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ABILITY OF TROPICAL FOREST SOILS OF FRENCH GUIANA AND REUNION TO DEPOLLUTE WOODS IMPREGNATED WITH BIOCIDES

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Abstract
Our study sought to fine-tune knowledge about those microorganisms, particularly wood-decaying fungi degrading pollutants in situ. With a view to the depollution or bioremediation of treated woods, wood-decaying microorganisms from tropical forest soils in French Guiana and the island of Reunion were assessed for their ability to degrade toxic biocides such as pentachlorophenol (PCP) or copper chromium arsenic compounds (CCA). The degradation of red pine (Pinus resinosa) test pieces was monitored and it was found that the soil from French Guiana was more efficient than the soil from Reunion in terms of microbial activity in relation to these two biocides. A significant difference in weight loss was found for the red pine test pieces treated with CCA and PCP, varying in a ratio of one to two (18% and 30%, respectively). In addition, a study of wood and soil fungus communities using D-HPLC and CE-SSCP, then analysed by a PCA, showed that biocide products leached into the soil had an impact on the fungus communities, which differed depending on the sampling time and on the wood treatment. Lastly, these results confirmed that CCA was less leachable and less degradable by microorganisms in these soils than PCP.

Key words*: depollution; treated woods; copper chromium arsenic (CCA); wood-decaying fungi; pentachlorophenol (PCP).*

INTRODUCTION
State of the art

As time goes by the substance of wood, an organic matter, is degraded, especially when exposed to pathogens such as fungi, bacteria and insects. In order to prevent this, several preservation treatments have been developed and applied thanks to the development of biocidal chemicals. The techniques used are notably surface application or impregnation, soaking and different deep impregnation techniques, vacuum and pressure, double vacuum (Fougerousse 1979). They are processes based on chemicals containing heavy metals or aromatic compounds that protect the material from biotic degradation. However, the products used are highly toxic. In 1998, the European Union issued the Biocide Directive 98/8/CE and in 2012 the biocidal products regulation BPR 582/0212, which came into force on 1st September 2013. The regulation seeks to harmonize the scientific assessment and marketing of biocides to ensure that they do not pose a risk for human and animal health and for the environment. However, these products raise some unsolved problems of toxicity, persistence, and their end-of-life fate in treated materials (MEDDE[1] 2013). For example, wood that is treated in this way cannot be burnt, because of the atmospheric pollution it would cause; nor can it be reused due to its toxicity (MDDEP[2] 2011). It is therefore often stored, leading to pollution of the underlying soils by leached toxic products. Although these products can be broken down in the soil by microorganisms, the degradation proves to be very slow. Among these microorganisms, wood-decaying fungi are known to degrade biocidal products and can be used as a biorestoration agent (Anastasi *et al*. 2008; Kamei *et al.* 2010). Gold and Alic (1993) discovered the degradation of a polychlorodibenzodioxin by the Basidiomycete *Phanerochaete chrysporium*. These authors were able to determine the degradation mechanism brought into play by this white rot fungus by characterizing the metabolites synthesized and the oxidation products generated from lignin, notably involving some manganese peroxidases (MnP). A review paper published by Chang in 2008 took stock of the degradation of dioxin-type compounds by microorganisms and, in particular, on the two possibilities of degradation by fungi, either by way of cytochrome P450, or by way of peroxidases and notably lignin peroxidase (liP) and MnP (Valli *et al.* 1992). Then, other authors confirmed the merits of these metabolic pathways in the degradation of certain chemical compounds: Sakaki and Munetsuna (2010) and Suhara *et al.* (2011) took a particular interest in cytochrome P450 while Manji and Ishihara (2004) focused their work on peroxidases. These enzymes, along with laccases, are enzymes that catalyse the process of the extracellular degradation of lignin by wood-decaying white rot fungi (Hiratsuka *et al.* 2005). For instance, in 2010, Sakaki and Munetsuna, published an article on the feasibility of bioremediation methods using different wood-decaying white rot fungi. In 2003, Andersson *et al.* showed that the degradation of chlorinated dioxins called for the use of white rot or stringy rot fungi, known to be involved in lignin degradation. Some other authors, such as Pointing (2001), found many other white rot fungi that could act as bioremediation agents, such as *Phlebia Lindtneri, Ceriporia, Phanerochaete sordida, Bjerkandera* sp.*, Cordyceps sinensis, Coriolus hirsutus, Phlebia radiata, Phlebia brevispora, Pseudallescheria boydii, Coprinellus* spp. However, the degradation of some heavy metals seems to be down to brown rot fungi, such as the species *Antrodia vaillantii* (Sierra-Alvarez 2009). In addition, although these fungi do not completely degrade treated woods or totally remediate the toxicity of polluted soils, they can be used as a pre-treatment with a view to reducing the quantity of organic substance prior to more radical treatment, such as incineration (Valentin *et al.* 2009).

