Studies on Isolation and Characterization of Plant Growth Promoting Endophytic Azospirillum from Roots of Wheat Plant (Triticum aestivum) of Semiarid Soil of Wardha District, Maharashtra.

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ABSTRACT

Among plant growth promoting rhizobacteria, *Azospirillum* is considered as an important genus which is closely associated with plants and shows potential to degrade organic contaminants, improve the plant health and increase crop yield. The present study deals with the isolation and characterization of *Azospirillum* strains from roots of wheat (at anthesis stage) plant grown under semiarid soil (15.80% soil moisture) and for different plant growth promoting traits. The isolate was identified on the basis of cultural, morphological and biochemical characteristics as *Azospirillum lipoferum*. The isolate was found to be Gram negative, plump rod, slightly curved, motile and able to ferment glucose, sucrose and lactose with production of acid and gas. The isolate demonstrated PGPR traits like IAA, siderophore, ammonia and HCN production and was also found to solubilize tricalcium phosphate. The enzyme profile of the isolate was also studied and found to produce cellulase, chitinase, catalase, oxidase enzymes. Moreover, *Azospirillum* (5.5×10^6/ ml) showed significant antifungal activity against plant pathogenic fungi viz; *Aspergillus niger Aspergillus flavus, Aspergillus terreus, Aspergillus glacus* and *Cladosporium* which helps to survive in competitive environment of rhizosphere. *Azospirillum* was evaluated for its seed germination efficiency and found to induce shoot length up to 23 cm in 10 days as compared to 17 cm in control. The survival of *Azospirillum* isolates from plants grown under semiarid soil and its potential PGPR traits have technological implications for inoculant formulation and improved growth of cereal crops.

Keywords: Endophytic, *Azospirillum,* PGPR, wheat, semiarid soil.

INTRODUCTION

Plant growth in soils is influenced by many factors. These factors can be biotic and abiotic factors. One of the most important environmental constraints is water stress, which can limit crop productivity (1). There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism, which is known as rhizosphere. The rhizosphere concept was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots where microbe populations

are stimulated by root activities (2). Rhizosphere soil is a "hot-spot" for microbial growth and major microbial activities (3). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (4). Root exudates are the substrate or fuel for the intense microbial (bacteria, fungi, algae, protozoa, nematodes and arthropods) activity within the rhizosphere.

In general, microbes commonly used as biofertilizers are *Azotobacter*, *Azospirillum*, *Rhizobium* as nitrogen fixing soil bacteria (5). Drought stress is one of the major agricultural problems reducing crop yield in arid and semiarid regions of the world. Abiotic environmental stress conditions (water stress, acidity, salinity, etc.) can affect the free-living (Azotobacter sp.), the associative (*Azospirillum* sp.), and the symbiotic (Rhizobium, Brady Rhizobium, and Syno Rhizobium) microorganisms (6). The adapted micro-symbionts can be used to protect their hosts from these stresses so as to maintain adequate food production and be used as possible biosensor of the soil functioning (7 & 8). *Azospirillum*–plant association is accompanied by biochemical changes in roots, which in turn can lead to plant growth and tolerance to low soil moisture (9 & 10). Changes in mean global air temperature and precipitation patterns are leading to longer drought periods and more extreme dry years, and more severe drought conditions may hinder food production in some countries (11). One alternative for growing plants under dry conditions is the use of plant growth promoting rhizobacteria. They can either directly or indirectly facilitate plant growth in optimal, biotic or abiotic stress conditions (12). Known mechanisms used by PGPR include nitrogen fixation, phytohormone production (including auxins, cytokinins and gibberellins), solubilization of mineral phosphates, and iron sequestration by bacterial siderophores (13).

