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Screening of Bacterial Endophytes for Osmotolerance and Improvement of Maize Seed Germination under Water Deficit Condition

Nakul Kale, N. Earanna and Tasmiya Imtiyaz
Department of Agricultural Microbiology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : shivajiputral@gmail.com

Authors Contribution

Nakul Kale :
Design of the work, data collection, data analysis and interpretation;
N. Earanna :
Critical revision and final approval;
Tasmiya Imtiyaz :
Data analysis, interpretation and drafting

Corresponding Author :
Nakul Kale
Department of Agricultural Microbiology,
College of Agriculture,
UAS, GKVK, Bengaluru

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Abstract

Bacterial endophytes present within plant tissues play an important role in maintaining host plant fitness, nutrient supply and mainly to mitigate water deficit stress. In this investigation, it was hypothesized that bacterial endophytes inhabiting the tissues of drought tolerant plants would ameliorate drought stress in maize seed germination. In this study 54 bacterial endophytes were isolated from drought adopted plants collected across different drought prone areas of Karnataka. These endophytes were screened for Osmotolerance using Polyethylene glycol (PEG MW-8000) at 15 and 20 per cent. Out of 54 isolates, five bacterial isolates (P7L1, P2L2, P6R1, P3L2 and P7R1) showed growth in the presence of 20 per cent PEG. These five bacterial isolates were inoculated to pre-germinated maize seeds and incubated in paper towel dipped with 15 per cent PEG. All the five endophytes inoculated seedlings showed increased growth of maize seedlings compared to un-inoculated seedling. However, the P7L1 isolate inoculated seedlings showed significantly increased seedling length than other isolates inoculated seedlings indicating its efficiency against water deficit stress This bacterium isolate was identified as *Pseudomonas tolaasii* using 16S rRNA gene sequence and its presence in the inoculated seedling was confirmed by re-isolation of bacterium after 10 days of seedling growth and comparing culture with inoculated mother culture both morphologically as well as by 16Sr RNA.

Keywords : Bacterial endophytes, *Pseudomonas tolaasii*, Drought stress, Polyethylene glycol (PEG MW-8000)

The Mysore Journal of Agricultural Sciences

In nature all plants have shown to inhabit diverse group of microbes, among which bacteria exists as dominant group. The bacteria inhabit within the tissues of healthy plant are referred to as bacterial endophytes (Hallmann *et al.,* 1997). Inside plant system these bacteria do not normally cause any substantial morphological changes like root-nodule as symbionts do. However they have significant impact on plant growth and survival under adverse conditions. In recent past drought stress has been one of the major crucial problem that negatively affects plant growth, development and more importantly yield (Ullah *et al.,* 2017). It has been reported that up to 40 per cent yield reduction in maize is mainly due to drought stress worldwide (Daryanto *et al.,* 2016). These effects of drought on plant system also depend on the duration and intensity of exposure. Under stress condition, plants execute a series of reactions in terms of stress responsive genes, activation or inactivation of functional proteins by secreting stress hormones and ROS, which functionally regulate physiology of the cell for its normal functioning in plant system.

Endophytes are microorganisms (bacteria and fungi) that infiltrate plants without creating disease symptoms. They populate practically every part of the plant, including the leaves, stems, roots, flowers and fruits. They found in a broad variety of plants, from grasses to higher order plants. They live symbiotically inside plant tissue could impart stress tolerance by various mechanisms. Many of them

create vital biochemical components that aid in the defence of plants against insect assault (Rabiey *et al.,* 2019) and diseases (Raveendra Reddy and Shivaprakash, 2018). Endophytes are extensively exploited for plant growth promotion and imparting stress tolerance when plants are exposed to a variety of abiotic stresses. Bacterial endophytes can confer benefit to plant fitness including increased biomass (root and shoot), yield and tolerance to abiotic stresses such as heat, salt and drought (Lata *et al*., 2018). In this context use of bacterial endophytes is the most feasible, reliable and sustainable option for enhancing growth of plants under drought stress conditions. Present study intended to screen and characterize the bacterial endophytes efficient against drought stress in maize crop.