STUDY OBJECTIVE

In 2013, an initial study was launched to explore microbial biodiversity in contact with woods and soils treated with pentachlorophenol (PCP) and with copper chromium arsenic based compounds (CCA) (Zaremski *et al.* 2012). The first culturing and molecular analyses led to the isolation and identification of forty strains of ascomycetes able to develop on wood and resist pollutants. In addition, the study indicated that biocides leached into the soil had an impact on the fungal communities, which differed depending on the sampling time**.** As a logical follow-on, the purpose of this study was to examine the ability of microorganisms in soils from French Guiana to degrade red pine, *Pinus resinosa*, and certain tropical woods, along with the ease with which they degraded the same woods treated with PCP or CCA. In terms of wood degradation, a comparison was made between soils from French Guiana and the island of Reunion, based on weight loss, in order to reveal fungal activity. Alongside these studies, the fungal communities of the woods undergoing degradation were monitored by D-HPLC (Denaturing High Performance Liquid Chromatography) and CE-SSCP (Capillary Electrophoresis Single-Strand Conformation Polymorphism), then analysed by PCA (Principal Component Analysis).

The whole study was carried out on red pine poles treated with PCP or CCA. The latter is particularly used to treat woods exposed to the elements. The copper is used as a fungicide, the arsenic as an insecticide and the chromium is used to fix the molecules in the wood. While copper as

[1] Ministry of Ecology, Sustainable Development and Energy, France.
[2] Ministry of Sustainable Development, the Environment and Parks, Quebec.

low toxicity for mammals, arsenic is known to be carcinogenic in humans (Sierra-Alvarez 2009). On the other hand, PCP is no longer permitted in constructions in contact with humans since the 1990s in many countries. Although its use is in decline, or even zero, in some European countries such as France, thanks to the Biocide Directive, some treated woods intended for industrial purposes, such as electricity poles or railway sleepers, were still being impregnated at the beginning of the 2000s. Indeed, PCP is a powerful biocide that affords strong resistance to degradation agents, although some fungi are known particularly to alter polychlorophenols, including PCP (Mileski *et al.* 1988), and others alter heavy metals including CCA (Sierra-Alvarez 2009); to date, no simultaneous treatment to depollute woods treated by and soils polluted by PCP and CCA has been developed. Given the wide diversity of species making up tropical ecosystems, it is highly likely that tropical soils are particularly rich in microorganisms (Berrin *et al*. 2012). In addition, the conditions of the humid tropical environment are ideal for good development of wood-decaying fungi. Three criteria essential for their life and their survival are inherent to the Guianese forest: mean temperatures that vary little around 26°C (Blancaneaux 1981), the ideal temperature for fungus growth (Kamei *et al.* 2010), high average humidity and abundant rainfall, with the soil litter being regularly renewed and thereby providing the nutrients needed by wood-decaying fungi.

MATERIALS AND METHODS

Biological material

The tests on polluted woods were carried out on core samples 1 cm in diameter and between 1.5 and 4cm long, taken from poles made of red pine, *Pinus resinosa,* treated with CCA or PCP (photograph 1). It needs to be said that PCP is known to migrate or be leached from wood to soil very rapidly. Consequently, in the case of woods treated with PCP, two types of samples were taken: one at the soil line and the second one metre above the soil line. For the pine core samples treated with CCA, samples were only taken one metre above the soil line. The experiment could thus show to what extent the distribution of the product in the pole affected biodegradation by fungus species. For the tests on polluted woods from French Guiana, the controls were red pine and wild nutmeg, *Virola surinamensis*. The former were 1 cm in diameter and were around 4cm long, the latter measured [5 mm × 10 mm × 100mm] (Fig.1). The polluted wood samples were taken from red pine electricity poles treated with CCA or PCP.