Azospirillum is one of the best-studied plant growth-promoting rhizobacteria (PGPR) that are normally associated with grasses, rice, wheat and sugarcane (14). *Azospirillum* is a free living plant growth promoting bacterium (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. The leading theory concerning its growth promotion lies in its ability to produce various phytohormones that improve root growth, absorption of water and minerals that eventually yield larger, and more productive plants (15). *Azospirillum* under stress conditions enhance plant growth by fixing atmospheric nitrogen and by the production of growth promoting substances and influencing root development, causing increased uptake of nutrients from the land, and inhibiting pathogenic fungi and bacteria in the rhizosphere (16). *Azospirillum* inoculation could significantly increase the growth in terms of height; number of leaf/plant; length and breadth of leaf; and fresh and dry weight/plant of rice plant (16). The mechanism of plant growth promotion known to be employed by bacterial endophytes are similar to the mechanisms used by rhizospheric bacteria (17).

Present research designed to isolate and characterize endophytic *Azospirillum* sp. from roots of wheat plant grown in semiarid soil region. This isolated *Azospirillum* from semiarid soil will be easy to use as biofertilizer because they easily adopt the ecological and environmental condition. However, utility of this approach under field conditions under diverse agro-climatic conditions need to be tested.

MATERIALS AND METHODS

1. Collection of sample

Root samples of wheat (*Triticum aestivum*) plant from the semiarid soil of Wardha district, Maharashtra were collected during the reproductive stage (anthesis stage) of the crop, placed in sterile plastic bags and stored at 4ºC in Laboratory, PG Department of Microbiology, J.B. College of Science, Wardha for further analysis.

2. Determination of moisture percentage of soil

Moisture percentage of soil was determined according to the following formula (18).

$$\text{Soil moisture (\%)} = \frac{\text{Weight of wet soil (g)} - \text{Weight of dry soil (g)} \times 100}{\text{Weight of dry soil (g)}}$$

Weight of wet soil (g) = 10g
Weight of dry soil (g) = 8.6353 g

$$\text{Soil moisture (\%)} = \frac{10 - 8.6353 \times 100}{8.6353}$$

$$= 15.80\%$$

3. Preparation of root samples and isolation of *Azospirillum*

The roots were thoroughly washed with tap water to remove loosely adhering soil particles. Roots were surface sterilized by immersion of roots in aqueous solution containing 1% Sodium hypochlorite and 70% Alcohol. After five successive rinses in sterile distilled water, the root samples were cut into pieces of 5 cm in length using sterile scalpel. These root samples were macerated in sterile mortars and serially diluted in sterile phosphate-buffered saline up to 10^{-8} dilutions (19). For the isolation of endophytic bacteria, one ml of diluted sample from 10^{-6} to 10^{-8} dilutions was taken and 0.1ml of aliquot was inoculated in test tube containing NFB (Nitrogen free bromothymol blue) semisolid media. Test tube was incubated at 32ºC for 48 h and observed for the growth by formation of pellicles. The pellicles were streaked on solidified Congo red –NFB medium. The plates were incubated at 37° C till the appearance of bacterial colonies. The colonies developed on Congo red –NFB medium were stored at 4 ºC (20).

4. Biochemical characterization of the isolates

The identification of isolates was done on the basis of cultural, morphological and biochemical tests (21). This identification was followed by studying production of oxidase, catalase, gelatinase, urease, indole, acetoin, methyl red test, production of hydrogen sulfide, citrate utilization, fermentation of glucose, lactose, mannitol, sucrose etc.

5. Characterization of isolates for PGPR traits

a) Assay for Indole acetic acid (IAA) Production

IAA production was detected by the method of Brick *et al*, (22). The isolate was freshly grown on their respective growth medium amended with tryptophan (500 µg/ ml) at 30^0C for 48 hours. Fully grown

culture was centrifuged at 8000 rpm for 10 minutes and was assayed for qualitative detection of IAA. 2 ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of salkowski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$). Formation of pink colour indicates IAA production.

b) Production of Ammonia

The isolate was tested for the production of ammonia by inoculating in 10 ml peptone broth and incubated for 24 hrs at 30 ^{0}C. The test was performed by adding 0.5 ml of Nesslers reagent in tube. Development of brown to yellow colour was a positive test for ammonia production (23).