Material and Methods

Isolation of Bacterial Endophytes from Drought Adapted Grasses

Grasses growing in the drought prone area of Koppal (Northern dry zone), Bellary (Northern dry zone), Chikkaballapur (Eastern dry zone) and Chitradurga districts (Central dry zone) of Karnataka were collected and the endophytic bacteria were isolated from root and leaf bits. The samples were surface sterilized using sodium hypochlorite (4%) solution followed by 70 per cent ethanol. Surface sterilized root and leaf bits were repeatedly washed using sterile water and last wash water was analyzed for lack of microbial growth by spread plate technique, this confirms surface sterilization of plant bits. These bits were placed on the petriplates dispensed with Nutrient Agar (NA) and incubated at 30 ºC for two days. The bacterial colonies developed were purified and maintained on nutrient agar slants in the refrigerator for further use.

Screening of Bacterial Endophytes for Drought Tolerance

The isolated bacteria were tested for their drought tolerance in liquid cultures. Ten ml of Nutrient broth (NB) supplemented with polyethylene glycol (PEG MW-8000) at 15 and 20 per cent concentrations which corresponds to -0.70 MPa, and -0.81 MPa (Control being -0.39 MPa) respectively were inoculated with each bacterial isolates. For the control nutrient broth was used. The plates were incubated for two days at 30 °C and the bacterial population was enumerated by serial dilution plate method.

Influence of the Bacterial Isolates on Maize Growth Under Drought Stress

Maize seeds (MAH 14-5) were surface sterilized and pre-germinated on sterile moist blotters. The pre-germinated seeds were treated with 48 h old bacterial cultures (~ 10^7 CFU) by soaking for 3 h as described by Wellington and Marcela (2004). The corresponding control was treated with sterile distilled water. These seeds were then subjected to drought stress by placing them on blotter paper dipped in the solution of 14.6 per cent (LC_{50}) PEG, LC stands for 'Lethal Concentration' value which refer to the concentration of a PEG chemical were at least 50 per cent of the seedlings can germinate during observation period (Roopashree, 2022). The growth of the seedling was recorded after 10 days.

Analysis of Plant Growth Promoting Traits of Bacterial Endophytes

Quantification of Indole acetic acid Gibberellic acid and Abscisic acid production using HPLC. The 24 h old cultures were inoculated in 20 mL nutrient broth containing with stress (15% PEG-8000) and without stress. For IAA, tryptophan was amended in the broth and incubated at 30 °C for 7 days. After incubation they were centrifuged at 6000 rpm for 10 minutes and the supernatant was collected, which was adjusted to a pH of 2.8 using 1 N HCl solution. The acidified supernatant was taken in 100 mL conical flask and equal volume of diethyl ether was added and incubated for 4 h at 4 °C. The solvent phase (upper layer) formed was collected and allowed to evaporate. To the evaporated samples 2-3 mL of HPLC grade methanol was added and stored at -20°C after membrane filtration to perform high performance liquid chromatography (Patten and Glick, 2002).

The Mysore Journal of Agricultural Sciences

Confirmation of the Endophytes in Inoculated Maize Seedlings

The inoculated maize seedlings were cut into one cm bits (root, stem and leaf), surface sterilized and placed on nutrient agar. The plates were incubated at 30 °C for 48 h. The bacterial colony emerged out of cut ends were sub-cultured and confirmed by comparing with the original colonies of respective mother culture.

Identification of Selected Isolate using 16S rRNA Gene Sequence

Total genomic DNA of the isolate was extracted by alkaline lysis method (Sambrook and Maniatis, 1989).The universal primers already reported (26 bp forward primer 5' GTTAGATCTTGGCTCA GGACGAACGC 3' and 24 bp reverse primer 5' GATCCAGCCGCACCTTCCGATACG 3') for 16S rRNA sequence from the NCBI (http:// www.ncbi.nlm.nih.gov) were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the Polymerase Chain Reaction (PCR). PCR was performed in 20 µl reaction mixture containing 2.0 µl of 1X PCR Taq buffer with $MgCl_2$ (1.5 mM), 2.0 µl of 10 mM dNTP's mix (200 µM), 0.5 µl of primers (both forward and reverse), 0.3 µl of Taq DNA Polymerase (1U Genei Bengaluru), 1.0 µl of Template DNA, 14.2 µl of Sterile distilled water. Amplification was carried out with an initial denaturation at 96 °C for four minutes followed by 35 amplification cycles consisting of 94 °C for one minute, 60 °C for 30 seconds and 72 °C for one minute and a final extension at 72 °C for 10 minutes. Then the amplified product of DNA was electrophoresed using one per cent agarose gel and documented using gel documentation system. The DNA was eluted by using gel elution kit (The Gene JET™ Gel Extraction Kit, Thermo Scientific) and the amplified product was got sequenced by Chromgene Biotech Pvt. Ltd., Bengaluru, Karnataka. The sequences obtained were analysed for homology using NCBI Gen Bank.