Fig. 1.
Samples of woods non treated and treated with PCP and CCA

Degradation of red pine test pieces in a soil from Reunion

Red pine test pieces treated with PCP or with CCA were placed in soil from the Bassin Plat station at Saint Pierre in Reunion. The test pieces were divided into three experimental designs, each corresponding to a treatment: CCA, PCP_{soil} or PCP_{1m}. For the tests on polluted woods in Reunion, all the controls were red pine with test pieces measuring one centimetre in diameter and around 4 cm long. They were examined after being left in the soil for a year.

Degradation of red pine test pieces in a Guianese soil

Two experimental designs were set up:

- Some red pine test pieces treated with PCP or CCA were placed in forest soil from the Point de Combi station, which is part of the CIRAD network of permanent plots in French Guiana. The test pieces were divided into three experimental designs, each corresponding to a treatment: CCA, PCP_{soil} or PCP_{1m}. Each design comprised three replicates which corresponded to three containers per treatment. Each container held 126 test pieces: between 70 and 80 treated test pieces and between 46 and 56 controls test pieces (Fig. 2). In all, 1,139 test pieces made up the experiment. The test pieces used to compare the degradation of the treatments were examined after two years in the soil.

- An identical design to that in Reunion was set up in the Guianese soil, near the Wood Sciences Laboratory in Kourou. The test pieces used to compare with the soil from Reunion were examined after five months in the soil.

Fig. 2.
Container WITH 126 test pieces: between 70 and 80 treated test pieces and between 46 and 56 controls test pieces

Weight loss measurement

The test pieces used to calculate weight loss were identified, brushed, washed and wiped, then weighed once at the moisture rate immediately after washing using a Sartorius CP224S balance and Software Wedge software. Then, the test pieces were placed in an oven at 103°C for 48 hours. Lastly, the test pieces removed from the oven were weighed a second time to obtain their anhydrous weight. Weight loss calculation was as follows: [$\Delta M = ((M_0 - M_1) / M_0)*100$] where: ΔM is weight loss as a percentage; M_0 is the anhydrous weight of the test pieces in grams before being placed in the tests; M_1 is the anhydrous weight of the test pieces taken from the experimental designs.

Statistical interpretation

The analysis of test piece weight losses sought to assess any possible significant differences in weight loss between several sites or treatments. It turned out that the variances of these data did no display a normal distribution, even after log transformation of the variables, which was the transformation best suited to the distribution, after the more general Box-Cox. Consequently the type of test chosen was a non-parametric Kruskal-Wallis one-way analysis of variance. Then, if that test made it possible to reject the hypothesis H_0 of unicity of the population by a P-value > 0.05, the data were subjected to a Siegel & Castellan multiple comparison test assessing the differences between groups; the statistical analyses were carried out with R 3.0.1. software.

Monitoring of fungus communities during degradation

The wood test pieces were taken randomly from the containers in September 2010, February and July 2011, and March 2012. The DNA of the wood and the soil were extracted by the “Fast DNA Spin Kit For Soil” (MP-Biomedicals) following the required recommendations. The wood was broken up beforehand with a rasp file prior to extraction. The extracted DNA was quantified by spectrophotometry (Nanodrop by Thermo Scientific). The ITS region (Internal Transcribed Spacer) of the fungi was amplified by the ITS 1 and ITS 4 primers (WHITE *et al.*, 1990) and the amplification products were analysed by D-HPLC following the method described by Zaremski *et al.* (2011). The profiles obtained were translated into digital values where each peak corresponded to an OTU

(Operational Taxonomic Unit). These profiles were compared by a standardized principal component analysis (Pearson) using R statistical software (FactoMineR package).

RESULTS AND DISCUSSION

Degradation of red pine test pieces in a soil from Reunion

Differences in the degradation of the red pine test pieces in a soil from French Guiana and a soil from Reunion were estimated from the weight loss differences of the samples in each soil. This variation (Fig. 3) showed the degrees of weight loss scatter for each type of soil. The weight loss of the test pieces tested in Reunion was lower than for the test pieces tested in French Guiana. For CCA, the difference was around 5% and, twice that figure for PCP at around 10%.

