Mycorrhiza (2011) 21:443–449
DOI 10.1007/s00572-011-0385-z

SHORT NOTE

The sterol biosynthesis inhibitor molecule fenhexamid impacts the vegetative compatibility of *Glomus clarum*

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Received: 23 December 2010 / Accepted: 19 April 2011 / Published online: 7 May 2011

Abstract The vegetative compatibility of the arbuscular mycorrhizal fungus (AMF) *Glomus clarum* MUCL 46238 was evaluated after continuous exposure to fenhexamid, a sterol biosynthesis inhibitor (SBI). Three lineages of this AMF were cultured in vitro for five generations in association with Ri T-DNA transformed carrot roots in the presence of 0, 5 or 10 mg l^{-1} of fenhexamid. Whatever the AMF generation, fenhexamid at 5 and 10 mg l^{-1} had no significant impact on the number of spores produced. However, vegetative compatibility tests (VCT) conducted with spores from the three lineages, in the presence of 10 mg l^{-1} of fenhexamid, impacted the anastomosis process. At this concentration, the morphology of the germ tubes was modified. In addition, nitrotetrazolium–trypan blue staining revealed that 10 mg l^{-1} of fenhexamid significantly reduced the probability of fusion between the germ tubes regardless of the culture conditions (i.e. absence or presence of fenhexamid) preceding the VCT. Our results demonstrated that spore production was not affected by fenhexamid, while anastomosis between germ tubes was decreased. This suggested that high concentrations, accumulation or repeated application of this SBI fungicide may impact the community structure of AMF in soil.

Keywords Vegetative compatibility test · Fenhexamid · Anastomoses · SBI fungicide · Arbuscular mycorrhizal fungi · Root organ culture

Mycothèque de l'Université catholique de Louvain (MUCL) is part of the Belgian Coordinated Collections of Micro-organisms (BCCM).

Electronic supplementary material The online version of this article (doi:10.1007/s00572-011-0385-z) contains supplementary material, which is available to authorized users.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that form a mutualistic symbiosis with the roots of most existing plants (Smith and Read 2008). They provide the plants with nutrients (e.g. phosphorus) in exchange of carbohydrates derived from the photosynthesis. Their impact on plant growth, resistance against biotic stresses such as the attack by pathogens (Akhtar and Siddiqui 2008) and abiotic stresses such as drought (Ruiz-Lozano 2003; Evelin et al. 2009) has been widely documented. Hence, these microorganisms are considered key players in research programmes oriented toward more sustainable agricultural practices.

The application of AMF in agriculture may be impaired by cultural practices such as the use of fungicides (Plenchette et al. 2005; Mukerji and Ciancio 2007). Several classes of molecules, among which the sterol biosynthesis inhibitors (SBI) have been reported to decrease AMF root colonization (Sukarno et al. 1993; Kjoller and Rosendahl 2000; Campagnac et al. 2008; 2009), spore production (Zocco et al. 2008; Campagnac et al. 2008; 2009), extraradical mycelium biomass (Kjoller and Rosendahl 2000; Campagnac et al. 2009) and phosphorus transport (Zocco et al. 2011).

The extraradical mycelium of AMF is responsible for the uptake of nutrients from soil and their subsequent transport to the plant. It also interconnects plants via large common mycelial networks (CMN) as reported by Newman (1988). These CMN may be formed by means of anastomosis (i.e. hyphal fusion) between independent individual mycelia (Giovannetti et al. 2004; Voets et al. 2006). Anastomoses are expected to facilitate the exchange of resources (e.g. nutrients) (Yao et al. 2003; Moyer-Henry et al. 2006) and genetic material between individuals (Giovannetti et al. 2004; Croll et al. 2009). Consequently, the anastomosis process could play a major role in the organization of genetic diversity in AMF (Bever et al. 2008) and in community structure (Croll et al. 2009). In AMF, the anastomosis is not a process exclusive to the extraradical mycelium. The germ tubes arising from spores also form anastomosis (Giovannetti et al. 1999; 2003), a process which has been proposed as a suitable mean to track vegetative compatibility between individuals (Giovannetti et al. 2003; Cárdenas-Flores et al. 2010). However, anastomoses in germ tube and extraradical mycelium may possibly be regulated differently and may not be completely analogues as reviewed in *Neurospora crassa* by Roca et al. (2005) who observed physiological and morphological differences in anastomosis between mature colonies and germ tubes of conidia.