Statistical Analysis

The data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and means were separated by Duncan Multiple Range Test (DMRT).

Results and Discussion

The details of the location and the plants used for isolation of endophytic bacteria are presented in Table 1. A total of 54 bacterial isolates were obtained from ten grasses. These isolates were designated serially based on the part of the plant tissue used (P=plant, L=leaf, R=root). Maximum number of isolates were obtained from the plant *Digitaria ciliaaris* (9) and minimum in the *Cyperus* sp. (3), in other plant samples number of isolates ranged from 4 to 7.

Screening of Bacterial Endophytes for Drought Tolerance

The isolated bacteria were tested for their drought tolerance ability in nutrient broth supplemented with PEG (MW-8000) at 15 and 20 per cent concentrations. All the 54 bacterial isolates showed growth at 15 per cent PEG (MW-8000). But at 20 per cent PEG concentration, only five isolates such as P7L1, P2L2, P6R1, P3L2 and P7R1 showed growth after 36 to 48 h. The population of these bacteria were 2.4×10^7, 1.2×10^7, 1.2×10^7, 1.6×10^7 and 1.2×10^7 (Colony forming units) CFU/ml respectively. The bacterial colonies developed were further purified by three way streaking (Fig. 1). This indicated that these bacteria can withstand drought stress at 20 per cent PEG. The ability of these isolates to serve under drought stress can be attributed to accumulation of osmolytes, which include amino acids like proline, glutamate, glutamine, alanine and sugars like sucrose, trehalose and quarternary amines like glycine betaine and polyglucosyl granules that improve cell growth under adverse osmotic conditions, as osmoprotectants (Potts, 1994). These osmolytes lowers the water potential in the cyto plasm, and maintains the cell turgor thus preventing cell death (Aswathy *et al*., 2020).

TABLE 1
Details of location of plants and endophytic bacterial isolates

District	Location	Longitude (N)	Latitude (E)	Plant sample	Number of isolates
Koppal (Northern dry zone)	Achalapur	15^0 25' 039"	76^0 34' 005"	*Brachiariamutica*	P1L1,P1L2,P1R1, P1R2,P1R3,P1R4
	Raghunata Halli	15^0 22' 045''	75^0 95' 016''	*Fimbristylis miliacea*	P2L1,P2L2,P2R1, P2R2,P2R3
Bellary (Northern dry zone)	Tondehal	15^0 38' 036''	76^0 59' 014''	*Panicum repens*	P3L1,P3L2,P3L3,P3R1, P3R2,P3R3,P3R4
	Desanur	15^0 36' 057''	76^0 50' 045''	*Digitaria ciliaaris*	P4L1,P4L2,P4L3,P4L4, P4R1,P4R2,P4R3, P4R4,P4R5
Chikkaballapur (Eastern dry zone)	Chintamani	13^0 23' 005''	78^0 03' 028''	*Dichanthium* sp.	P5L1,P5L2,P5L3,P5R1, P5R2
	Kurubur	13^0 19' 020''	78^0 04' 044''	*Eleusine indica*	P6R3,P6L1,P6L2,P6R1, P6R2
	Shidlaghatta	13^0 24' 009''	77^0 52' 007''	*Sorghum halepense*	P7L1,P7L2,P7L3,P7R1
Chitradurga (Central dry zone)	Hiryuru	13^0 56' 001''	76^0 37' 005''	*Tragus* sp.	P8L1,P8L2,P8L3,P8R1, P8R2
	Ramajogihalli	14^0 17' 019''	76° 32' 036''	*Cyperus* sp.	P9L1,P9R1,P9R2
	Challakere	14^0 18' 038''	76^0 39' 026''	*Urochloa* sp.	P10L1,P10L2,P10R1, P10R2,P10R3
				Total isolates	54