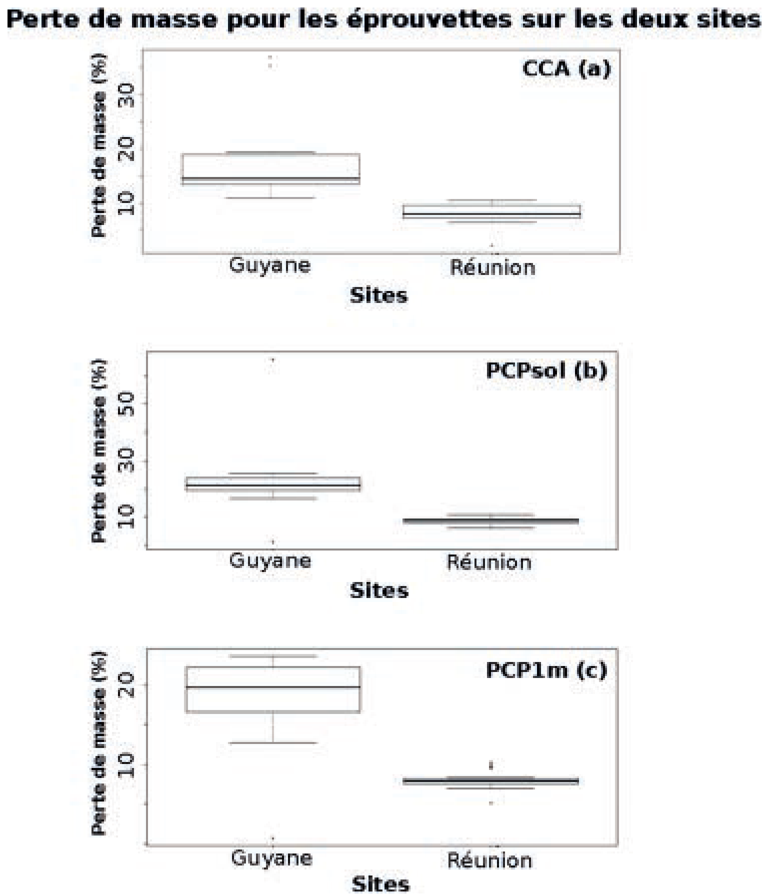

Fig. 3.

Comparison of weight losses for red pine test pieces having undergone different treatments, CCA (a), PCPsoil (b) and PCP1m (c), between two sites in two geographical regions (French Guiana and Reunion)

Degradation of red pine test pieces in a soil from French Guiana

- **Weight loss of treated test pieces**

The differences in degradation of the red pine test pieces for the three types of treatments (CCA, PCP_{soil}, PCP_{1m}) were estimated from the differences in sample weight loss for each treatment. This variation (Fig. 4) showed the different degrees of scatter in weight loss for each treatment. The weight loss of the test pieces treated with CCA was slightly scattered around 18%, while for the test pieces treated with PCP it was almost twice as great, at 30%. Thus, the differences in weight loss were significant between the test pieces treated with CCA and those treated with PCP. On the other hand, the difference was not significant between the two treatments PCP_{soil} and PCP_{1m}.

