Evaluation of the Microbial Viability of Soil Samples from Maize Crops in Freeze-Storage under Different Management Conditions in a Semi-Arid Climate

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Abstract: The effect of freeze-storage on culturable soil microorganism viability was determined for soil samples from three agricultural maize crop systems (under conventional management, direct seeding, and direct seeding with a cover crop). Most culturable soil bacteria were unaffected by the freeze-thaw stress or the prolonged freeze-storage, but the viability of mold and *Bacillus mycoides* populations decreased drastically after eight months of freeze-storage, limiting the process sustainability to this period. However, these changes did not significantly affect either the total microorganism biodiversity index or the biodiversity differences between treatment. Based on the available results, freeze-storage seems to be a sustainable process for up to eight months that can be allowed in analyses of culturable microbial population biodiversity.

Keywords: microbial viability; maize crop soil; freeze-storage; conservation management

1. Introduction

When the immediate assay of soil samples is unfeasible, recommendations for soil handling often include refrigeration or freezing in the interim between collection and assay, even if the storage period is likely to be short, although this may perturb soil microbial community [1]. Whereas the ISO standard [2] explicitly warns against freezing as a soil storage method for the laboratory assessment of aerobic microbial processes, the Organisation for Economic Cooperation and Development (OECD) guideline [3] suggests that soils can be freeze-stored ($-20\,^\circ$C $\pm\ 2\,^\circ$C) for one year. Some studies have shown that freeze-storage is a more sustainable process than cold storage for monitoring microbial biomass (i.e., Stenberg et al. [4]). In this case, it is important to determine the effect of freeze-storage on the survival of culturable soil microorganisms for later microbial population analysis. This effect has been studied indirectly by focusing on particular microbial activities such as methidathion degradation [5], nitrification and denitrification [6–8], and hydrocarbon biodegradation [9]. Also, the soil microbial biomass has been measured by the substrate-induced

respiration (SIR) method [10] after frozen and cold storage. All these studies showed that freeze-thaw stress affects soil microbial populations, reducing or increasing some biological activities [8,11], and changing microbial diversity [5,12]. In addition, other studies have indicated preservation methods should be accurately evaluated for each soil before DNA extraction [13,14]. In this sense, Wang et al. found that while soil storage at $-20\,^{\circ}$C without reconditioning was the best for drained soils, storage at $4\,^{\circ}$C with reconditioning was the best for flooded soils [15] and the study of Rubin et al., clearly states that the storage time and temperature affect the bacterial community composition and its structure, pointing to the importance of our own study [16].

It is generally believed that only a small proportion of the species within soil microbial communities can be cultured and isolated using standard laboratory media [17]. Several non-culturing procedures that depend on nucleic acid extraction and PCR gene amplification are being widely used to overcome that experimental limitation, and indeed molecular techniques have become the preferred approach to analyze soil microbial community structure and dynamics [18,19]. However, discrepancies have been found between culture-dependent and culture-independent methods. Both techniques result in a certain bias, resolving different fractions of bacterial communities [20,21]. The results of the two strategies are complementary and can be highly useful to assess microbial community stability in soil [21–23]. Also, it has been proposed that culturable bacteria are very important to the soil ecosystem because they are alive and have high metabolic activity [18]. Moreover, it might be possible that the proportion of viable-but-non-culturable soil bacteria is less than it generally appears, and that most dominant non-culturable bacteria were actually non-viable forms of the culturable ones. These non-viable bacteria accumulate in the soil after vegetative growth, waiting for, or already involved in, an eventual degradation process. The accumulation of their ribosomal DNA (rDNA) in the soil leads to the false-positive identification of non-existing bacteria because of the synthesis of a high proportion of rDNA chimeras in the 16S rDNA gene amplification process [24]. Culturable bacteria may therefore be considered as responsive indicators of physical, chemical, and biological changes in the soil environment.

