

# Maize Seedlings Colonization with *Serendipita indica* and Its Colonization Efficiency Analysis

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## Abstract

Maize is one of the most important crops in the world, and ensuring its successful growth and productivity is crucial for global food security. One way to enhance maize growth and productivity is by improving the colonization of its roots by beneficial microorganisms. In this regard, *Serendipita indica*, a plant growth-promoting fungus, has gained attention for its ability to enhance plant growth and productivity, especially in cereal crops and medicinal plants. Previous studies have shown that *S. indica* can colonize various plant species, including maize, but the efficiency of the colonization process in maize seedlings has not been extensively characterized. This protocol outlines a method for efficient colonization of maize seedlings with the beneficial fungus *S. indica*. The protocol includes the preparation of stock solutions, maintenance and growth of *S. indica*, surface sterilization and germination of seeds, preparation of *S. indica* chlamydospores, and colonization of maize plants with *S. indica*. The advantages of this protocol include the use of surface sterilization techniques that minimize contamination, the production of a large number of viable chlamydospores, and efficient colonization of maize seedlings with *S. indica*. This protocol may be useful for researchers studying the role of *S. indica* in promoting plant growth and combating biotic and abiotic stress. Additionally, this protocol may be used in the development of biofertilizers using *S. indica* as a means of increasing crop yields and reducing dependence on synthetic fertilizers. Overall, this protocol offers a reliable and efficient method for colonizing maize seedlings with *S. indica* and may have potential applications in the agricultural industry. This study also provides a valuable tool for researchers interested in studying plant-microbe interactions in maize and highlights the potential of *S. indica* as a biocontrol agent to enhance maize productivity under adverse conditions.

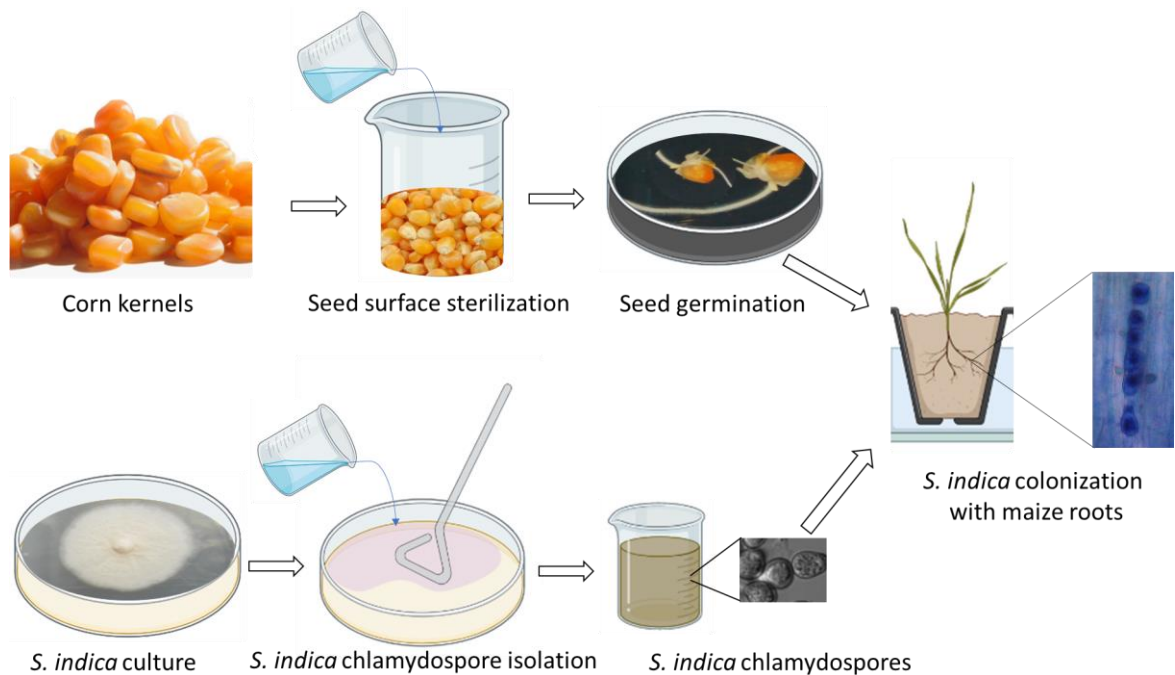
## Key features

- This protocol builds upon the method developed by Narayan et al. (2022), and its application optimized for the root endophytic symbiotic fungus *S. indica*.
- This protocol also allows for histochemical analysis to visualize the colonized fungal spores in the root cells of host plant species.
- This protocol helps in mathematical calculation of the percent colonization or efficiency of colonization.
- This protocol utilizes readily available laboratory equipment, including a light microscope, autoclave, and laminar flow hood, ensuring ease of reproducibility in other research laboratories.