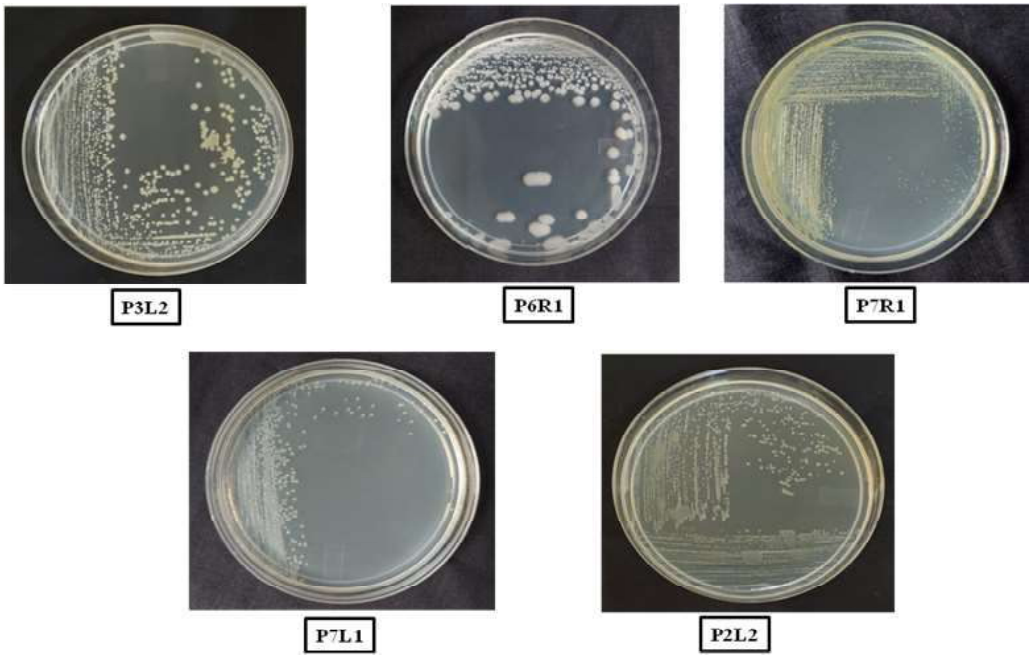

Fig. 1 : Pure cultures of selected bacterial isolates

TABLE 2
Bacterial growth at 20 per cent PEG in Nutrient broth medium

Sl.No.	Isolate	CFU/ml	Sl.No.	Isolate	CFU/ml	Sl.No.	Isolate	CFU/ml
1	P1L1	0.0	19	P4L1	0.0	37	P6R3	0.0
2	P1L2	0.0	20	P4L2	0.0	38	P7L1	2.4×10^{7}
3	P1R1	0.0	21	P4L3	0.0	39	P7L2	0.0
4	P1R2	0.0	22	P4L4	0.0	40	P7L3	0.0
5	P1R3	0.0	23	P4R1	2.0×10^{6}	41	P7R1	1.2×10^{7}
6	P1R4	2.0×10^{6}	24	P4R2	0.0	42	P8L1	0.0
7	P2L1	0.0	25	P4R3	1.0×10^{6}	43	P8L2	0.0
8	P2L2	1.2×10^{7}	26	P4R4	0.0	44	P8L3	0.0
9	P2R1	5.3×10^{6}	27	P4R5	0.0	45	P8R1	0.0
10	P2R2	2.3×10^{6}	28	P5L1	0.0	46	P8R2	0.0
11	P2R3	0.0	29	P5L2	0.0	47	P9L1	0.0
12	P3L1	3.0	30	P5L3	0.0	48	P9R1	0.0
13	P3L2	1.6×10^{7}	31	P5R1	4.5×10^{6}	49	P9R2	0.0
14	P3L3	0.0	32	P5R2	4.0	50	P10L1	4.6×10^{6}
15	P3R1	6.0	33	P6L1	0.0	51	P10L2	0.0
16	P3R2	2.0	34	P6L2	0.0	52	P10R1	1.0×10^{6}
17	P3R3	0.0	35	P6R1	1.2×10^{7}	53	P10R2	0.0
18	P3R4	0.0	36	P6R2	0.0	54	P10R3	0.0

Note: The values are presented as mean. CFU=Colony forming unit.