Even though the impact of fungicides on the AMF morphology and physiology has been widely investigated in the last decade (e.g. Kjoller and Rosendahl 2000; Zocco et al. 2008; Campagnac et al. 2008; 2009), no study has ever reported the influence of these molecules on the anastomosis process.

In the present study, we tested the hypothesis that fenhexamid, a SBI fungicide, may impact the anastomosis process in AMF but that the continuous exposure to this molecule may result in the selection of tolerant phenotypes which are not further affected in their potential to anastomose. The effect of fenhexamid was investigated on the vegetative compatibility in *Glomus clarum*. Successive lineages of these AMF were subcultured over several generations in the absence/presence of fenhexamid and the vegetative compatibility was tested on germinating spores within and between the lineages and in the absence/presence of fenhexamid.

Materials and methods

Biological material

Transformed carrot (*Daucus carota* L.) roots DC1 and *G. clarum* Nicolson and Schenk MUCL 46238 were purchased from the Glomeromycota in vitro collection (GINCO—http://www.mycorrhiza.be/ginco-bel/collection.php). The AMF were provided in the third generation of a root organ culture (ROC) established from a single spore isolated from pot culture in 2004. The AMF were further subcultured in ROC during four generations before the start of the experiment.

Fungicide

Fenhexamid (FHM; CAS No. 126833-17-8), a SBI molecule, was kindly supplied by Bayer CropScience (Germany) in technical grade. The molecule was dissolved in 200 µl of acetone and added to the Modified Strullu–Romand (MSR—Declerck et al. 1998) medium (sterilized at 121°C for 15 min) cooled at 80°C to obtain a concentration of 5 or 10 mg l^{-1} of FHM (Zocco et al. 2008). The treatments without FHM (i.e. the controls) were also amended with 200 µl l^{-1} acetone. This concentration of acetone was below the concentration reported by Zocco et al. (2008) to be non-toxic for *Glomus intraradices* MUCL 43194 and carrot roots.

Lineages of *G. clarum* under fenhexamid stress

Starting from the same mother ROC of *G. clarum*, three distinct lineages were set up and subcultured over five successive generations in MSR medium. The first lineage (termed L1) was subcultured in the absence of FHM but

amended with 200 μl of acetone. In the second lineage (termed L2), the AMF were subcultured in the presence of 5 mg l^{-1} of FHM added to the medium, and in the third lineage (termed L3), the AMF were subcultured in the presence of 10 mg l^{-1} of FHM added to the medium. Each lineage was started with ten randomly selected spores sampled from the mother ROC. Using a sterile dissecting needle, the spores were carefully sampled and inoculated close to the apex of an 8 cm long transformed carrot root clone DC1, placed on a Petri plate (90 mm diameter) containing 50 ml MSR medium with sucrose (10 g l^{-1}) and solidified with 3 g l^{-1} of Phytagel (CAS 71010-52-1, Sigma-Aldrich, USA) supplemented with 0, 5 or 10 mg l^{-1} of FHM.

Fifteen Petri plates were started per lineage and incubated in an inverted position, in the dark at 27°C during 10 weeks to produce a first generation. After 10 weeks culture, five Petri plates per lineage were randomly selected to estimate the total spore production under stereomicroscope (Declerck et al. 1998). For each lineage, the Petri plate with the number of spores closest to the mean of the five randomly selected Petri plates was used to start the next generation of 15 replicates. This process was rigorously repeated five times to produce five successive generations.

Vegetative compatibility tests

Vegetative compatibility tests (VCT) were conducted with the spores produced in the fifth generation of each of the three lineages. The ROC with the number of spores closest to the mean of the five selected ROC in each lineage was used and the spores were recovered following solubilization of the MSR medium with 0.1 M citrate buffer (Bécard and Fortin 1988). The spores were subsequently rinsed with sterile distilled water and separated into single spores with a dissecting needle under a stereomicroscope (Olympus SZ40-CTVT, Japan).