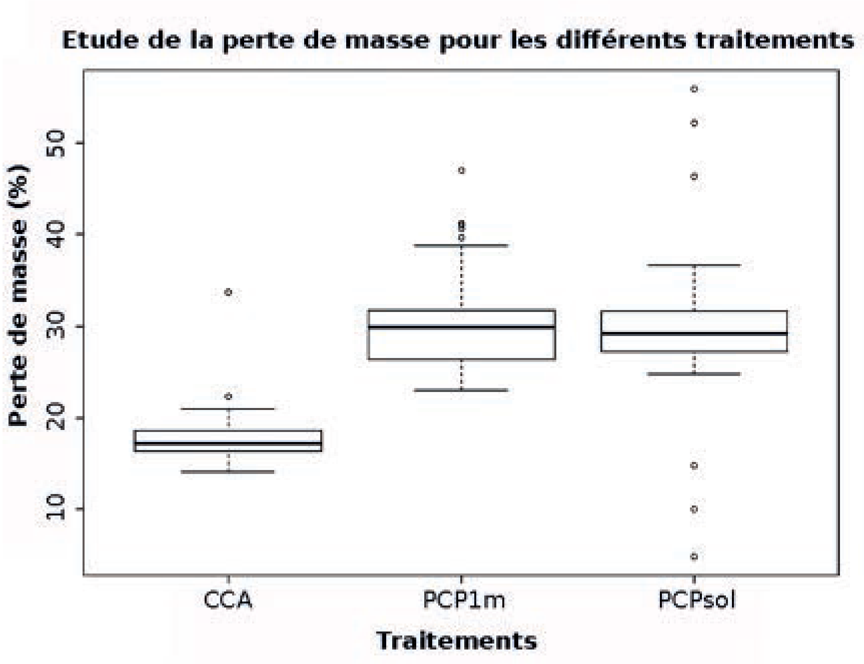

Fig. 4.
Comparison of weight losses for red pine pieces with three types of treatments (CCA, PCP1m, PCPsol)

- **Weight loss of the control test pieces**

The containers whose test pieces were treated with CCA and PCP_{soil} also contained some red pine and wild nutmeg control test pieces. For those containing the test pieces treated with PCP_{1m}, they only contained wild nutmeg control test pieces. The comparison of weight losses (Fig.5) showed that in the containers of the CCA and PCP_{soil} treatments, the wild nutmeg test pieces were totally degraded, while not all those in the PCP_{1m} containers were so. These control test pieces had an average weight loss of 88% (Standard deviation = 2.829 and Variance = 5.337). The red pine control test pieces were never totally degraded. In fact, those in the CCA containers had a weight loss that varied between 65% and 85%, while those in the PCP_{soil} containers varied between 45% and 65%.

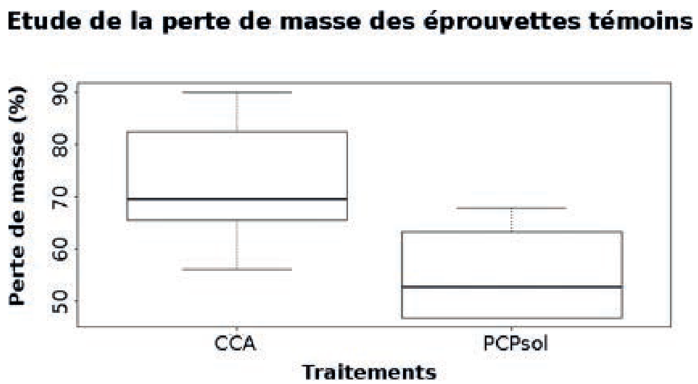

Fig. 5.
Comparison of weight losses for red pine control test pieces for the CCA and PCPsoil

The tests set up in a soil of French Guiana and a soil of the island of Reunion showed a significant difference in degradation of the test pieces for red pine, with a better degradation capacity in favour of the Guianese soil. The time spent in the soil strengthened these results accordingly since the test pieces installed in Reunion (10 months) spent twice as much time in the soil as those installed in French Guiana (5 months). These results highlight the merits of carrying out tests on the degradation of woods treated with CCA and PCP in French Guiana (Point de Combi), rather than at the Bassin Plat site in Reunion.

The tests carried out on the woods showed a significant difference in weight loss for the red pine test pieces treated with CCA and PCP, within a ratio of around one to two (18% and 30%

respectively). According to the weight loss scale in the laboratory tests (table I), these weight loss percentages, of 18% and 30%, would class the test pieces treated with CCA or PCP as non-durable and perishable respectively, after three years in contact with the soil, yet they were treated at the outset to be highly durable. This differentiation between the woods treated with CCA and PCP was also found during the analyses of the wood-decaying fungus communities. It appeared that the CCA treatment greatly inhibited the fungus communities at the start of the experiment. Conversely, even though the statistical tests did not enable us to conclude that there was a significant difference between the CCA and PCP_{soil} treatments, the weight loss of the control test pieces in the containers holding test pieces treated with CCA was greater than that of the control test pieces in the containers holding test pieces treated with PCP_{soil}. It should be noted that the P-value was quite low, barely over 0.05 (P-value = 0.0550 for df = 1 and $\chi 2$ = 3.681), though it was enough to signify this relation, which revealed the greater leachability of PCP compared to CCA. In addition, the wild nutmeg control test pieces were totally degraded for both treatments, whereas they were not entirely degraded in the containers of the PCP_{1m} treatment.