Influence of Bacterial Endophytes on Maize Seedling Growth Under Drought Stress

The drought stress mainly leads to over production of ROS (Reactive oxygen species) and it must be managed homeostatically therwise it results in denaturation of protein structure, lipid peroxidation, nucleotide disruption by affecting plant physiology which ultimately leads to death of plants. In the present study, five selected bacterial endophytes (P7L1, P2L2, P6R1, P3L2 and P7R1) were inoculated to surface sterilised pre-germinated maize seeds and then subjected to drought stress by placing them on blotter paper dipped in polyethy lene glycol (PEG MW-8000) solution of 14.6 per cent (LC_{50}) concentration. Among the five bacterial endophytes, the P7L1 inoculated seedlings showed significantly higher growth (34.1 cm) which is followed by P7R1, P2L2, P3L2 and P6R1 (Table 3). The least growth was observed in un-inoculated seedlings (Fig. 2). These endophytes also increased the growth of maize under normal conditions compared to control and this may be attributed to plant growth promoting substances produced by the endophytes (Santoyo *et al.,* 2016). Naveed *et al*. (2014) reported the improved seedling growth and water availability in maize cultivars inoculated with *Burkholderia phytofirmans* and *Enterobacter* sp. under drought stress. Similarly, Tasmiya and Earanna (2021) also reported that the bacterial endophyte

TABLE 3
Effect of bacterial endophytes on seedling growth of maize

Treatments	Seedling length (cm)	
	Without drought stress	Drought stress (-0.69MPa)
Control	36.3 [b]	22.3 [c]
P7L1	41.7 [a]	34.1 [a]
P2L2	41.46 [a]	28.9 [b]
P6R1	41.8 [a]	25.8 [bc]
P7R1	41.22 [a]	29.1 [b]
P3L2	37.5 [b]	28.4 [b]
C.D. (5%)	2.416	3.6

Note: Means with the same superscript in a column do not differ significantly as per uncan Multiple Range Test (DMRT) @ p=0.05

Fig. 2 : Effect of bacterial endophytes on seedling length of maize under normal as well as in drought condition

Stenotrophomonas maltophilia isolated from Himalayan cold desert plants increased the seedling length of rice (var. IR-64) compared to uninoculated seedlings.

Determination of Indole Acetic Acid, Gibberellic Acid and Abscisic Acid Production by Endophytes

Phytohormones play a vital role on growth and development of plants. The quantified data of Indole acetic acid (IAA), gibberellic acid (GA) and abscisic acid (ABA) production using high performance liquid chromatography (HPLC) is presented in the Table 4. The bacterial endophytes grown on the medium amended with precursor L-tryptophan showed the highest production of IAA under abiotic (Water deficit) stress compared to bacteria grown under normal medium. This envisaged that the tryptophan amendment in medium can enhance the IAA production. Indole acetic acid is the most prevalent kind of auxin, that influences many aspects of plant growth and development. Many bacteria generate and release IAA as a secondary metabolite by exploiting L-tryptophan found in root exudates (Fu *et al*., 2015). Gibberellic acid helps plant in stimulating cell division and elongation. Abscisic acid also regulates abiotic stress by stomatal closure and lowering transpiration water loss and inducing acquired resistance in plants against diseases. In this study, bacterial endophytes P7L1 and P7R1 grown under abiotic (Water deficit) stress condition

TABLE 4

Plant growth promoting traits of bacterial endophytes (HPLC data)

