated microorganisms have a more general role in caffeine degradation than was previously considered. Specifically, *Trichoderma hamatum,* acting in cooperation with sage plants by degrading the allelochemical caffeine, suggests a function of this fungus that has not yet been taken into account. The caffeine-induced growth inhibition, due to the high sensitivity of the root tips to this compound, might be explained by the not yet established colonization of young tissues by microorganisms and a lack of interactions with endophytic microorganisms.

Depending on the dosage, volatile compounds of aromatic plants can have beneficial or harmful effects on neighbored plants. Low concentrations of some monoterpenes can promote plant growth whereas higher ones led to stomata opening and desiccation of receiver plants[15, 30]. A possible influence of caffeine on monoterpene synthesis in sage and how *T. hamatum* is affected by the sage monoterpenes or involved in the crosstalk is under investigation.

5. Conclusions

In the agro-ecosystem "coffee plantation", a high level of colonization of the soil with diverse, active microorganisms, along with suitable ground-cover vegetation, should contribute to a reduction of the deleterious effects caused by caffeine. Shaded coffee plantations are predestined for a better degradation of caffeine because of their high biodiversity[31] including a high richness in microorganisms and plant species[32]. Using sage with its endophyte *Trichoderma hamatum* as an intercrop may be a strategy to reduce caffeine accumulation in the soil. A possible role of the local ground cover vegetation in caffeine degradation with the aid of endophytes has still to be unraveled. A general function of endophytic microorganisms in supporting the plant´s efforts in the detoxification and degradation of xenobiotics has to be elucidated in future.

ACKNOWLEDGEMENTS

REFERENCES

[1] A.L. Anaya, G. R. Waller, O. Owuor, J. Friedman, C. H. Chou, T. Suzuki, J. F. Arroyo-Estrada, and R. Cruz-Ortega. 2002. The role of caffeine in the production decline due to autotoxicity in coffee and tea plantations. In: Reigosa, M., Pedrol, N. (Eds.), Allelopathy - from Molecules to Ecosystems. Science Publishers Inc., Enfield USA, Plymouth UK, pp71-9.

[2] A.L. Anaya, R. Cruz-Ortega, and G. R. Waller. Metabolism and ecology of purine alkaloids. Front Biosci. 2006, 11: 2354-2370.

[3] T. Suzuki and G.R. Waller. Allelopathy due to purine alkaloids in tea seeds during germination. Plant Soil 1987, 98:131-136.

[4] H. H. Ashihara, Sano, and A. Crozier. Caffeine and related purine alkaloids: biosynthesis, catabolism, function and genetic engineering. Phytochemistry 2008, 69: 841-856.

[5] V. Lakshim and N. Das. Biodegradation of caffeine by Trichosporon asahii isolated from caffeine contaminated soil. Int. J. Engin. Sci. Technol. 3: 7988-7997.

[6] S. K. Gokulakrishnan, Chandraraj, N. Sathyanarayana, and N. Gummadi. Microbial and enzymatic methods for the removal of caffeine. Enzyme Microb. Technol.2005, 37:225-232.

[7] A.G. Trovo, T.F.S. Silva, O. Gomes Jr., A.E.H. Machado, W. BorgesNeto, P.S. Muller Jr. and D. Daniel. Degradation of caffeine by photo-Fenton process: optimization of treatment conditions using experimental design. Chemosphere 2013, 90: 170-175.

[8] R. T. Caspi, Altman, J. M. Dale, K. Dreher, C. A. Fulcher, F. Gilham, P. Kaipa et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. 2010, 38: D473-D479.

[9] S. N. Gummadi, S. S. Dash, and S. Devarai, S. 2009. Optimization of production of caffeine demethylase by Pseudomonas sp. in a bioreactor. J. Industr. Microbiol. Biotechnol. 2009, 36:713-720.

[10] R.M. Summers, T.M. Louie, C.L. Yu, and M. Subramanian. Characterization of a broad-specificity non-haem iron N-demethylase from Pseudomonas putida CBB5 capable of utilizing several purine alkaloids as sole carbon and nitrogen source. Microbiology 2011, 157: 583-592.

[11] R. M. Summers, T.M. Louie, C. L. Yu, L. Gakhar, K. C Louie and M. Subramanian. Novel highly specific N - demethylations enable bacteria to live on caffeine and selected purine alkaloids. J. Bacteriol. 2012, 194: 2041-2049.

[12] P. Mazzafera. Catabolism of caffeine in plant and microorganisms. Front. Biosci. 2004, 9:1348-1359.

[13] S. A. Roussos, M. de los Angeles, M. del Refugio Trejo - Hernandez, I. Gaime Perraud, E. Favala, M. Ramakrishna, M. Raimbault, and G. Viniegra-Gonzales.1995. Biotechnological management of coffee pulp-isolation, screening, characterization, selection of caffeine-degrading fungi and natural microflora in coffee pulp and husk. Appl. Microbiol. Biotechnol. 1995, 42:756-762.