The culture-based approach to measuring microbial parameters is still used by soil microbiologists, including soil dilution and plate colony counts [25], rates of utilization of the carbon sources in plates [26], measurements of some functional groups such as ammonifying and nitrifying bacteria [27], and even the utilization of molecular techniques to measure culturable microorganisms [28]. Frequently, however, it is impossible to perform these analyses immediately after sampling, and freeze-storage is required for the process to be sustainable. In order to obtain a considerable quantity of these microorganisms for the optimum development of this study, we opt for a culture with a high Carbon/Nitrogen (C/N) ratio as, under these conditions, microorganisms with high energy available use nitrogen and other essential elements to develop and multiply, since microorganisms develop by mineralizing organic matter. According to Silva et al., the highest C/N ratio crops that incite an optimum microorganism development are monocultures of sorghum and maize [29] and, for cultural reasons, we opted for the latter. Dignam et al. stated that soil microorganisms must be thoroughly studied in order to develop plans of action that lead to the understanding of the functional potential and properties of soil microbiomes, as this affects pasture productivity [30]. Meanwhile, Bahadur et al.states that using microorganisms as a bio-inoculant is a good practice in agricultural biotechnology for sustainable crop production [31]. Also, Kumari et al. comment that microorganisms solubilize K, making it more available to plant life [32]. The study of Ciancio et al. demonstrated that, among other microbiological life, specific *Bacillus subtilis* strains interfere with fungi, *Trichoderma* sp., and the soil-borne *Ralstonia solanacearum* pathogen colonization, thus defending the installed culture [33]. Additionally, the latter study stated that bacterial metabolites may also protect plants by inhibiting herbivores. Agricultural activity is responsible for shaping the microbial communities in the agro-ecosystem, as the study performed by Embarcadero-Jiménez et al. [34] illustrates, and, as stated by Bender et al., agricultural ecosystem sustainability can be restored by stimulating soil life [35] (in that soil biodiversity supports several ecosystem functions simultaneously,

underpinning its crucial role in ecosystems worldwide). We therefore conclude, along the same lines as Keesstra et al. [36] and the United Nations Sustainable Development Goals (Convention on Biological Diversity) [37], that the microorganisms present in soil contribute largely to the soil's sustainability and that, thus, they should be thoroughly studied. Accordingly, we evaluated the effects of freeze-storage and thawing on the viability and biodiversity of the culturable microbial population of maize crop soils and the overall process sustainability under three different management conditions over a period of 44 months.

2. Materials and Methods

2.1. Experimental Site

The study was carried out on a stony district luvisol with 210 g·kg^{-1} clay, 300 g·kg^{-1} silt, 490 g·kg^{-1} sand, organic C 19 g·kg^{-1}, total N 1.23 g·kg^{-1}, aggregate stability 44.9%, soil penetration resistance 7.5 MPa, actual water content 0.30 cm^3, and pH 5.3. Particle size distribution was determined by sedimentation using the pipette method after organic carbon destruction with H_2O_2 and chemical dispersion using $Na_4P_2O_7$. The organic carbon was determined by dichromate oxidation. The pH was measured in 1:1 (*w/v*) soil/water using a combination electrode. Aggregate stability was determined in 1–2 mm air-dry aggregates using a single 0.250-mm sieve and an apparatus with a stroke length of 1.3 cm and frequency of 35 cycles min^{-1}, and sodium hydroxide as a dispersant. The soil penetration resistance was measured using a hand penetrometer with a 1-cm^2 conical tip. The experimental field was located in south-western Spain (39°06′N; 5°40′W), where the climate is Mediterranean with a mean annual precipitation of 480 mm, and very hot and dry summers, and an aridity index of 0.49 according to UNESCO [38]. Before beginning this research, the experimental area (3500 m^2) was already cropped with maize (*Zea mays* L.) under irrigation using conventional tillage management (deep ploughing prior to planting). After harvesting the maize at the beginning of this study, the field was divided into twelve plots of approximately 200 m^2 (20 × 10 m) each, that were subjected to three different management regimes: conventional tillage (CT), and two no-till management systems: direct seeding without a cover crop (DS) and direct seeding with a lopsided oat (*Avena strigosa*) cover crop (DSC). Each treatment was replicated four times in a completely randomized design. The soils from the three management regimes had low K (<0.5 cmolc·kg^{-1}, extracted with 1 M NH$_4$OAc at pH 7, and assayed by atomic absorption spectrophotometry) and low pH. They mainly differed at the end of the sampling period in total organic carbon content (10.9, 13.1, and 13.2 g·kg^{-1}), volumetric soil water content (0.19, 0.23, and 0.25 cm^3·cm^{-3}), total N (1.23, 1.30, and 1.46 g·kg^{-1}), and Olsen available P (37.2, 32.5, and 39.9 mg·kg^{-1} for the CT-, DS-, and DSC-soils, respectively). The differences were significant for all these parameters in the three soils, and positive correlations had previously been found between the amount of soil microorganisms and the organic carbon or water content [39].