Treatments	IAA mg/l		GA mg/l		ABA mg/l	
	Without stress	With stress	Without stress	With stress	Without stress	With stress
P7L1	2.87 ^{a}	0.53 ^{a}	86.96 ^{a}	49.11 ^{a}	0.62 ^{c}	2.67 ^{a}
P2L2	1.00 ^{b}	0.20 ^{b}	20.26 ^{d}	17.43 ^{c}	3.79 ^{a}	1.2 ^{b}
P6R1	0.41 ^{c}	0.28 ^{b}	9.10 ^{e}	5.85 ^{e}	1.44 ^{b}	0.79 ^{bc}
P3L2	0.22 ^{c}	0.27 ^{b}	36.77 ^{c}	13.14 ^{d}	1.33 ^{b}	0.64 ^{c}
P7R1	0.76 ^{b}	0.56 ^{a}	63.31 ^{b}	33.68 ^{b}	0.68 ^{c}	1.13 ^{bc}
C.D(1%)	0.35	0.077	0.759	0.76	0.489	0.5

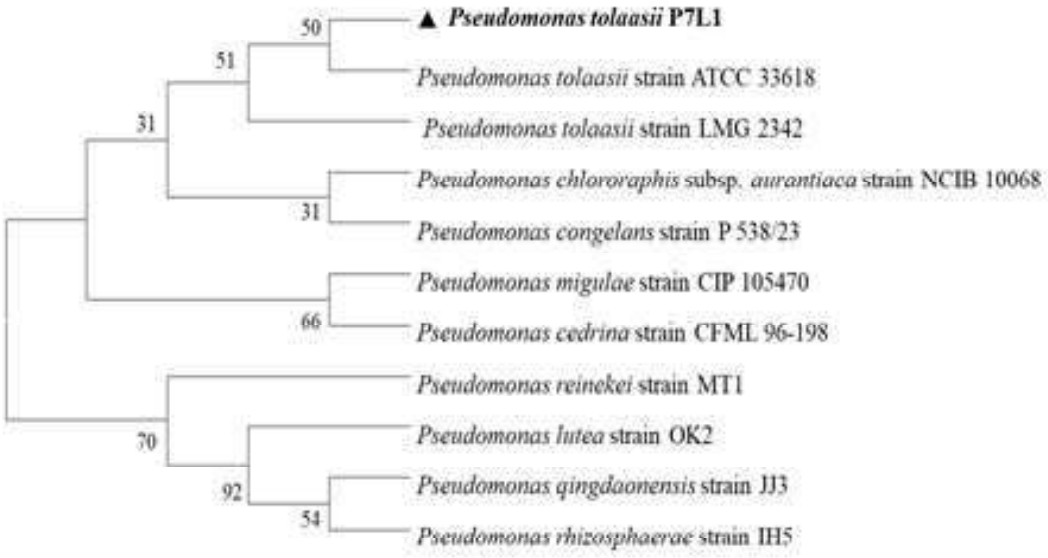

Fig. 3 : Phylogenetic tree of *Pseudomonas species*

showed the significantly difference in production of IAA, GA and ABA (Table 4). These results are in agreement with those of Goswami *et al.* (2014) who reported bacteria *Kocuria turfanensis* produced the IAA in presence of tryptophan under abiotic stress. Pathade (2015) reported that *Bacillus siamensis* BE 76 isolated from the banana plant (Musa spp.) produce gibberellic acid of 0.108 $\mu g\ mL^{-1}$.

Identification of the Selected Bacterial Endophyte using 16S rRNA Gene Sequence

Bacteria can be identified using morphological as well as molecular tools. The genes encoding for 16S rRNA in prokaryotes have been used extensively for sequence based evolutionary analysis because, they are (1) Universally distributed, (2) Functionally constant, (3) sufficiently conserved and (4) Have adequate length to provide a view of evolution encompassing all living microorganisms (Madigan *et al.,* 2009). Molecular methods such as 16S rRNA/ 18S rRNA gene sequence is extensively used for identification of microorganisms (Nandan *et al.,* 2021). In the present study, drought tolerant bacterial endophyte (isolate P7L1) was identified by 16S rRNA gene sequence. The amplified product having sequence length of 1130 bp showed 99.4 per cent homology with *Pseudomonas tolaasii* available at NCBI database. The phylogenetic tree constructed with the sequences of 10 *Pseudomonas species* revealed that the isolate P7L1 is closely related to *Pseudomonas tolaasii* ATCC 33618 and the bacterium was confirmed as *Pseudomonas tolaasii* (Fig. 3).

This study envisaged that the, *Pseudomonas tolaasii* as an efficient bacterium for mitigating drought stress in maize can be used for seed biopriming.

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