c) Hydrogen Cyanide (HCN) production

Qualitative HCN determination was carried out by Lorck (1948) method modified by Alstrom and Burns (1989). Isolate was cultured on Nutrient agar medium supplemented with glycine (4 / 4 gl-1). The production of HCN was detected after 48 hrs by using whatman filter paper no. 1 soaked in 2 % sodium carbonate and 0.5% picric acid fixed to the underside of the petri-dish lid which were sealed with parafilm before incubation at 28 to 30 ^{0}C. A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate or strongly cyanogenic potential respectively (24).

d) Siderophore Production

Siderophore production was determined on Chrome azurol S agar (CAS) by the method given by Schwyn and Neilands (1987). CAS agar plate divided into equal sectors and spot inoculated with test organisms and incubated at 28 – 30^{0}C. Development of orange halos around the colonies indicates positive siderophore production (25).

e) Phosphate solubilization activity

For the study of phosphate solublizing ability, the isolates were first screened on Pikovaskaya's agar plate for solubilization of insoluble inorganic phosphate as described by Gaur (26).Cultures were inoculated on center of agar plate under aseptic condition. Plates were incubated for 3 days at 30°C. Presence of clear zone (halo zone) around the colony indicates presence of phosphate solubilization ability.

f) Heavy metal tolerance

The isolate was studied for its heavy metal tolerance potential on Nutrient agar supplemented with varying concentrations of four heavy metals (Cu, Hg, Zn, Pb). Plates were incubated at 37°C for 24 h for diffusion of the metal into the agar, after incubation the plates were observed for the growth of isolate (27).

6. Antibiotic Susceptibility test

Antibiotic susceptibility of the isolate was studied by using Ampicillin (10 µg/disc), Penicillin (10 µg/disc) and Streptomycin (10 µg/disc) on Mueller-Hinton agar. 0.1ml (5.5×10^6/ ml) of culture was spread on to the media. Antibiotic discs were placed on the agar plate and plates were incubated at 37°C for 24-48 hours. Zone of inhibition were recorded in mm.

7. Effect on Seed Germination

For testing the plant growth promoting efficiency of isolated *Azospirillum*, its effect was observed on seed germination of wheat in vitro. Pot culture experiment was conducted to study seed germination efficiency for a period of 10 days. Wheat seeds were surface-sterilized with $HgCl_2$ (0.1%) and successively washed

several times with sterilized water. Then seed was soaked overnight in 7-day-old culture of *Azospiillum* isolate (5.5×10^6/ ml). Wheat seeds not soaked in *Azospiillum* culture was used as control. Both the seeds were sown in two separate plastic pots and filled with autoclaved soil. Pot sowed with inoculated seed was labeled as experimental and pot sowed with uninoculated seed was labeled as control. Pots were incubated for 10 days. Then these two pots were observed for germination of seeds after regular interval of time (18).

8. Antifungal activity of *Azospirillum* against plant pathogenic fungi

Azospirillum was evaluated for its antifungal activity against plant pathogenic fungi viz; *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Aspergillus glacus* and *Cladosporium.* These fungal cultures were obtained from the Post Graduate Department of Botany, J.B. College of Science, Wardha and stored for the further use at 4ºC. For the evaluation of antifungal activity, broth culture of *Azospirillum* was centrifuged at 8000 rpm for 15 min. 0.1ml of each fungal broth culture spread on the respective plates using Czapek Dox agar. Then 100µl of *Azospirillum* supernatant was added in wells and plates were incubated for 4-5 days at 30ºC. Inhibition of fungal mycelium (halo zone) around the well was noted as positive and the inhibition zone was measured.

9. Cell wall degrading enzyme production

a. Cellulase activity

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC (Carboxy Methyl Cellulose) agar containing (g L-1) KH2PO4-1.0, MgSO4.7H2O-0.5, NaCl-0.5, FeSO4.7H2O- 0.01, MnSO4.H2O- 0.01, NH4NO3-0.3, CMC-10.0, Agar-12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to secrete cellulase. The agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. Clear zone around the colony was indicative of cellulase enzyme production (28).

b. Chitinase activity

A minimal salt medium containing colloidal chitin as sole carbon and energy source was used. The medium consisted of Na2HPO4-6g, KH2PO4-3.0g, NH4Cl-1g, NaCl-0.5g, yeast extract-0.05g, colloidal chitin-1.0 % (w/v), agar-15g and distilled water-1000 ml and incubated at 30ºC. Colloidal chitin was prepared by the method of Hsu and Lockwood (29). Colonies showing zones of clearance against the creamy background were recorded as chitinase producing PGPR.

