Endophytic bacterial flora in root and stem tissues of black pepper (Piper nigrum L.) genotype: isolation, identification and evaluation against Phytophthora capsici

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ORIGINAL ARTICLE

Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: isolation, identification and evaluation against *Phytophthora capsici*

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Keywords
Bacillus megaterium, bacterial endophytes, black pepper, *Phytophthora capsici, Pseudomonas aeruginosa, Pseudomonas putida,* 16S rDNA.

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Abstract

Aim: To isolate and identify black pepper (*Piper nigrum* L) associated endophytic bacteria antagonistic to *Phytophthora capsici* causing foot rot disease.
Methods and Results: Endophytic bacteria (74) were isolated, characterized and evaluated against *P. capsici*. Six genera belong to *Pseudomonas* spp (20 strains), *Serratia* (1 strain), *Bacillus* spp. (22 strains), *Arthrobacter* spp. (15 strains), *Micrococcus* spp. (7 strains), *Curtobacterium* sp. (1 strain) and eight unidentified strains were isolated from internal tissues of root and stem. Three isolates, IISRBP 35, IISRBP 25 and IISRBP 17 were found effective for *Phytophthora* suppression in multilevel screening assays which recorded over 70% disease suppression in green house trials. A species closest match (99% similarity) of IISRBP 35 was established as *Pseudomonas aeruginosa* (*Pseudomonas* EF568931), IISRBP 25 as *P. putida* (*Pseudomonas* EF568932), and IISRBP 17 as *Bacillus megaterium* (*B. megaterium* EU071712) based on 16S rDNA sequencing.
Conclusion: Black pepper associated *P. aeruginosa*, *P. putida* and *B. megaterium* were identified as effective antagonistic endophytes for biological control of *Phytophthora* foot rot in black pepper.
Significance and Impact of the Study: This work provides the first evidence for endophytic bacterial diversity in black pepper stem and roots, with biocontrol potential against *P. capsici* infection.

Introduction

Foot rot disease caused by *Phytophthora capsici* is among the serious production constraints in black pepper in India and other countries, which necessitates frequent application fungicides in pepper plantations. In order to mitigate the use of toxic chemicals, several safe management options such as resistant varieties, biocontrol agents and cultural practices have been suggested. Several antagonistic rhizosphere microorganisms have been reported to combat foot rot (Anith *et al.* 2003; Diby *et al.* 2005) and few of them are endophytic bacteria (Van Buren *et al.* 1993; Chen *et al.* 1995; Chanway 1996; Zinniel *et al.* 2002). Diverse populations of endophytes have been identified in potato (Sturz *et al.* 1999; Garbeva *et al.* 2001), maize (McInroy and Kloepper 1995), cotton (Misaghi and Donndelinger 1990; McInroy and Kloepper 1995) and cucumber (Mahafee and Kloepper 1997). Endophytes may either become localized at the point of entry or spread throughout the plant (Hallmann *et al.* 1997), in the intercellular spaces (Patriquin and Döbereiner 1978) or in the vascular system (Bell *et al.* 1995). Bacteria may colonize and persist on the roots, enter inside the plant tissues like tubers (Hollis 1951), fruits (Samish *et al.* 1961), stems (Misaghi and Donndelinger 1990) and seeds and ovules (Mundt and Hinkle 1976), thereby providing protection (Chen *et al.* 1995). The prime objectives of our work are to isolate antagonistic endophytic bacteria from black pepper against *P. capsici*, for evaluation *in vitro* and *in vivo* and identification using 16s rDNA sequencing.

Materials and methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from the internal tissues of roots and stem of apparently healthy black pepper varieties (10 cultivars) collected from major Indian black pepper growing regions such as Calicut, Idukki and Wyanad districts in Kerala State and Kodagu District in Karnataka State. Samples were surface sterilized with 2% sodium hypochlorite for 10 min and 70% alcohol for a minute and rinsed five times in sterile distilled water. Surface sterility checks were carried out for each sample to monitor the efficiency of the disinfection procedure. For this, 0·1 ml of the last wash was either transferred to 9·9 ml TSB (TSB-Hi Media, Code No. M011) incubated at 28°C on a shaker or spread onto TSA plate (TSA-Hi Media, Code No. M290) for a sterility check. The tissue samples (1 g) were ground aseptically in phosphate buffer saline (PBS) (g l^{-1} NaCl 8, KCl 0·2, Na_2HPO_4 1·44 and KH_2PO_4 0·24, pH 7·4) and were centrifuged (60 ***g***) at 4°C for a minute. The supernatant was serially diluted up to 10^{-5}, pour plated on TSA and incubated at 28°C. The population of the bacteria in the tissue samples was expressed as colony forming units (CFU g^{-1}) of tissue. The individual bacterial colonies from each tissue were selected and sub-cultured on TSA and stored at –80°C in 20% sterile glycerol for further studies.