2.2. Soil Sampling and Analysis

Three years after the initiation of each soil management, five soil sub-samples from each plot were taken randomly from a 10-cm depth with a sterile trowel, and placed into sterile bags. The sub-samples from each plot were composited (roughly 2 kg). A total of four composited samples were obtained from each soil management, thus giving a total of twelve composited samples (four replications for each soil management). Samples were taken in October, after harvest, when the microbial population was the greatest (unpublished previous results). Samples were returned to the lab under refrigeration, and passed through a 2-mm sterile sieve for four hours before sampling. A before-freeze-storage microbial population analysis was performed for each sample (see below). Then, 50-g aliquots were freeze-stored at −20 °C. The aliquots were thawed at room temperature every 2 months. Properly diluted (10^{-4}–10^{-7}) soil aliquots were inoculated onto two rich culture media, TSA (Triptone Soy Agar) and YEPD (Yeast Extract Peptone Dextrose), for the microbial population analysis. The highest soil sample dilution (10^{-6} and 10^{-7}) facilitated the detection of slow-growth microorganisms by reducing

the effect of overgrowth by fast-growing microorganisms. The plates were incubated at 25 °C for up to 10 days to detect slow-growth microorganisms. After colony counting, the data from the two culture media were combined prior to analysis.

2.3. DNA Extraction from Cultured Bacteria, PCR Amplification, Sequencing of 16S Ribosomal DNA, and Bacteria Identification

Bacteria were cultured in YEPD and TSA media, and five colonies of each bacterial morphological type were independently lysed by freeze-thawing [40]. The PCR was performed directly from lysed bacteria or purified soil DNA with the kit pReTaq Ready-To-Go PCR Beads (Amersham, Biosciences), with the 16S rDNA specific bacterial primers 27F (fD1) and 907R [41]. The thermocycler protocol was an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 15 s, annealing at 55 °C for 15 s, an extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min [42]. As a preliminary step for the DNA sequencing, samples were purified with the "Jetquick PCR purification Spin Kit" (Genomed, Löhne, Germany) following the manufacturer's recommendations. The purified rDNA PCR fragment from each isolated microorganism was sent to a sequencing service (Secugen S.L., Madrid, Spain) on a BigDye Terminator v3.1 sequencer. The partial 16S rDNA sequences were checked with the software Chromas v. 1.45 (http://www.technelysium.com.au/chromas.html) in order to correct sequencing mistakes, and were screened against those in GenBank/EMBL by using BLAST [43]. Sequences with >99% similarity to previously published data available at NCBI (http://ncbi.nlm.nih.gov) were clustered into the same operational taxonomic unit (OTU). Sequences with <99% similarity to previously published data were less than 10% and were not considered for further analysis.

2.4. Biodiversity and Statistical Analysis

Simpson's reciprocal index of diversity [44] was used as a measure of the soil microbial diversity. Analysis of variance (ANOVA) and the Duncan test (at $p < 0.05$) for equality of means were used to detect significant differences. Normality and homogeneity of variances were checked by using Levene's test.

3. Results and Discussion

A total of 41 bacterial morphological types were identified de visu according to colony (form, margin, texture, opacity, color) and cell morphology (shape, sporulation capability, Gram staining). After 16S ribosomal DNA sequencing of five isolated colonies of each bacterial morphological type, they were regrouped into 25 bacterial OTUs (operational taxonomic units), in addition to one common group for all the unidentified filamentous fungi (molds) in the sum of all culture media plates inoculated with the soil samples (Table 1). To validate the morphological-type differentiation, ten new isolates of each OTU (a total of 250 newly isolated colonies) were identified by 16S ribosomal DNA sequencing, obtaining a fairly low total error of 4.02% in the assignment of each colony to one of the previously defined OTUs. The genera with the greatest number of different species were *Streptomyces* (five species), *Pseudomonas* (seven species), and *Bacillus* (five species). The most relatively abundant bacteria were *Acinetobacter* (9.3%), *Arthrobacter* (13.2%), and *Stenotrophomonas* (14.4%); and the least frequent were *Staphylococcus*, *Microbacterium*, and *Janthinobacterium* (less than 1%). The amount of cultured mold was almost 2% of the total cultured microorganisms.