RESULTS AND DISSUCION

Azospirillum isolated from root of Wheat plant (*Triticum aestivum*) at anthesis stage of the crop from semiarid soil having moisture content 15.80% from Nagapur village of Wardha district. The isolate was selected on the basis of their ability to grow better and faster in Congo red-NFB medium. This selected isolate was characterized on the basis of cultural, morphological and biochemical characteristics and

identified as *Azospirillum lipoferum*. This isolate was found to form pellicles in NFB (Nitrogen free bromothymol blue) semisolid media which is a characteristic feature of *Azospirillum.* The isolate was found to be Gram negative, plump rod, slightly curved, motile and able to ferment glucose, sucrose and lactose with production of acid and gas. Isolate showed positive reaction for MR and found to utilize citrate as sole source of carbon. The isolate was found to produce IAA, siderophore (22 mm orange halos), ammonia and showed moderate cyanogenic potential in terms of HCN production and was also found to solubilize tricalcium phosphate (11 mm clear zone). The enzyme profile of the isolate was also studied and found to produce cellulase, chitinase, catalase, oxidase, urease enzymes which helps to survive in the competitive environment of rhizosphere. *A. lipoferum* was found to be resistant towards penicillin as well as ampicillin whereas sensitive for streptomycin demonstrating zone of inhibition of 15 mm. Thus, this isolate demonstrated variation with respect to antibiotic sensitivity. The organism was also tested for its heavy metal tolerance ability and found to tolerate four heavy metals Cu, Zn, Pb up to 1000 mg/L. On the other hand the isolate was found to be very sensitive against Hg.

The important aspect of this investigation was to study the effect of *Azospirillum* on seed germination of wheat plant in vitro. Seeds coated with *Azospirillum* showed significant increase in length of shoot. After 10 days the height of the experimental plant was found to be 23 cms whereas control plant demonstrated height of 17 cms. *Azospirillum* was evaluated for its antifungal activity against plant pathogenic fungi *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Aspergillus glacus* and *Cladosporium. Azospirillum* was found to inhibit the growth of all the five pathogenic fungi. Among the five pathogens, *Aspergillus niger* was found to be most sensitive against *Azospirillum* (zone of inhibition 21 mm). The results of antifungal activity of *Azospirillum* are depicted in table 3.

Table 1: Biochemical characterization of *A. lipoferum* and its PGPR traits

Sr. No.	Tests	Isolate KD 1	Sr. No.	Tests	Isolate KD 1
1	Grams Reaction	-	**12**	Catalase	+
2	Motility	+	**13**	Oxidase	+
3	Glucose fermentation	+	**14**	Urease	+
4	Sucrose fermentation	+	**15**	Amylase	-
5	Lactose fermentation	+	**16**	Gelatinase	+
6	Mannitol fermentation	-	**17**	Ammonia	+
7	H_2S Production	-	**18**	Siderophore	+
8	Indole test	-	**19**	HCN	+
9	Methyl Red	+	**20**	Phosphate solubilization	+
10	Vogues- Proskauer test	-	**21**	Cellulase	+
11	Citrate utilization test	+	**22**	Chitinase	+

Table 2: Antibiotic susceptibility test of *A. lipoferum*

Sr No.	Antibiotic used (10 µg/disc)	Zone of inhibition (mm)	Interpretation
1	Penicillin	Nil	Resistant
2	Streptomycin	15	Sensitive
2	Ampicillin	Nil	Resistant

Table 3: Antifungal activity of *A. lipoferum*

Sr No.	Fungi used	Zone of inhibition (mm)
1	*Aspergillus flavus*	16
2	*Aspergillus niger*	21
3	*Aspergillus terreus*	17
4	*Aspergillus glacus*	18
5	*Cladosporium*	17

