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Phosphate solubility and biocontrol activity of *Trichoderma harzianum*

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Abstract: Biological control of soilborne plant pathogens is a possible alternative to the use of chemical pesticides, which are harmful to the environment. Under greenhouse and field conditions, several isolated strains of the fungus *Trichoderma* have been found to be effective biocontrol agents of various soilborne plant pathogenic fungi. The use of phosphate solubilizing microorganisms (PSMs) has a considerable synergistic effect on the growth and development of crop plants. In the present study, *T. harzianum* was isolated from 10 different agro-climatic zones of Karnataka and tested for the potential of antagonistic activity against the plant pathogen *Xanthomonas* and the ability of phosphate solubility under *in vitro* conditions. Variations within the 10 isolates of *T. harzianum* were assayed using the Rapid Amplification of Polymorphic DNA (RAPD) method.

Key words: *Trichoderma*, biocontrol activity, P solubility, RAPD

Introduction

Most plant species exploit soil with the help of beneficial microorganisms such as fungi and bacteria, some of which are important in nitrogen fixation and phosphate solubilization. Occurrence and distribution of phosphate solubilizing microorganisms (PSM) have been found in almost all the soils tested, although their populations vary with different soils, climate, and cropping history (1).

Trichoderma is a fungus that exists in almost all soils and a wide range of habitats. In soil, they are the most widespread, culturable fungi. They prefer locations with a large supply of plant roots, which they promptly colonize. Additionally, the *Trichoderma* species attack, parasitize or derive nutrition from other fungi. Because the species flourishes best when there are copious amounts of healthy roots, they have developed many mechanisms for attacking other fungi and for improving plant and root growth (2). Intricate chemical reactions in the soil convert applied phosphate fertilizers into highly insoluble forms. However, the soil also has fungi that convert these insoluble forms into soluble forms. Such fungi are said to possess a mineral phosphate solubilizing ability that is an alternative to chemical fertilizers.

Nonavailability of resistant crop varieties, nondesirability of applying huge quantities of fungicides to soil because of residue problems, and the development of resistance in soilborne plant pathogens have lead to increased research efforts on biological control of soil plant pathogens all over the world.

Trichoderma has some antibiotic producing and biocontrolling properties. Thus, it is used as a bioagent. Isolates of *Trichoderma*, including isolates from the same species, produce a range of volatile

antibiotics that have different effects on different test fungi. It is possible that the isolates produced either the same blend of metabolites in different proportions or completely different chemical compounds (3).

Nutrient competition, mechanical barriers, or pH changes are some of the antagonistic effects. Both fungi and bacteria are able to synthesize a wide range of metabolites with fungicidal and bactericidal capabilities. These antibiotics are an alternative biological protection to conventional fungicides (4).

The *Trichoderma* species release antibiotics and other chemicals that are harmful to pathogens and inhibit growth (antibiosis). The potential use of the *Trichoderma* species as a biocontrol agent was suggested more than 70 years ago by Weindling (5), who was first to demonstrate the parasitic activity of a member of this genus against soilborne fungal or bacterial pathogens.

The mechanisms proposed to explain the biocontrol of plant pathogens by *Trichoderma* are presumptive. The suggested mechanisms for biocontrol are antibiosis, lysis, competition, and mycoparasitism (6). These may act alone or in combination. However, in biological systems, a single simple action is most unlikely. Many studies on the interaction of PSMs have been conducted in different crop plants. However, the information on the capability of these organisms in localized soil conditions, in promoting plant growth and the biochemical characterization of phosphate solubilizing bacteria and fungi isolated from different soil types of different zones, is very much limited. Furthermore, screening of the isolates, either individually or in combination, is needed to select efficient isolates to improve the plant growth and biomass (7).

Various types of molecular markers are utilized to evaluate DNA polymorphism (8). Species and varietal characterization based on DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. The Rapid Amplification of Polymorphic DNA (RAPD) is a novel and very powerful DNA fingerprinting technique for DNA of any origin or complexity. It has been widely used for the analysis of genetic diversity and was recently found to be effective for developing linkage maps in fungi (9). Hence, the present study was undertaken to prove the effective biological control of *Trichoderma harzianum*, isolated from 10 different agro climatic zones of Karnataka and tested for the potential of the antagonistic activity on the plant pathogen *Xanthomonas* and the ability of phosphate solubility under in vitro conditions. Variations within the 10 isolates of *T. harzianum* were assayed using the RAPD method.