Characterization of endophytic bacteria

Isolates were tentatively grouped based on phenotypic characteristics such as colour, form, elevation margin, surface, opacity, texture, motility, cell morphology, size, Gram reaction and spore formation. Routine biochemical tests such as Indole; Methyl red; Voges-Proskauer; Citrate; succinic acid; hydrogen cyanide; production the presence of oxidase and catalase; hydrolysis of casein and starch; growth at 4°C, 28°C, 37°C, 41°C, 50°C and 60°C; growth at 1%, 2%, 5%, 7% and 10% salt concentration and ammonia production were assessed for each endophyte as described by Zvyagintsev (1991) (Table 1).

Screening for antagonism on *P. capsici*

Dual plate assay

Isolates were screened for their antagonism against *P. capsici* by adopting the confrontation assay of Dennis and Webster (1971). The inhibition of radial growth of *P. capsici* was measured and percent inhibition was determined.

Cut shoots assay

Bacterial isolates were tested for their ability to inhibit the pathogenesis of *P. capsici* on stem cuttings of black pepper according to the method of Dinu *et al.* (2007). Briefly, healthy shoots (cv. IISR-Subhakara) of about 8 cm length with at least one nodal primordia were washed thoroughly with tap water, surface sterilized with 1% sodium hypochlorite solution for 10 min and washed five times with sterile distilled water. The lower cut end (2–3 cm) was dipped in bacterial suspension ($\sim 10^9$ CFU ml^{-1}) for 30 min (shoot bacterization). A mycelial plug of *P. capsici* was inoculated on the bacterized cut end of the shoot and incubated in a moisture chamber 28 ± 1°C. A dark brown to black lesion developed after 96 h was measured and percent lesion inhibition over untreated control was calculated, normalized by angular transformation and statistically analysed using MSTATC.

Rapid in vivo *assay*

Evaluation of endophytic bacterial isolates was conducted on rooted cutting of cultivar, Panniyur 1 in a completely randomized design (CRD). Briefly, roots were treated (root bacterization) with bacterial suspension ($\sim 10^9$ CFU ml^{-1}) for 30 min (root bacterization), planted in bags (2:1:1 Soil : Sand : Farmyard manure) and observed. The treated rooted cuttings were challenge inoculated with *P. capsici* (10^4 zoospores g^{-1}) after 2 weeks and observed for about 2 months for rotting of roots and collar region and the eventual wilting. The percentage of the wilted plants was calculated, normalized by angular transformation and statistically analysed using MstatC.

Green house assay

Six highly antagonistic endophytic bacteria were further evaluated in the greenhouse on two varieties of black pepper (Karimunda type: *IISR-Subhakara* and Panniyur type: *Panniyur-1*). A two factor CRD was adopted with varieties as a first factor and bacterial treatments as a second factor. The treatments consisted of six endophytic bacteria and a existing *Phytophthora* suppressing rhizobacteria (*Pseudomonas fluorescens* MTCC-5178), a fungicide (Metalaxyl-Mancozeb 1·25 g l^{-1}) and untreated control. Rooted cuttings were treated with bacterial suspension ($\sim 10^9$ CFU ml^{-1}) for 30 min, and planted in soil (2:1:1 mixture of soil: sand: farmyard manure) and were challenged with *P. capsici* (10^4 zoospores g^{-1}) after a month and observed for rotting of roots and collar region and the eventual wilting. The percentage of the wilted plants was calculated, normalized by angular transformation and statistically analysed using MstatC. The trial was repeated in triplicate and pooled data presented.