Four square millimeter sterilized (121°C for 15 min) cellophane membranes (Z377600-1PAK, Sigma, USA) were placed in Petri plates filled with 20 ml of MSR medium supplied with sucrose (10 g l^{-1}), solidified with Phytagel (3 g l^{-1}) and supplemented with 0 or 10 mg l^{-1} of FHM as prepared above. Two spores from either the same or different lineages were paired approximately 5 mm apart on each membrane, with their subtending hyphae oriented toward each other. The following combinations were tested: L1 × L1, L2 × L2, L3 × L3, L1 × L2, L1 × L3 and L2 × L3. For each combination, half of the pairings were tested on the MSR medium supplemented with 10 mg l^{-1} of FHM (i.e. the highest concentration of FHM used in the lineage establishment —affix 10-[L]), while the other half was tested on the MSR medium without FHM (i.e. 0 mg l^{-1} of FHM—affix 0-[L]). Thirty-six replicates of each combination were prepared for VCT on both FHM regimes (0 and 10 mg l^{-1}). The Petri plates were incubated in the dark at 27°C in normal position.

After 21 days of incubation, the number of total contacts and the number of fusions between the germ tubes of spores in the given pairings were scored. Additionally, the number of germinated spores was scored in the same lineage pairings (i.e. L1 × L1, L2 × L2, L3 × L3) to estimate the germination rate of each lineage. The frequency of anastomosis formation was evaluated by determining the probability of fusion between the two lineages involved in each pairing (see the "Statistical analysis" section). To confirm hyphal fusion and protoplasm continuity between fusing hyphae, the membranes supporting the pairings were treated using the methodology reported by Cardenas-Flores et al. (2010). In addition, to observe nuclei in hyphal bridges, a different set of three pairing replicates per combination was prepared on both FHM concentrations (0 and 10 mg l^{-1}). For these pairings, 14- to 21-day incubated membranes supporting mycelium with active cytoplasmic flow were stained with 4',6-diamidino-2-phenylindole (DAPI) and analyzed as described by Cardenas-Flores et al. (2010).

Statistical analysis of VCT

Using the XLSTAT software v.2008.5.01 (Addinsoft, 1995–2008), a two-way ANOVA (α=0.05) was performed on the spore production taking the FHM concentration and the number of generation as explanatory variables. Additionally, a two-way ANOVA (α=0.05) was conducted on the number of contacts between germ tubes (dependent variable) considering the FHM concentration (0 and 10 mg l^{-1}) and each of the pairings as explanatory variables.

The anastomosis frequency data were analysed by means of a logistic regression using, for each contact, the presence/absence of fusion as a response variable, two factors, (a) FHM concentration and (b) lineage pairings and their interaction as categorical independent variables. This analysis models the probability of fusion following contact in each of the six pairings in the presence of the two different concentrations of fungicide. The effect of the categorical variable was assessed with a likelihood ratio test, taking the equality of the 12 fusion probabilities as a null hypothesis. Contrasts were computed to compare the probability of fusion of the same stressed lineage pairings to that of different lineage (Wald Chi-squared test; α=0.05). This analysis was performed with the LOGISTIC procedure of the SAS/STAT software 9.1.3 (SAS Institute Inc., USA).

Results

Patterns of hyphal development and sporulation under different FHM regimes

Whatever the lineage and generation, the spores used to initiate the successive generations germinated within 3–5 days following association with the carrot roots. The first runner hyphae were visible within 7–10 days following association with the carrot root and the first incipient spores appeared approximately 14 days following inoculation whatever the lineage and generation.

The number of spores (counted at week 10) produced in each lineage was recorded during the five generations. No significant difference (p=0.053) was observed between the lineages within a same generation. However, a significantly lower (p=0.011) spore production was observed in the first generation (402±31.4) as compared to the fourth (678 spores±102.1) and fifth (731±101.3) generations (supplementary Fig. S1).

Germ tube fusions

During the VCT, the spore germination started after 3 days of incubation on the cellophane membrane whatever the pairing was tested. No significant difference was observed in the germination rate between spores plated in the absence of FHM and FHM at 10 mg l^{-1} whatever the lineage was. For lineage L1, the rate of spore germination was 100% and 91% on 0 and 10 mg l^{-1} of FHM, respectively and for L3, 96% and 94%, respectively. The germination rate for lineage L2 was 100% on both FHM concentrations.