Materials and methods

Soil samples, weighing 500 g each, were collected from the top 15-cm layer of each agro-climatic zone of Karnataka. Samples were sieved through 1000-µ mesh and used for the isolation of fungi by the serial dilution method (10). Dilutions of 1 mL were plated on Martin's rose bengal agar (MRBA) and incubated at 30 °C for 5 days. Based on the colony morphology, the fungal colonies were selected and cultured separately to obtain pure cultures. The genomic DNA was isolated from fungi following the protocol of Hegedus and Khachatourians (11) with certain modifications. For 5 min, 3 g of fungal mat was homogenized with 2% sodium dodecyl sulfate (SDS). To the above solution, 6 mL of lysis buffer (2.5 Mm EDTA, 1% Triton X-100, and 50 mM Tris-HCl, pH 8.0) was added. The suspension was extracted with equal volumes of phenol:chloroform (1:1) and centrifuged at 10,000 rpm for 10 min. Added to the supernatant at room temperature was 1/10th of the volume of 3M sodium acetate and 0.54 mL of isopropanol, which was then was mixed by gentle inversion and kept for 30 min at 2 °C. The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4 °C and the pellet was washed with 70% ethanol and quantified by agarose gel electrophoresis stained with ethidium bromide using different concentrations of DNA (Bangalore Genei) as standard. The PCR amplification was carried out (12). The DNA was amplified in 25 mL reaction tubes containing 150 ng of genomic DNA, 250 mM of each dNTP, 3mM of $MgCl_2$ and 1 unit of Taq Polymerase (Genei, Bangalore), and 0.5 mM of arbitrary primer (MWG-Biotech AG, Bangalore). Each amplification included a negative control in which 5 mL of sterile water was substituted for the DNA template. A total of 8 arbitory primers were tested for RAPD analysis. These included primers 203, 211, 220, 230, 232, 238, 241, and OPA 13 (Table 1). A dendrogram

was constructed using UPGMA software (13). The dissimilarity was evaluated through simple association and the genetic distances as the Euclidean distance. The binary matrix was built pair wise and the presence or absence of a determined RAPD band scored 1 and 0, respectively. The hierarchal groupings were based on the unweighed pair group using arithmetic averages (UPGMA).

The *Xanthomonas* isolate was tested in vitro for the efficiency of biocontrol activity using antagonistic microorganism *T. harzianum* isolates 1 to 10. As a preliminary experiment, we tested the growth response of the bioagents *T. harzianum* and the pathogen *Xanthomonas* on different media, such as potato dextrose agar and nutrient agar, in order to verify the suitability of the growth medium for the study of antagonistic activity. Experiments were then conducted in vitro on potato dextrose agar to learn the antagonistic activity of *T. harzianum* isolates against the *Xanthomonas* isolate. Lawn cultures of *T. harzianum* and *Xanthomonas* were allowed to grow for 4 days using the spread plate method (14).

Table 1. Sequence of primers tested for *T. harzianum*.

Primers used	Sequence 5'-3'
203	CAC GGC GAG T
211	GAA GCG CGA T
220	GTC GAT GTC G
230	CGT CGC CCA T
232	CGG TGA CAT C
238	CTG TCC AGC A
241	GCC CGA CGC G
OPA 13	CGA CAC CCA C

The antagonistic potential of *T. harzianum* against soilborne bacterial pathogen *Xanthomonas* isolates was tested using the dual culture method on potato dextrose agar (PDA) medium. Plates without *T. harzianum* served as the control for the pathogen *Xanthomonas*. The plates were incubated at 30 °C for 7 days. Each treatment was replicated 3 times.

The extent of antagonistic activity by *Trichoderma harzianum* was recorded on the 5th day by measuring the growth of the pathogen on the dual culture plate and on the control plate. The percent inhibition of the pathogen was calculated (15) as follows:

$$I = \frac{(C-T)}{C} \times 100$$

where

I = Percent inhibition

C = Growth of the pathogen in the control plate (mm)

T = Growth of the pathogen in dual culture plate (mm)

The phosphate solubilizing efficiency of different isolates of *T. harzianum* was spotted on Sperber's medium (16). The plates were incubated at 30 °C for 36 h. The zone of solubilization produced by these isolates around the colonies due to utilization of insoluble phosphate present in the medium was measured.