The leaching capacity of the two treatments was different: CCA displayed low leachability whereas PCP was much more leachable (Fougerousse, 1979). The wood-decaying organisms had greater difficulty degrading the test pieces treated with CCA, which was much more present in the wood, whereas they more easily degraded the wood treated with PCP, which was more easily leached. In addition, as the CCA was consequently not widely spread within the soil of the containers, the microorganisms were fairly well developed there and considerably degraded the control test pieces. On the other hand, as the PCP was widely distributed throughout the soil of the containers, the microorganisms had greater difficulty in maintaining themselves and degraded the test pieces less. An analysis of the soil fungus communities in contact with the test pieces (data not shown) in February 2011 showed that the three types of treatments could be distinguished from each other. This difference was also found between the two PCP treatments, as the wild nutmeg test pieces in the PCP_{1m} containers were not totally degraded, whereas wild nutmeg is a species with very low durability. There was probably more PCP in the PCP_{1m} test pieces than in the PCP_{soil} test pieces. Indeed, the latter treatment corresponded to the bottom of the electricity poles in contact with the soil where leaching and biological activity were much greater when they were used as poles. Comparing these results with those obtained by (Andersson *et al.*, 2003), dealing with the degradation of certain toxins by microorganisms, a question can be raised: Might this decrease in degradation by wood-decaying microorganisms has been due to the by-products of PCP degradation? It would be wise to identify them to estimate their toxicity. The differences between the containers for the same treatment confirmed that there was a difference in weight loss between the test pieces treated with CCA and those treated with PCP, and unicity in the weight losses for the PCP_{soil} and PCP_{1m} treatments.

Table 1

Weight loss in the laboratory

Weight loss in the laboratory	Durability class
Over 25%	Perishable
Between 15 and 25%	Slightly durable
Between 10 and 15%	Moderately durable
Between 5 and 10%	Durable
5% or under	Very durable

CONCLUSION AND PROSPECTS

Wood is a material that can be highly susceptible to its environment depending on the species used. It is therefore useful to strengthen its natural durability by chemical or natural procedures. Up to the 1980s, a first generation of biocides was used, notably polychlorophenol (PCP) formulated in petroleum solvents. PCP is a coal-tar derivative that is easily leached by water and, through its run-off, it pollutes the soil it infiltrates. PCP has therefore been banned by the authorities and replaced by a second generation biocide, copper chromium arsenic (CCA) compounds, which are less toxic and more effective, but they have now been banned by so-called European "biocide" directives. At the moment, some third generation biocides are being marketed, such as the Propiconazole/Tebuconazole combination or other different combinations of fungicides depending on the uses.

The issue of recycling or destroying woods treated with 1st and 2nd generation biocides is raised today with particular acuteness due to changes in the regulations, which oblige wood manufacturers to manage these products when they reach the end of their life span. This problem concerns not just woods at the end of their life and withdrawn each year, but also stocks that have accumulated over years, sometimes stored in unsuitable conditions, and the impacts on the environment are considerable: pollution of the air, soil and water tables, etc.

The microorganisms in Guianese soils offer a major advantage: unlike chemical or thermal techniques, they break down pollutants rather than just extracting them. In addition, unlike bacteria that are specific to a pollutant, these microorganisms, such as wood-decaying fungi, are capable of degrading a broad panel of toxic substances using numerous extracellular enzymes that they secrete, such as lignin peroxidase, manganese peroxidase and cytochrome P450.

However, two questions remain unanswered as regards PCP: Was the reduction in microbial activity, revealed through the weight loss of the control test pieces, only due to PCP leaching? Might the microbial communities have degraded the leached PCP? In order to answer that question, other tests need to be programmed at the Kourou site as part of future studies. It will be a matter of identifying these microorganisms and characterizing them using culturing and molecular methods. Other molecular and biochemical studies will be undertaken on the involvement of these enzymes in degrading chemical compounds that are highly toxic for both humans and the environment. Another prospect will be the further use of woods following their depollution, meaning that they can be recycled and go to sectors using reject wood, such as energy production from biomass. Other types of use are also being studied for ecological purposes, such as the treatment of wastewater sludge, the depollution of storage sites or wood-based compost activators.

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