The first contacts between germ tubes were observed within the first week of incubation on both FHM concentrations (0 and 10 mg l^{-1}) tested in VCT. Incidentally, the germ tubes of spores isolated from L1, L2 and L3 presented an undulated morphology in absence of FHM (Fig. 1a). In contrast, the germ tubes of the spores incubated in the presence of 10 mg l^{-1} of FHM, showed a straighter morphology with few traces of undulation (Fig. 1b).

Compatibility and hyphal fusions were observed in the six pairings grown either in the absence (0 mg l^{-1}) or presence (10 mg l^{-1}) of FHM. Both pairing sets with spores developing on medium containing 0 and 10 mg l^{-1} of FHM showed normal hyphal fusions with cytoplasm continuity and nuclei appearance as revealed by the SDH and DAPI staining. No evidences of incompatible reactions were observed among the pairings from the same or different lineages on any of the FHM concentrations tested.

As revealed by the statistical analyses, the FHM concentration in the VCT media did not affect the number of total contacts between germ tubes (supplementary Table S1). However, the probability of fusion (anastomosis frequency) was significantly lower for the spores paired over the medium containing 10 mg l^{-1} of FHM (47.2%) as compared to the spores paired over medium without FHM (i.e. 64.2%). Conversely, the different pairings tested showed significant differences in the number of contacts (L2 × L3 and L3 × L3 the lowest, and L1 × L1 the highest), but the probability of fusion between germ tubes remained the same whatever the pairing (Table 1).

Finally, in some pairings, the number of contacts and the probability of fusion varied according to the FHM concentration on which the VCT were conducted (supplementary Table S1 and Table 1). Accordingly, the pairings formed by the same lineage under no fenhexamid (0-[L1 × L1], 0-[L2 × L2], 0-[L3 × L3]) had a significantly higher probability of anastomosis, while the pairings formed between lineages L2 and L3 under fenhexamid (10-[L2 ×

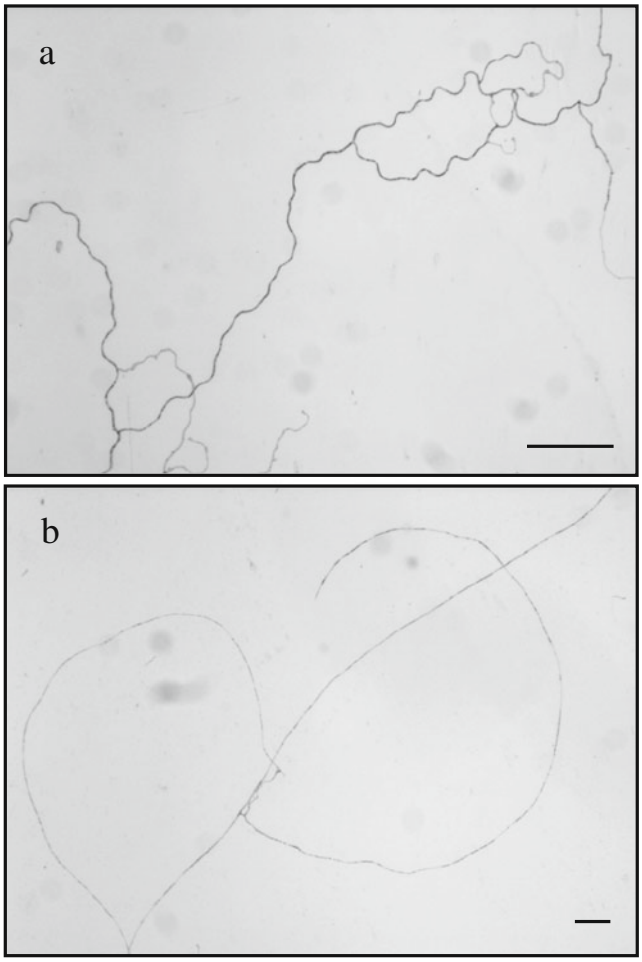

Fig. 1 Bright field micrograph of *G. clarum* MUCL 46238 spores plated on cellophane membranes. Germ tubes were stained with nitrotetrazolium–trypan blue to observe hyphal fusions. **a** Undulated growth of germ tubes on the modified Strullu–Romand medium in the absence of fenhexamid and **b** straight growth of germ tubes on the modified Strullu–Romand medium in presence of 10 mg l^{-1} fenhexamid. *Bar*=50 μm