Results and discussion

T. harzianum can be recognized by its distinct spore arrangement on the conidial heads as that of the standard reference strain, showing repeatedly branched conidiophores that are irregularly verticillate and bear clusters of divergent, often irregularly bent, flask-shaped phialides (17). All of the investigated isolates and the standard strains initially formed white fungal colonies that turned to a greenish-black color after sporulation, due to the color of the spores. Variations within the species were further categorized using RAPD markers.

T. harzianum grew well on the potato dextrose agar but no growth was observed on the nutrient agar. *The Xanthomonas* isolate grew well on both the potato dextrose agar and the nutrient agar.

Different methods of inoculation were followed to learn the efficiency producing zone of inhibition and

to select the best in vitro inhibition of *Xanthomonas* by *T. harzianum*. No evidence of inhibition of growth of *Xanthomonas* by *T. harzianum* was observed when grown in dual culture on PDA medium when they were inoculated on the same day. Inhibition of the *Xanthomonas* growth by *T. harzianum* isolates 1 to 10 was observed in the plates where inoculation discs of 4-day-old cultures were placed side by side on PDA medium and further incubated.

The effect of placing 10 *T. harzianum* isolates over a stabilized colony of *Xanthomonas* isolates was studied and the growth of *T. harzianum* was also observed. The growth of *T. harzianum* isolates grew over the colony of *Xanthomonas* and covered the entire plate within 3 days. All of the isolates of *T. harzianum* caused complete lysis of *Xanthomonas* and showed suppression of the growth of *Xanthomonas.*

Among the 10 isolates used, isolates 2 and 10 (Figure 1) showed a better percentage of inhibition (88% and 89%, respectively) on the *Xanthomonas* strain used in this experiment. Isolate 9 also inhibited the growth of *Xanthomonas* by 82%. The effect was less when compared with *T. harzianum* isolates 2 and 10. The lowest response was noticed with isolate 6 (68%). When *Xanthomonas* and *T. harzianum* were grown together, absolutely no bacterial growth was observed in either of the plates. The data on percentage of inhibition of bacterial growth by *T. harzianum* are shown in Figure 2.

The inhibitory effect of *T. harzianum* against the soilborne bacterial pathogen was probably due

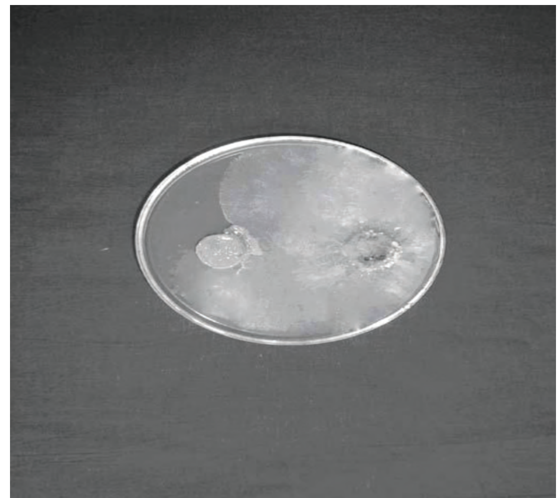


Figure 1. Xanthomonas growth suppressed by *T. harzianum*.

to competition and antibiosis (18). *Xanthomonas*, a member of the family Pseudomonadaceae, is always found in association with living plants, causing diseases like canker in citrus and black rot. *Xanthomonas* closely resembles *Pseudomonads* (small, motile, gram-negative rods), which is aerobic and produces a yellow pigment. Different strains are subdivided into pathovers based on factors like host range, geographical distribution, or named using “the plant from which it was first isolated” method (19).