Table 1 Characterization of endophytic bacteria isolated from black pepper

Isolates	Characters	Tentative identification
IISRBP 3, IISRBP 9, IISRBP 7, IISRBP 17, IISRBP 21, IISRBP 23, IISRBP 27, IISRBP 52, IISRBP 53, IISRBP 54, IISRBP 56, IISRBP 67, IISRBP 70, IISRBP 71, IISRBP 77, IISRBP 94, IISRBP 104, IISRBP 123, IISRBP 128, IISRBP 136, IISRBP 138, IISRBP 141	G+, long rods, Motile, spore formers, Indole (–), Methyl red (–), Voges-Proskauer (–), Citrate (–), Catalase (+), Oxidase (–), Succinic acid (–), Casein hydrolysis (+), Starch hydrolysis (+), Ammonia production (+/–), HCN production (–), Growth in NaCl-2% (+), 5% (+), 7% (+), 10% (–), Growth at 4°C (–), 28°C (+), 37°C (+), 40°C (+), 45°C (+), 55°C (+)	*Bacillus* spp.
IISRBP 4, IISRBP 10, IISRBP 12, IISRBP 13, IISRBP 14, IISRBP 15, IISRBP 16, IISRBP 18, IISRBP 25, IISRBP 26, IISRBP 28, IISRBP 30, IISRBP 35, IISRBP 49, IISRBP 69, IISRBP 76, IISRBP 88, IISRBP 115, IISRTC 5, IISRTC 9	G-, short rods, Motile, Non spore formers, Indole (–), Methyl red (–), Voges-Proskauer (–), Citrate (+), Catalase (+), Oxidase (+), Succinic acid (+), Casein hydrolysis (+), Starch hydrolysis (–), Ammonia production (+), HCN production (+), Growth in NaCl 2% (+), 5% (+), 7% (–), 10% (–), Growth at 4°C (–), 28°C (+), 37°C (+), 40°C (+), 45°C (–), 55°C (–)	*Pseudomonas* spp
IISRBP 6, IISRBP 11, IISRBP 19, IISRBP 24, IISRBP 29, IISRBP 44, IISRBP 47, IISRBP 50, IISRBP 66, IISRBP 68, IISRBP 74, IISRBP 90, IISRBP 139, IISRBP 140, IISRTC 16	G+, long rods, Non motile, Non spore formers, Indole (), Methyl red (–), Voges-Proskauer (–), Citrate (–), Catalase (+), Oxidase (–), Succinic acid (–), Casein hydrolysis (+), Starch hydrolysis (+), Ammonia production (+/–), HCN production (–), Growth in NaCl 2% (+), 5% (–), 7% (–), 10% (–), Growth at 4°C (–), 28°C (+), 37°C (+), 40°C (+), 45°C (+), 55°C (–)	*Arthrobacter* spp
IISRBP 40, IISRBP 41, IISRBP 42, IISRBP 51, IISRBP 55, IISRBP 75, IISRTC 8	G+, Coccus, Non motile, Non spore formers, Indole (–), Methyl red (–), Voges-Proskauer (–), Citrate (–), Catalase (+), Oxidase (+), Succinic acid (–), Casein hydrolysis (–), Starch hydrolysis (–), Ammonia production (+/–), HCN production (–), Growth in NaCl 2% (+), 5% (+), 7% (+), 10% (–), Growth at 4°C (–), 28°C (+), 37°C (+), 40°C (+), 45°C (+), 55°C (+)	*Micrococcus* spp
IISRBP 73	G-, Rods, Motile, Non spore formers, Indole (–), Methyl red (–), Voges-Proskauer (+), Citrate (+), Catalase (+), Oxidase (–), Succinic acid (–), Casein hydrolysis (+), Starch hydrolysis (–), Ammonia production (–), HCN production (–), Growth in NaCl 2% (+), 5% (+), 7% (+), 10% (+), Growth at 4°C (+), 28°C (+), 37°C (+), 40°C (+), 45°C (+), 55°C (–)	*Serratia* sp
IISRTC 10	G+, short rods, Motile, Non spore formers, Indole (–), Methyl red (–), Voges-Proskauer (–), Citrate (–), Catalase (+), Oxidase (–), Succinic acid (–), Casein hydrolysis (+), Starch hydrolysis (–), Ammonia production (+/–), HCN production (–), Growth in NaCl 2% (+), 5% (+), 7% (–), 10% (–), Growth at 4°C (–), 28°C (+), 37°C (+), 40°C (+), 45°C (–), 55°C (–)	*Curtobacterium* sp

IISRBP 2, IISRBP 72, IISRBP 97, IISRBP 125, IISRBP 133, IISRBP 135, IISRBP 137, IISRTC 17 were not identified.