Table 1 Effect of fenhexamid and pairing over the probability of fusion between germ tubes of *G. clarum* MUCL 46238 lineages in vegetative compatibility tests

Variable	Probability of anastomosis (%)[d]
FHM concentration (mg l^{-1})	p=0.0002
0	64.2[a]
10	47.2[b]
Lineage pairing[b]	
L1 × L1	63.4
L2 × L2	56.5
L3 × L3	59.2
L1 × L2	60.0
L1 × L3	50.9
L2 × L3	44.4
FHM conc. × pairing	p=0.0003
0-[L1 × L1][c]	73.7[a]
0-[L2 × L2]	73.4[a]
0-[L3 × L3]	77.3[a]
0-[L1 × L2]	66.3[a, b]
0-[L1 × L3]	43.5[c, d]
0-[L2 × L3]	51.1[c, d]
10-[L1 × L1]	53.1[b, c, d]
10-[L2 × L2]	39.6[d]
10-[L3 × L3]	41.1[c, d]
10-[L1 × L2]	53.7[b, c, d]
10-[L1 × L3]	58.3[a, b, c]
10-[L2 × L3]	37.7[d]

FHM fenhexamid (SBI fungicide)

[b] Lineages treated with 0 (L1), 5 (L2), or 10 mg l^{-1} (L3) of fenhexamid during five successive generations

[c] Affix 10-[L] refers to pairings tested under 10 mg l^{-1} of FHM; 0-[L] refers to parings tested in the absence of FHM

[d] Mean probabilities in the same column followed by a different letter are significantly different at the Wald Chi^2 test, p indicated in the column heading (α=0.05)

L2], 10-[L3 × L3], 10-[L2 × L3]), presented the lowest values (Table 1).

Discussion

Sterol biosynthesis inhibitors such as fenhexamid are fungicide molecules broadly used for the control of undesired plant fungal pathogens. Their effects on non-target beneficial organisms such as AMF have been explored in the last decade and recently Zocco et al. (2008) demonstrated in vitro the negative impact of fenhexamid on the AMF extraradical mycelium (i.e. development and spore production) at concentration above 20 mg l^{-1}, and an impact on P transfer to the host plant at concentrations of 2 mg l^{-1} (Zocco et al. 2011). Here, we reported for the first time the effects of fenhexamid on the capacity of the AMF *G. clarum* MUCL 46238 to form anastomoses. Even if no significant impact over the spore production was found, we demonstrated that in presence of this SBI molecule, the probability of hyphal fusion between germinating spores decreased markedly.

Our findings about spore production corroborate earlier results by Zocco et al. (2008) and Campagnac et al. (2008) who reported that fenhexamid at concentrations below 20 mg l^{-1} did not impact the in vitro spore production of *G. intraradices*. It is to be noted that summed over the three lineages, the first generation produced significantly less spores than the fourth and fifth generations, while no differences were observed between generations F2 to F5. No firm explanation could be found to elucidate the difference with the first generation but it may be hypothesized that the age of the spores used as initial inoculum affected the spore yield because the mother culture (i.e. F0) was more than 25 weeks old as compared to the 10-week-old spores used to start the F2 to F5.

Strikingly, *G. clarum* presented a high degree of intrinsic tolerance to FHM because germination and growth were possible in every generation at both FHM concentrations (5 and 10 mg l^{-1}). This suggested, as earlier observed in *Botrytis cinerea* (grey mould), that *G. clarum* possesses enzymatic complexes in the SB pathway poorly sensitive to FHM or that they have the capacity to avoid the toxicity of FHM (Fillinger et al. 2008; Leroux et al. 2002).