The phosphate solubilizing efficiency of different isolates of *T. harzianum* was tested on modified Sperber’s medium. All the isolates were found to have good P solubilizing ability. However, the isolates

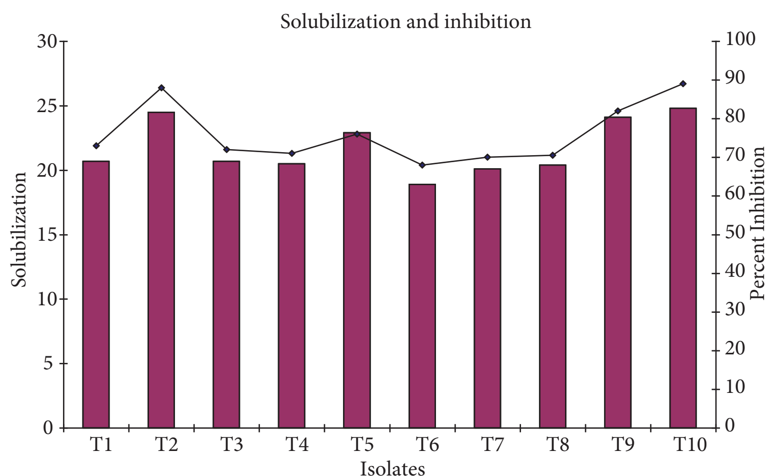


Figure 2. Efficiency of P solubility and biocontrol activity of *T. harzianum* isolates.

showed variability in the solubility zones around the colonies, with isolates 2 and 10 being the highest (Figure 2). The *Aspergillus awamori* strain was maintained as a standard to compare the zone of solubility.

The amplified product of arbitory primers showed a considerable degree of polymorphism between them. The dissimilarity matrix constructed based on the polymorphic bands showed that the *T. harzianum* isolates, which were clustered together, were less dissimilar (Table 2). Therefore, *T. harzianum* isolates 8 and 10 were the most dissimilar, while *T. harzianum* isolates 6 and 7 were the least dissimilar. The polymorphism among the *T. harzianum* isolates was observed as 61.95%. The average number of bands found was 10, while the average number of polymorphic bands per primer was found to be from 4 to 6 (Table 2).

In view of the results of the present study, RAPD patterns were obtained using different primers and the qualitative data obtained enabled us to identify 10 isolates of *T. harzianum*, whose taxa had been uncertain based on the morphological criteria. Eight arbitory primers for 10 isolates were used. Of these, primer combinations 230, 232, and 241 were found to give better amplification and polymorphism (Table 3).

Isolate 3 did not give any results when used with primer 232 (Figure 3). The banding pattern could easily distinguish the isolates from different zones (Figures 4 and 5). This suggests a wide range of difference in the genomic sequence in isolates from different

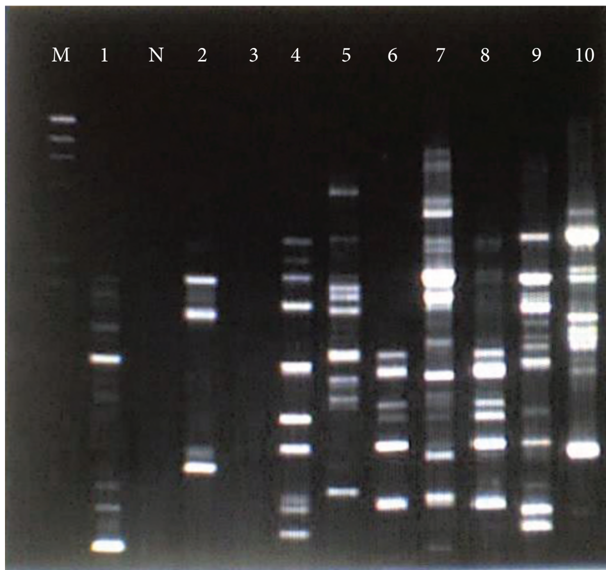
Table 2. Dissimilarity matrix of *T. harzianum* isolates based on RAPD analysis (1-10 *T. harzianum* isolates).

Isolates	1	2	3	4	5	6	7	8	9	10
1	0	11	18	15	19	16	18	20	18	15
2		0	17	18	16	15	17	19	11	19
3			0	17	15	12	18	14	12	10
4				0	14	15	19	17	16	18
5					0	9	19	21	20	19
6						0	16	18	14	14
7							0	18	16	17
8								0	20	14
9									0	19
10										0

Table 3. Effectiveness of primers tested for *T. harzianum*.