Fig1: A. *lipoferum* on Congo red–NFB medium

Fig 2: Production of siderophore

Fig 3: Phosphate solubilization

Heavy metal tolerance ability

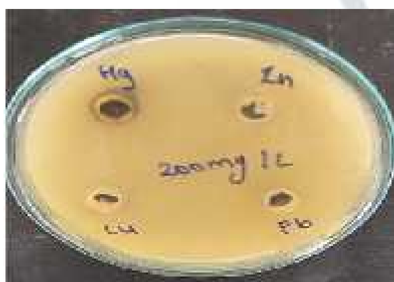

Fig 4: Heavy metal Concentration 200 mg/l

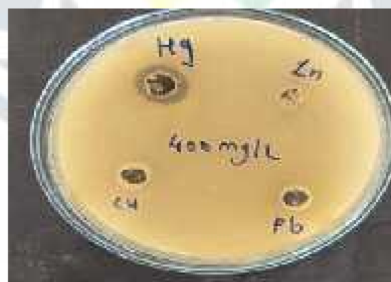

Fig 5: Heavy metal Concentration 400 mg/l

Fig 6: Heavy metal Concentration 600 mg/l

Fig 7: Heavy metal Concentration 800 mg/l

Fig 8: Heavy metal Concentration 1000 mg/l

Antifungal activity of *A. lipoferum*

Fig 9: ***Aspergillus flavus***

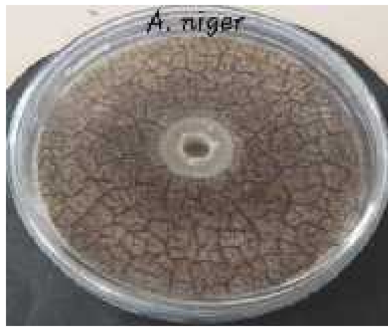

Fig 10: ***Aspergillus niger***

Fig 11: ***Aspergillus terreus***

Fig 12: ***Aspergillus glacus***

Fig 13: ***Cladosporium***

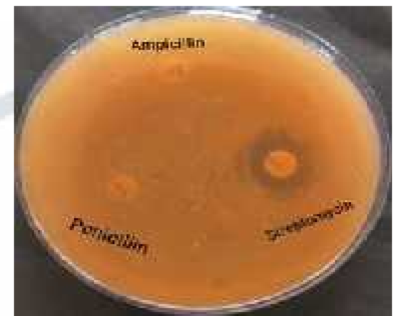

Fig14: Antibiotic susceptibility test.