Sequencing of 16S rDNA and identification

Genomic DNA from the endophytic bacteria was isolated according to the protocol of Kumar *et al.* (2004). Amplification of 16s rDNA gene of endophytic bacteria was performed with universal primer set pA (Fp) (5′-AgAgTTTgATCCTggCTCAg-3′) and pH (Rp) (5′-AAggAggTgATCCAgCCgCA-3′) (Woese 1987; Stackebrandt and Goebel 1994) in 25 μl of reaction containing 1X Taq buffer, 100 μmol l^{-1} dNTPs mix, 3 mmol l^{-1} $MgCl_2$, 10 μg BSA, 5 pM each primer, 0·5 U of Taq DNA polymerase and 100 ng template DNA. The thermocycling conditions consisted of an initial denaturation at 94°C for 2 min, 35 amplification cycles of 94°C for 1 min 10 s, 48°C for 30 s, 72°C for 2 min 10 s and a final polymerization step of 72°C for 6 min 10 s with Eppendorf master thermal cycler. The final PCR product was resolved in 0·8% agarose gel, excised and purified with Sigma elution kit. DNA sequencing was performed and sequences were subjected to a BLAST analysis and nucleotide sequence similarities were determined with the NCBI (National Center for Biotechnology Information databases) and their identity was established by closest match.

Results

Characterization of endophytic bacteria

A total of 74 bacteria were isolated from 10 varieties of black pepper (Table 2) among them 66 were from roots

Table 2 Endophytic bacterial isolates isolated from ten varieties of black pepper

Varieties	Bacterial strains isolated		Genus
	Root	Stem	
Panniyur-5	16	7	*Pseudomonas* spp., *Bacillus* spp., *Arthrobacter* spp. & *Micrococcus* spp.
IISR-Sreekara	19	1	*Pseudomonas* spp., *Bacillus* spp., *Arthrobacter* spp., *Serratia* & *Micrococcus* spp.
Panniyur-1	10	–	*Pseudomonas* spp., *Bacillus* spp., *Arthrobacter* spp., *Serratia* & *Micrococcus* spp.
Panniyur-2	3	–	*Bacillus* spp. & unidentified
IISR-Panchami	6	–	*Pseudomonas* spp., *Bacillus* spp., *Arthrobacter* spp. & *Micrococcus* spp.
Karimunda	4	–	*Pseudomonas* spp. & *Micrococcus* spp.
Karimundi	2	–	*Bacillus* spp.
IISR-Thevam	2	–	*Bacillus* sp. & *Pseudomonas* sp.
Wild (756)	3	–	*Bacillus* spp. & unidentified
IISR-Shakthi	1	–	*Pseudomonas* sp.
Total	66	8	

and 8 were from stem. These isolates were tentatively grouped into *Pseudomonas* spp. (20 strains), *Serratia* sp. (1 strain), *Bacillus* spp. (22 strains), *Arthrobacter* spp. (15 strains), *Micrococcus* spp. (7 strains), *Curtobacterium* sp. (one strain) and eight unidentified strains based on the keys provided in the Bergey's manual.

Antagonistic assays

Three independent assays such as dual plate assay, cut shoot assay and rapid *in vivo* assay were adopted to screen the isolates. These assays allowed us to confirm 14, 17 and 16 antagonistic bacterial isolates based on mycelial growth inhibition, lesion inhibition and foot rot suppression, respectively. Among them six isolates viz., IISRBP 17, IISRBP 25, IISRBP 35, IISRBP 71, IISRBP 104 and IISRTC 10 were selected for foot rot suppression trials in green house as they recorded 100% disease protection in a rapid *in vivo* assay (Fig. 1). These isolates were tested on two varieties (*IISR-Subhakara* and *Panniyur-1*) of black pepper in green house experiments. The data indicated that the endophytic bacterial isolates IISRBP 17, IISRBP 25 and IISRBP 35 recorded over 70% suppression of the pathogen in both the varieties, which is on par with the chemical treatment and *P. fluorescens* MTCC-5178, the existing biocontrol agent for foot rot management. The disease protection was more prominent in *IISR-Subhakara* than *Panniyur* (Table 3).