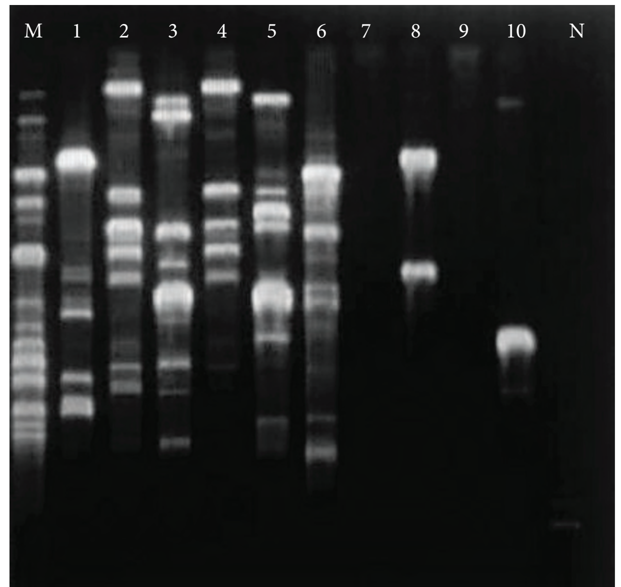
Primer no.	Sequence 5'-3'	Total no. of bands amplified	No. of polymorphic bands	% polymorphism
230	CGT CGC CCA T	9	6	66.67
232	CGG TGA CAT C	11	7	63.64
241	GCC CGA CGC G	9	5	55.55
Total no. of bands amplified		29	18	61.95

Average number of bands per primer: 10
Average number of polymorphic bands per primer: 6
Average % of polymorphism: 61.95



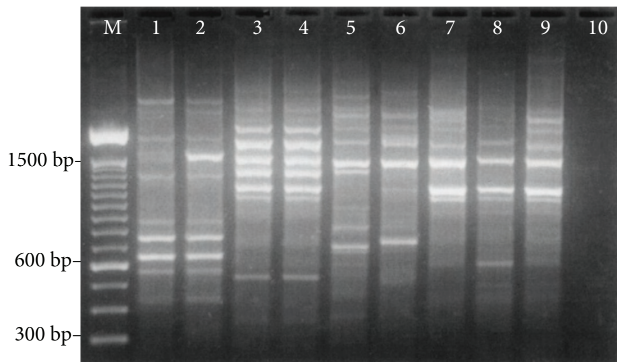
M-100 Bp DNA ladder
N-Negative control
1-10 DNA sample of ten isolates of *T. harzianum*

Figure 3. RAPD banding pattern of *T. harzianum* isolates with primer 232.



1-10 Isolates of *T. harzianum*
M-100 Bp DNA ladder
N-Negative control

Figure 5. RAPD banding pattern of different *T. harzianum* isolates with primer 241.



M-100 Bp DNA ladder
1-10 *T. harzianum* isolates

Figure 4. RAPD banding pattern of different *T. harzianum* isolates with primer 230.

zones. Molecular markers are being increasingly used to characterize fungal populations. They are versatile and highly informative tools for fungal pathogen identification and diagnosis in population genetic studies. The dendrogram constructed using the RAPD data enabled the classification of isolates into 3 major groups (Figure 6). Among the 3 groups, *T. harzianum* isolate 1 formed a separate group of its own. *T. harzianum* isolates 2, 5, 6, 7, and 10 formed