Effect of *A. lipoferum* on Seed Germination

Fig 15: Seed germination study

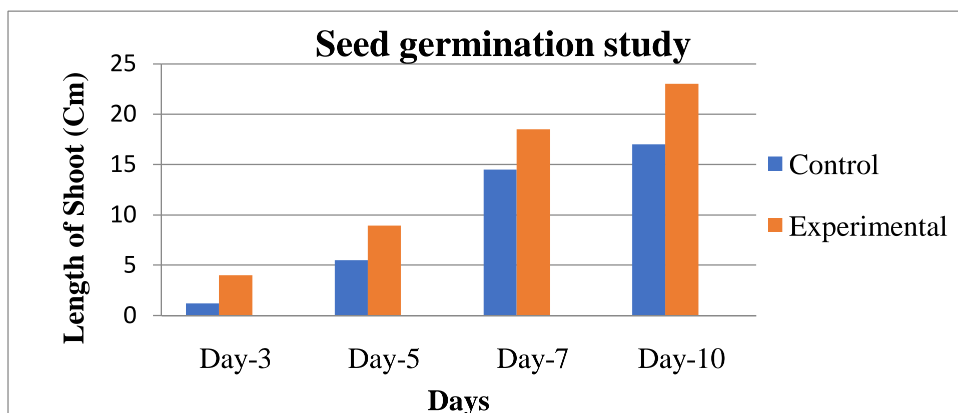

Fig 16: Effect of *A. lipoferum* on seed germination

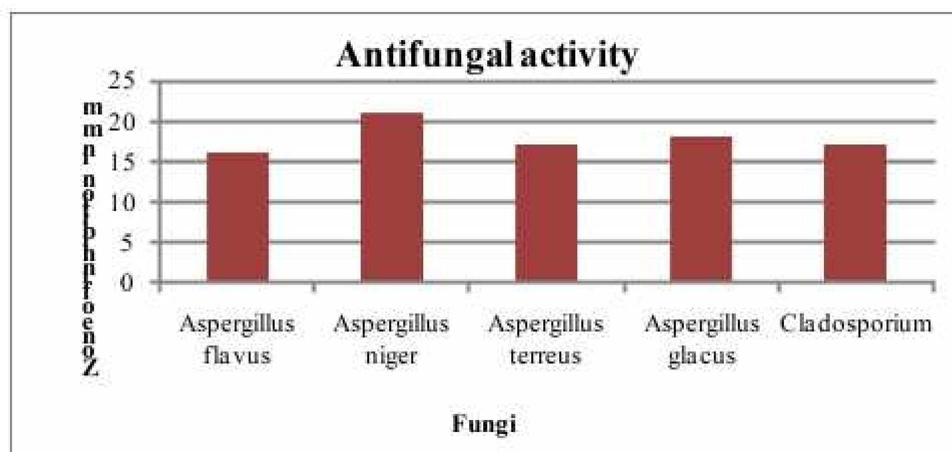

Fig 17: Antifungal activity of *A. lipoferum* against plant pathogenic fungi

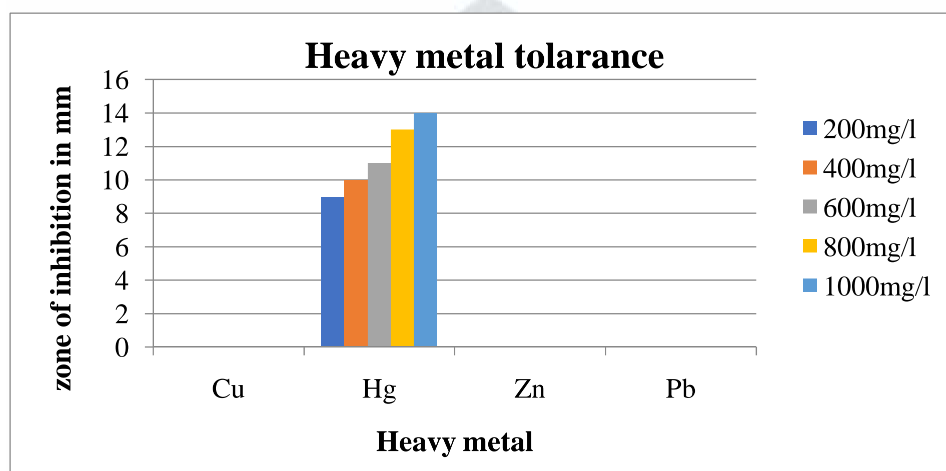

Fig 18: Heavy metal tolerance of *A. lipoferum*

CONCLUSION

The results of present investigation assumed a significant role of *A. lipoferum* in the improvement of fertility of soil in semi arid habitats. The soil with low moisture content adversely affects the survival of rhizobacteria. There is decrease in root colonization of microbes in semiarid soils. The *Azospirillum lipoferum* isolated from wheat root from semiarid condition have strong potential for production of PGPR traits like IAA, siderophore, ammonia, HCN and also found to solubilize phosphate. This isolate also demonstrated antifungal activity against plant pathogenic fungi. *A. lipoferum* demonstrated better potential for PGPR traits which are very beneficial for increase in crop productivity in semiarid soil regions of wheat plant. Drought stress is one of the major agricultural problems reducing crop yield in arid and semiarid regions of the world. These adapted micro-symbionts can be used to protect their hosts from these stresses and may be used for the production of biofertilizer for semi arid soil. The survival of *Azospirillum* isolates from plants grown under semiarid soil and its potential PGPR traits have technological implications for inoculant formulation and improved growth of cereal crops.

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