[14] F. E. Vega, A. Simpkins, M. C. Aime, F. Posada, S. W. Peterson, S. A. Rehner, F. Infante, A. Castillo, A. E. Arnold. 2010. Fungal endophyte diversity in coffee plants from Columbia, Hawaií, Mexico, and Puerto Rico. Fungal Ecol. 2010, 3, 122-138.

[15] A. H. Pacheco Bustos, J. Pohlan, and M. Schulz. Allelopathic

effects of aromatic species intercropped with coffee: investigations of their growth stimulation capacity and potential of caffeine uptake in Puebla, Mexico. Allelopathy J. 2008a, l 21, 39-56.

[16] A. H. Pacheco Bustos, J. Pohlan, and M. Schulz. Interaction between coffee (Coffea arabica L) and intercropped herbs under field conditions in the Sierra Norte of Puebla, Mexico. J. Agricult. Rural Develop. Tropis and Subtropis 2008b, 109:85 A.M -93.

[17] T. J. White, T. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Shisky, J.J., White, T.J. (Eds.) PCR protocols: a guide to methods and applications, Academic press, San Diego, pp 315-322.

[18] Z. F. Yu, M. Qiao, Y. Zhang, and K. Q. Zhang. Two new species of Trichoderma from Yunnan, China. Antonie van Leeuwenhoek 2007, 92:101-108.

[19] M. N. Widjojoatmodjo, A. D. C. Fluit, and J. Verhoef, J. Rapid identification of bacteria by PCR-single strand conformation polymorphism. J. Clin. Microbiol. 1994, 32:3002-3007.

[20] K. E. Nelson, C. Weinel, I. T. Paulson, R. J. Dodson, H. Hilbert et al. Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440. Envir. Microbiol. 2002, 4:799-808.

[21] S. S. Dash, N. S. Sailaja, and S. N. Gummadi. Chemotaxis of Pseudomonas sp. To caffeine and related methylxanthines. J. Basic Microbiol. 2008, 48:130-134.

[22] C. L. Yu, T. M. Louie, R. Summers, Y. Kale, S. Gopishetty, and M. Subramanian. Two distinct pathways for metabolism of theophylline and caffeine are co-expressed in Pseudomonas putida CBB5. J. Bacteriol. 2009, 191: 4624-4632.

[23] I., Samolski, A. de Luis, J. A. Vizcaino, E. Monte, and M. B. Suarez. Gene expression analysis of the biocontrol fungus Trichoderma harzianum in the presence of tomato plants, chitin, or glucose using a high-density oligonucleotide microarray. BMC Microbiol. 2009, 9: 217.

[24] G. E. Harman, C. R. Howell, A. Viterbo, I. Chet, and M. Lorito. Trichoderma species - opportunistic, avirulent plant symbionts. Nature Rev. Microbiol. 2004, 2: 43-56.

[25] P. Hohmann, E.E. Jones, R.A. Hill, and A. Stewart. Understanding Trichoderma in the root system of Pinus radiata: associations between rhizosphere colonisation and growth promotion for commercially grown seedlings. Fungal Biol. 2011, 115: 759–767.

[26] P. Hohmann, E. E. Jones, R. A. Hill, and A. Stewart. Ecological studies of the bio-inoculant Trichoderma hamatum LU592 in the root system of Pinus radiata. FEMS Microbiol Ecol.2012, 80: 709–721.

[27] A. Schuster, and M. Schmoll, M. Biology and biotechnology of Trichoderma. Appl. Microbiol. Biotechnol. 2010, 87: 787-799.

[28] H. Bae, R.C. Sicher, M.S. Kim, S.H. Kim, M. D. Strem, R. L. Melnick, and B. A. Bailey. The beneficial endophyte Trichoderma hamatum isolate DIS219b promotes growth and delays the onset of the drought response in Theobroma cacao. J. Exp. Bot. 2009, 60: 3295-3295.

[29] H. C. Evans, K. A. Holmes, and S.E. Thomas. Mycobiota of an indigenous Theobroma species (Sterculiaceae) in Ecuador: assessing its potential for biological control of cocoa diseases. Mycol. Progr. 2003, 2:149-160.

[30] Schulz M., P. Kussmann , M. Knop, B. Kriegs, F. Gresens, T. Eichert, A. Ulbrich, F. Marx, H. Fabricius , H. Goldbach, G. Noga. 2007. Allelopathic monoterpenes interfere with Arabidopsis thaliana cuticular waxes and enhance transpiration. Plant Signal. Behav. 24: 231-239.

[31] J. I. Vandermeer, J., I. Perfecto, and S. Philpott. Ecological complexity and pest control in organic coffee production; uncovering an autonomous ecosystem service. Bioscience 2010, 60: 527-537.

[32] C. Potvin, C. T. Owen, S. Melzi, and P. Beaucage. Biodiversity and modernization in four coffee-producing villages of Mexico. Ecol. Society 2005, 10, 18.