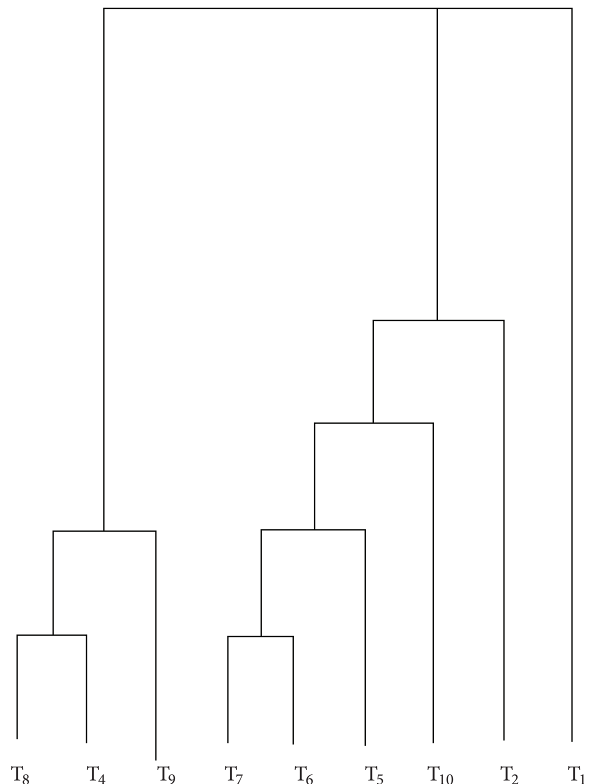


Figure 6. Dendrogram of *T. harzianum* isolates obtained by RAPD analysis.

another group and *T. harzianum* isolates 4, 8, and 9 formed a third group. The 4 minor clusters differ in their genetic distance with the least genetic distance noticed between isolates 1 and 7. Among the 3 groups, it is interesting to note from the cluster analysis that the isolates from adjacent zones clustered together. This is evident from the dendrogram constructed using the UPGMA method.

Sadfi (20) isolated a total of 83 spore-forming bacteria belonging to the genus *Bacillus* from Tunisian salty soils, which produces crystalline insecticidal protein. Because of this, it is usually used as a biopesticide. Recent studies have shown that it can also control the growth of pathogenic fungi like *Fusarium* that causes wilt in plants.

Lorito (21) isolated DNA fragments containing gene encoding for different chitinolytic enzymes from 5 different species and cloned them for mechanistic studies and biocontrol purposes. They studied the role of lytic enzymes in processes of biocontrol and strongly suggested the use of lytic enzymes and genes for biological control of plant diseases.

Plants inoculated with *Glomus mosseae* and *Bacillus coagulans* (G + B) and *Glomus mosseae* with *Trichoderma harzianum* (G + T) had produced significantly higher shoot biomass, root biomass, and total plant biomass compared to the uninoculated plants. Triple inoculation with *Glomus*, *Bacillus*, and *Trichoderma* did not significantly improve plant biomass compared to inoculation with *Glomus* alone. This is probably because *T. harzianum* inhibited the growth of *Bacillus*, as it has been reported to be antagonistic to several soil microorganisms in previous studies.

The *Trichoderma* species releases antibiotics and other chemicals that are harmful to the pathogen and inhibit the growth (antibiosis). Dennis and Webster (22) studied the production of a nonvolatile (diffusible) antibiotic substance by the *Trichoderma* species using an agar layer technique. They noticed that many isolates produced the nonvolatile antibiotics active against a range of bacteria. The ability to produce such a substance varied between the isolates. The susceptibility of pathogenic bacteria also varied widely. Specific strains of fungi in the genus *Trichoderma* colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes considered to be part of the plant defense response.

A significant increase in N and P uptake and green manure was observed during the first stage of vegetation in sunflower plants due to soil inoculation with phosphate dissolving microorganisms (23). Today, biological control of soilborne pathogens by the *Trichoderma* species is a vital area of plant pathological research all over the world. It is regarded as being important because the use of chemicals against plant pathogens has become too expensive and ecologically dangerous, creating serious health problems in human beings, animals, and also polluting soil and water with chemical residues. Diverse environmental conditions existing in different agro-climatic zones could have contributed to the biological and molecular variability in *T. harzianum,* which was evident in this study. However, not much work has been done, especially with fungi. Much of the work has been done mainly to identify the pathogenic strains within a species. The present study can be further improved upon by making an attempt to identify more effective 'P' solubilizing and biocontrolling strains. Further studies for utilization for commercial exploitation need to be carried out.

Genomic DNA from fungi could be amplified provided the time and duration of the program is standardized. RAPD is a useful diagnostic tool to analyze isolates or populations of individual species (24), and possibly to distinguish among species if enough primers are used. Analysis of the clustering pattern using the UPGMA method indicates that *T. harzianum* isolates, isolated from geographically adjacent soil zones, are more closely related than *T. harzianum* isolates from more distant zones. The present study reports that *T. harzianum* isolates 2 and 10 were potential phosphate solubilizers and biocontrolling agents.

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