Identification of bacteria

Antagonistic endophytic bacterial isolates IISRBP 35, IISRBP 25 and IISRBP 17 were identified based on 16s RNA gene sequencing. The 1500 bp amplicon obtained

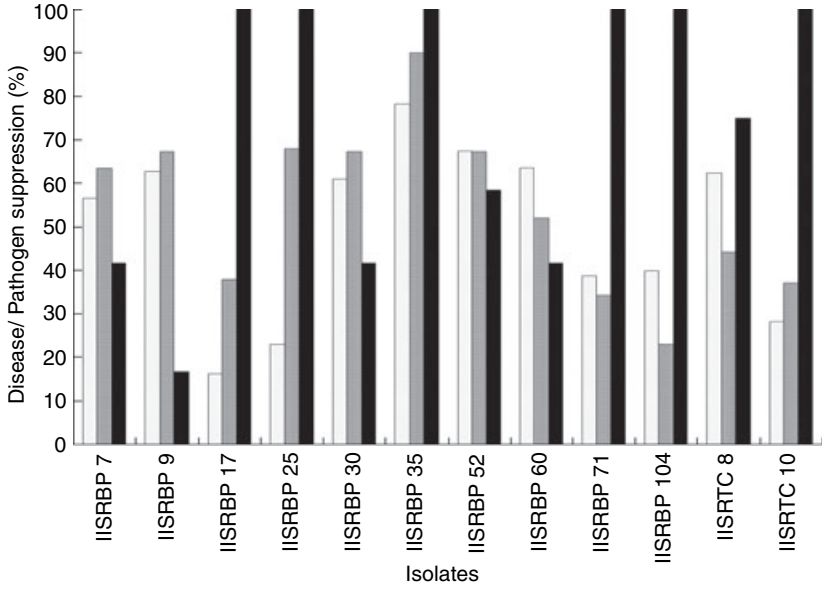

Figure 1 Selection of antagonistic endophytic bacteria based on multiple screening procedures such as dual plate assay, cut shoot assay, and rapid *in vivo* assay. Values presented are the mean of three replicates. LSD (P = 0·05): □ Mycelial inhibition 1·84; ■ Lesion inhibition 8·46; ■ Foot rot suppression 25·2.

Table 3 Performance of bacterial endophytes for suppressing of *P. capsici* infection on two varieties of black pepper

Treatments	Suppression of *P. capsici* (%)		
	IISR-Subhakara	Panniyur-1	Mean
IISRBP 17	77·7 (69·9) ab	88·8 (76·9) ab	83·3 (73·4) a
IISRBP 25	77·7 (62·2) b	94·4 (87·9) ab	86·1 (75·0) a
IISRBP 35	83·3 (73·2) ab	94·4 (87·9) ab	88·8 (80·6) a
IISRBP 71	11·1 (16·2) c	44·4 (41·7) c	27·7 (28·9) bc
IISRBP 104	5·5 (8·4) c	44·4 (37·1) c	25·0 (22·7) bc
IISRTC 10	16·6 (15·3) c	66·6 (55·2) bc	41·6 (35·3) b
Pseudomonas fluorescens MTCC 5178	77·7 (62·2) b	88·8 (76·9) ab	83·3 (69·5) a
Metalaxyl Mancozeb (1·25 g l^{-1})	100·0 (99·0) a	100·0 (99·0) a	100·0 (99·0) a
Control	0·0 (0·57) c	0·0 (0·57) d	0·0 (0·57) c
Mean	50·0 (45·2) a	69·1 (62·6) a	

Values in the indices are Arc Sine transformed value. Mean followed by the same letter designation are not significantly different according to the Duncan's multiple range test at the $P = 0{\cdot}05$.

by the PCR was purified using an elution kit, its quantity determined and sequenced. Partial sequence data for the 16S rDNA gene have been deposited in the GenBank (NCBI) nucleotide sequence data base library. Data for endophytic strains have been deposited under the following accession numbers: *Pseudomonas aeruginosa* IISRBP35-*Pseudomonas* EF568931, *Pseudomonas putida* IISRBP25-*Pseudomonas* EF568932 and *Bacillus megaterium* IISRBP17-*B. megaterium* EU071712.