Table 1. Identification of the bacterial morphological types isolated in the culture media plates inoculated with the soil samples.

| Type	Colony Morphology					Spores	Cell Shape	Gram	Microorganism (OTU) [†]
	Form	Margin	Texture	Opacity	Color				
T1	Circular	Entire	Shiny	Opaque	White	No	Cocci-rods	+	*Arthrobacter dextranolyticus*
T2	Circular	Entire	Shiny	Opaque	Yellow	No	Cocci-rods	+	*Arthrobacter nicotinovorans*
T3	Rhizoid	Curled	Wrinkled	Opaque	Beige	Yes	Rods	+	*Bacillus mycoides*
T4	Irregular	Entire	Granular	Opaque	White	Yes	Rods	+	*Bacillus megaterium*
T5	Irregular	Entire	Mucoid	Opaque	Beige	Yes	Rods	+	*Bacillus simplex*
T6	Irregular	Lobate	Mucoid	Translucent	Beige	Yes	Rods	+	*Bacillus subtilis*
T7	Irregular	Lobate	Granular	Translucent	Beige	Yes	Rods	+	*Bacillus thuringiensis*
T8	Circular	Entire	Rough	Opaque	Beige	Yes	Rods	+	*Bacillus weihenstephanensis*
T9	Irregular	Entire	Smooth	Opaque	Beige	No	Rods	-	*Chryseobacterium indologenes*
T10	Circular	Curled	Wrinkled	Opaque	Violet	No	Rods	-	*Janthinobacterium lividum*
T11	Circular	Entire	Smooth	Opaque	White	Yes	Rods	+	*Paenibacillus polymyxa*
T12	Circular	Entire	Shiny	Translucent	White	No	Rods	-	*Pantoea agglomerans*
T13	Irregular	Entire	Smooth	Transparent	n.a.	No	Rods	-	*Pseudomonas cedrella*
T14	Circular	Entire	Shiny	Opaque	White	No	Rods	-	*Pseudomonas filiscindens*
T15	Irregular	Entire	Rough	Opaque	Beige	No	Rods	-	*Pseudomonas fluorecens*
T16	Circular	Entire	Shiny	Opaque	Brown	No	Rods	-	*Pseudomonas jessenii*
T17	Circular	Entire	Shiny	Translucent	Brown	No	Rods	-	*Pseudomonas mediterranea*
T18	Circular	Entire	Shiny	Opaque	White	No	Rods	-	*Pseudomonas mosselii*
T19	Circular	Entire	Shiny	Opaque	Beige	No	Rods	-	*Pseudomonas synxantha*
T20	Circular	Entire	Dull	Opaque	White	No	Cocci	+	*Staphylococcus epidermidis*
T21	Irregular	Undulated	Wrinkled	Opaque	Beige	Yes	Large rods	+	*Streptomyces scabrisporus*
T22	Circular	Entire	Dusty	Opaque	Beige	Yes	Large rods	+	*Streptomyces flavovirens*
T23	Circular	Entire	Dusty	Opaque	Grey	Yes	Large rods	+	*Streptomyces xanthophaeus*
T24	Circular	Entire	Dusty	Opaque	White	Yes	Large rods	+	*Streptomyces ciscaucasicus*
T25	Circular	Entire	Dusty	Opaque	Brown	Yes	Large rods	+	*Streptomyces griseoaurantiacus*

[†] OTU = operational taxonomic unit.