In the VCT, the spores from the three lineages (L1, L2 and L3) incubated on the MSR medium containing 10 mg $l^{-1}$of fenhexamid had identical germination rates than the spores incubated in the absence of fenhexamid. However, the germ tube morphology in the presence of fenhexamid consisted of straight hyphae compared to the undulated morphology of the spores incubated in the absence of fenhexamid. In previous studies, a reduction in spore germination rates caused by SBI fungicides was observed with *G. intraradices* (Zocco et al. 2008), *Glomus monosporum* or *Glomus mosseae* (Dodd and Jeffries 1989). In these studies, it was postulated that the sterol biosynthesis pathway is active during germination, and that sterols play an essential role in AMF cell membrane and cell wall synthesis. Even though we did not observe any impact on germination rates, the modification in germ tube morphology may possibly also be attributed to a modification in the germ tube sterol profile (composition and/or quantity). Evidences that SBI fungicides modify AMF hyphal architecture (Zocco et al. 2008) and sterol profiles (Campagnac et al. 2009) were recently provided. These last authors reported modifications of the sterol profiles of the extraradical mycelium of *G. intraradices* MUCL 43194 caused under the increasing concentrations of

fenpropimorph. Unfortunately, even though a negative impact on spore and fungal biomass production was recorded, no precisions about hyphal morphology were provided.

The presence of fenhexamid at 10 mg l^{-1} in the MSR medium affected the probability of fusion between germinating spores but not the total number of contacts between the germ tubes. Contrastingly, although the lineages were produced during five generations on different concentrations (i. e. 0, 5 or 10 mg l^{-1}) of fenhexamid, the pairings that were tested showed the same probability of fusion. Such results indicate that the vegetative compatibility between lineages was only affected by the SBI molecule and not affected by the subcultivation process (over a period of five generations). Interestingly, on the 10-[L] VCT, the probability of hyphal fusion was affected in pairings involving the three lineages but more deeply in those involving L2 and L3. These results pointed out that the continuous exposure to the SBI fungicide during subcultivation did not induce an increased tolerance of *G. clarum* to this molecule because when anastomosis was tested in VCT with 10 mg l^{-1} of FHM, the germ tubes of spores from L2 and L3 lineages had the same or lower probability to form hyphal fusions than those obtained from the L1 lineage.

Since no incompatibility reactions were observed between the germ tubes and since the morphology of the germ tubes in the presence of fenhexamid did not inhibit the hyphal fusions, the results suggested that the decreased probability of fusion in the presence of 10 mg l^{-1} of FHM could be related to perturbations during the pre-contact phase (i.e. before any contact between hyphae takes place; Glass et al. 2004). As described by Leslie and Zeller (1996), it is probable that the hyphal homing-fusion process was affected in the presence of fenhexamid because the recognition signaling between hyphae was perturbed. It has been shown that sterols and sphingolipids form microdomains of cellular membranes called lipid rafts (Simons and Ikonen 1997; Mukherjee and Maxfield 2004) which may function as signaling platforms (Simons and Ikonen 1997; Simons and Toomre 2000; Golub et al. 2004). Indeed, in a recent paper, Jin et al. (2008) demonstrated by means of SBI molecules, that the decrease of ergosterol seriously inhibited the pheromone signaling and cell membrane fusion in yeast mating. Thus, it could be hypothesized that the fenhexamid molecule caused a similar effect in the germ tubes of *G. clarum* reducing the probability of fusion between them. It is obvious, however, that deeper analyses on the sterol synthesis and composition of the *G. clarum* germ tubes need to be conducted to corroborate this hypothesis.

In this study, we showed evidences by means of a two-dimensioned in vitro cultivation system that fenhexamid, a broadly used SBI molecule, may impact AMF by decreasing the formation of germ tube fusions. This suggested that, under field conditions, the repeated application and/or accumulation of SBI molecules may potentially result in the disruption of hyphal fusions thus representing a serious risk for the shaping of proper community genetic structures. It has been revealed that AMF possess a wide genetic variability (Kuhn et al. 2001; Koch et al. 2004; Cárdenas-Flores et al. 2010) and several reports indicated that anastomoses play a key role in AMF genetic diversity (Bever and Morton 1999; Giovannetti et al. 2003; 2004; Bever et al. 2008; Croll et al. 2009; Cárdenas-Flores et al. 2010). Therefore, impacting the capacity of anastomoses between individuals may have severe consequences on AMF community structure in agro-environments.

Acknowledgements This work was supported by the CONACyT (Mexico) grant no. 217083 and by the Bourse de Cooperation au Développement (UCL/ADRI, Belgium) UCL register no. 8108-05-00. We are grateful to Bayer Crop Science for kindly providing the fenhexamid. S.C. gratefully acknowledges the financial contribution of the Belgian Federal Science Policy Office (contract BCCM C4/00/001).

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