Discussion

In the recent years endogenous microorganisms from the internal plant tissue have attracted the attention of researchers as biological control agents of plant pathogens due to their plant colonizing ability. As many as 74 strains of bacteria belonging to six genera were isolated from healthy black pepper roots and stem at an average population of 3–4 log and 2–3 log (CFU g^{-1}), respectively. The roots harboured more diverse population of endophytic bacteria than stem. The fact that endogenous bacterial population is higher in roots may reflect the fact that the root is the primary site where bacteria gain entry in the plants (Lodewyckx *et al.* 2002). Close proximity of soil would have contributed to the more diverse population of endophytes in the root tissues than stem tissues. Most of the endophytic bacteria isolated were Gram positive (80%) and Gram negative constituted only 20%. Among the Gram positives, the dominant ones were *Bacillus* spp., followed by *Arthrobacter* spp., *Micrococcus* spp. and *Curtobacterium* sp. Among the Gram negative *Pseudomonas* spp dominated followed by *Serratia* sp. Endophytic association of *Bacillus*, *Pseudomonas, Arthrobacter, Micrococcus* and *Curtobacterium* is reported in potato (Sturz *et al.* 1999). Other bacterial genera reported as endophytes are *Agrobacterium, Bacillus, Bradyrhizobium, Cellulomonas, Clavibacter, Corynebacterium, Enterobacter, Erwinia, Escherichia, Klebsiella, Microbacterium, Micrococcus, Pseudomonas, Rothia* and *Xanthomonas* (Gardner *et al.* 1982; Van Peer *et al.* 1990; Kobayashi and Palumbo 2000; Lodewyckx *et al.* 2002; Zinniel *et al.* 2002).

Selection of antagonists should be based on multiple factors such as direct antagonism on pathogens, competitive fitness in the environment and induction of resistance in the host. Han *et al.* (2000) suggested a rapid bioassay for screening microorganisms and their ability to induce systemic resistance. Our multilevel screening of the endophytic bacteria yielded three promising endophytes (IISRBP 17, IISRBP 25 and IISRBP 35) for foot rot management in black pepper. When evaluated the isolates IISRBP 35, IISRBP 25, and IISRBP 17 recorded over 70% disease suppression in greenhouse. The performance of IISRBP 35 was superior to other endophytic bacterial isolates, IISRBP 25 and IISRBP 17. A variety of endophytes showing superior antagonistic activities against bacterial and fungal pathogens are reported (Van Buren *et al.* 1993; Chen *et al.* 1995; Sturz *et al.* 2000; Lodewyckx *et al.* 2002; Reiter *et al.* 2002). Bacterial endophytes are known to protect the plants from pathogens by production of antibiosis, out competition of pathogens and induced systemic resistance (Hallmann *et al.* 1997; Sturz *et al.* 1999; Sessitsch *et al.* 2004). Due to intimate contact between endophytes and plant cells, induced systemic resistance may be an important mechanism of biocontrol (Chen *et al.* 1995; Benhamou *et al.* 1996; Hallmann *et al.* 1997; Nejad and Johnson 2000; Reiter *et al.* 2002). The highly effective bacteria were identified by sequence

comparison of conserved 16s rDNA. Reiter *et al.* (2003) has reported that the sequence analysis of endophytic bacteria revealed a high *Pseudomonas* species diversity in potato plants. Based on the sequence data the endophytes, IISRBP 35 was identified as *P. aeruginosa*, IISRBP 25 as *P. putida* and IISRBP 17 as *B. megaterium*, which showed nucleotide identity more than 99%.

Three black pepper associated endophytic bacteria identified as *P. aeruginosa* IISRBP 35, *P. putida* IISRBP 25 and *B. megaterium* IISRBP 17 were found promising for suppression of *P. capsici*. This work provides the first evidence of black pepper associated endophytic bacteria against *P. capsici*.

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