Most bacterial populations were unaffected by the freezing time or the freeze-thaw stress. After 44 months of freeze-storage, the number of colonies remained the same for 24 out of the 25 bacterial OTUs analyzed (Figure 1a). The exception was the sporulating *Bacillus mycoides*, extensively present in the three studied soils, of which the colony number decreased after eight months of freeze-storage to 25% of the population compared to before freeze-storage (Figure 1b), and the molds, which decreased to become undetectable after 44 months (Figure 1c). The loss of viability of *B. mycoides* and mold populations may be because they have no time to sporulate before freezing, with the vegetative cells being more sensitive to freeze-storage than their spores or the rest of the cultured bacteria. The decreasing viability trend was similar for the *B. mycoides* and mold populations in the three soils, independently of their different total organic carbon content, which was lower in the conventional management (10.9 g·kg^{-1}) than in the direct seeding and direct seeding with cover crop soils (13.1 and 13.2 g·kg^{-1}, respectively). In agreement with our results, inactivation of vegetative *B. subtilis* cells upon -25 °C freezing has been reported previously [45,46]. This sensitivity might be reduced if soil samples were dehydrated before storage, allowing cells to live on a much reduced metabolism [47]. However, with this procedure sample handling would take longer, losing part of the advantage of rapid sample freeze-storage. Despite the fact that irreversible changes in microbiological soil characteristics can occur after freezing and thawing, and that freezing soil samples prior to analysis has not been recommended for the investigation of specific microbial communities such as Archaea [5], it seems that the overall situation in our soils is rather satisfactory after freeze-storage. This is, although a decrease was observed in the cell viability of culturable *B. mycoides* and filamentous fungi, they only represented <1% and <2%, respectively, of the total microbial populations of the three treatments. On the contrary, the viability of the rest of the culturable bacteria, which represented the great majority, did not change throughout the freeze-storage period. Consequently, the slight decrease in the Simpson's diversity index of the total microbial populations after 44 months of freeze-storage was in no case statistically significant ($p > 0.05$), and the biodiversity remained significantly greater in the soil under direct seeding with a cover crop than in the soil under conventional management or the soil under direct seeding (Figure 2). As in most other soil microbial analyses, we might be

missing a fraction of the soil culturable bacteria, i.e., those that grow extremely slow, are present at an extremely low frequency, or are not culturable in the media we used. This caveat notwithstanding, for a soil microbiologist it is very interesting to observe that the most frequent culturable bacteria could be freeze-stored for further analysis without any major loss of cell viability, and that this was unaffected by the regime of soil management applied. Due to its high C/N ratio, the chosen crop (corn) allowed microorganisms to develop in full. Other crops could have been used (i.e., sorghum or a gramineae/leguminosae consociation) but, because they have an equal or lower C/N ratio, according to Silva et al. [29], than that of the chosen crop, the number of obtained microorganisms would be expectantly lower than that obtained. Optimum microorganism development is crucial, as it contributes in a decisive way to improve soil quality and sustainability according to previous studies [30–37] and, as we stated before, the freeze-thaw process did not affect most of the bacterial populations for a period of 44 months. However, one should keep in mind that, while one might expect the results to be very reproducible when working with similar soil microbial communities and environmental conditions, somehow different results could be obtained when studying very different soil microbial communities, principally because the effect of freeze-storage on the average cell viability of a given microbial community may well depend on its species composition.
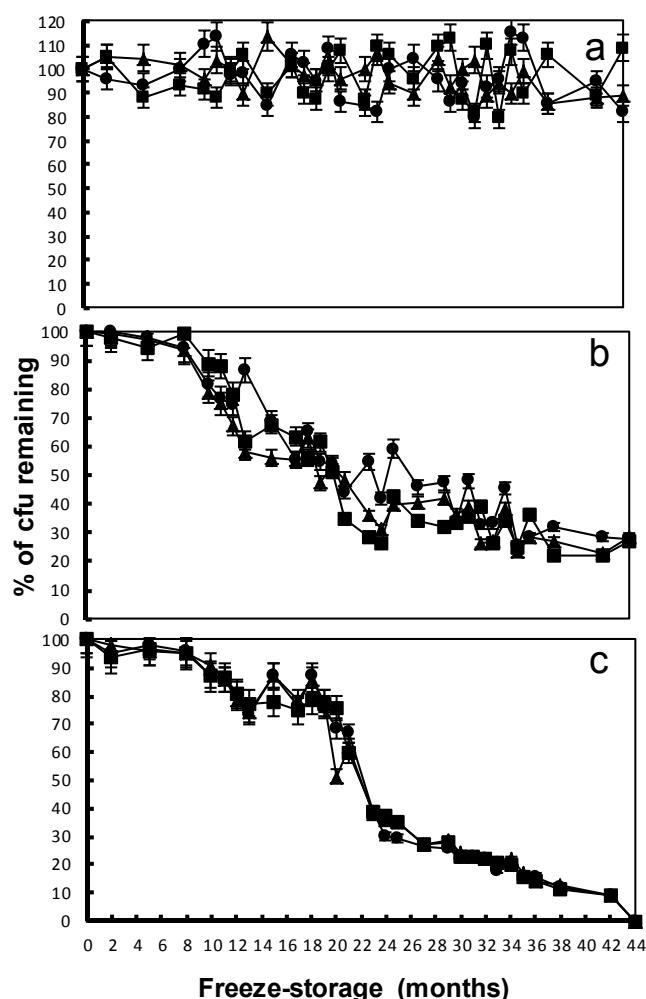
Figure 1. Percentage of the original colony-forming units (cfu) remaining after freeze-storage. Bacterial populations excepting *Bacillus mycoides* (**a**); *Bacillus mycoides* (**b**); and filamentous fungi (molds) (**c**). Conventional soil management (CT-soil) (●), direct seeding (DS-soil) (▲), and direct seeding with a cover crop (DSC-soil) (■). Data are shown as mean ± standard error of four replicates for each soil management regime.
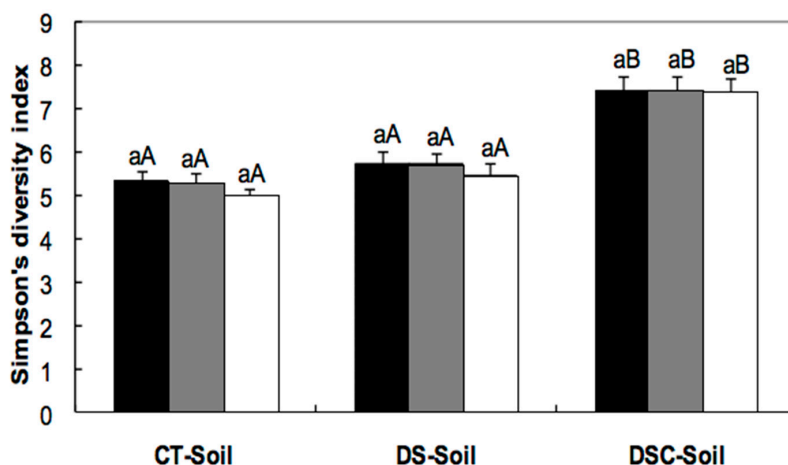
Figure 2. Simpson's diversity index of total microbial populations of the soil under the three management regimes: before freeze-storage (■); after eight months of freeze-storage (▥); and after 44 months of freeze-storage (□). Columns in the same treatment with the same lower case letter were not significantly different at the $p < 0.05$ level. Columns for the same freeze-storage time in different soils with the same capital letter were not significantly different at the $p < 0.05$ level.

4. Conclusions

Freeze-storage at $-20\ °C$ was suitable for the storage of the most abundant cultured bacteria of the three soils studied. However, it was inappropriate for preserving the filamentous fungi and *B. mycoides* populations for longer than eight months, which can be mitigated if the soil samples are dehydrated before storage. Therefore, freeze-storage for up to eight months seems to be a sustainable process allowable in analyses of microbial population biodiversity, and should be reasonably acceptable for comparisons of different treatments within the same soil, given that the diversity indices of the total microorganism populations in the present study were not significantly affected. Although other studies [5] have suggested that the freeze-thaw process of soil samples should be avoided, our study did not find evidence for such a claim and, as the United Nations Sustainable Development Goals (Convention on Biological Diversity) [37] and other studies [33–36] have stated, the microorganisms present in the soil contribute largely to the soil's sustainability and, thus, more studies on the preservation of this biological life should be perpetrated.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CT	conventional tillage
DS	direct seeding
DSC	direct seeding with a lopsided cover crop
OUT	operational taxonomic unit
rDNA	Ribosomal DNA
SIR	Substrate-Induced Respiration
TSA	Triptone Soy Agar

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