**Keywords:** Maize, *Serendipita indica*, Colonization, Plant microbe interaction, Endophytic fungus, Plant biotic stress, Symbiosis, PGPF

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## Graphical overview



## Background

Maize is an important cereal crop and an essential source of food, feed, and biofuel worldwide. However, maize is highly susceptible to biotic and abiotic stresses, such as pests, diseases, and adverse environmental conditions, which can significantly reduce crop yield and quality (Erenstein et al., 2022; Chávez-Arias et al., 2021). Therefore, it is becoming increasingly important to create sustainable and eco-friendly agricultural methods that can improve the productivity and resilience of maize crops. One such strategy is the use of plant growth-promoting fungi (PGPF) to improve plant growth and health (Adedayo and Babalola, 2023).

*S. indica* (formerly known as *Piriformospora indica*) is a PGPF that has been reported to enhance the growth and stress tolerance of various plant species, including maize (Singhal et al., 2017; Narayan et al., 2021). The colonization of maize seedlings with *S. indica* has been shown to increase root growth, nutrient uptake, photosynthetic efficiency, and resistance to various biotic and abiotic stresses (Varma et al., 1999; Narayan et al., 2017, 2021 and 2022; Prasad et al., 2019; N. Verma et al., 2021). Therefore, the optimization and standardization of the maize seedlings' colonization protocol with *S. indica* are crucial to ensure the reproducibility and reliability of the results.

The protocol described in this paper can help explore the mechanisms underlying the beneficial effects of *S. indica* on maize growth and stress tolerance and identify potential molecular and biochemical markers of plant-fungi symbiosis. Moreover, this protocol can be used to evaluate the performance of different *S. indica* strains and inoculation methods and to assess the effects of environmental factors on colonization efficiency, and plant growth and development.

Several methodologies have been developed to study the interactions between plant growth-promoting fungi (PGPF) and plants such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and chickpea (*Cicer arietinum*) using *S. indica* (Rocha et al., 2019; Wahid et al., 2019; El-Maraghy et al., 2020; Mahdi et al., 2022; Verma et al., 2022). One common approach is the use of in vitro culture systems, such as agar dishes or hydroponic cultures, to assess the effects of *S. indica* on plant growth and stress responses under controlled conditions (Osman et al., 2020). Another approach is the use of field trials to evaluate the performance of *S. indica* in enhancing crop productivity and quality under natural conditions.

Additionally, various molecular and biochemical techniques, such as quantitative polymerase chain reaction (qPCR), metabolomics, and proteomics, were used to analyze the gene and protein expression levels and metabolic pathways involved in the plant–fungi interaction (Gouda et al., 2018).

The protocol described in this paper has several advantages over other published methods (Kumar et al., 2009; Hosseini et al., 2018; Zhang et al., 2018). Firstly, this protocol provides a standardized and reproducible procedure for the colonization of maize seedlings with *S. indica*. Secondly, this protocol allows for the evaluation of the colonization efficiency and distribution of *S. indica* in different plant tissues, which provides insights into the spatial and temporal dynamics of the plant–fungi interaction. Thirdly, this protocol can be combined with various physiological, biochemical, and molecular analyses to study the mechanisms underlying the plant–fungi symbiosis. In addition to the research applications, this protocol has several other possible applications. Firstly, this protocol can be used to develop inoculum production and delivery methods for *S. indica*, which can be used for commercial purposes, such as biofertilizer or biostimulant production. Secondly, this protocol can be used to screen and identify potential *S. indica* strains with improved plant growth–promoting and stress tolerance properties, which can be used to develop new and more effective PGPF-based products. Thirdly, this protocol can be used to investigate the interaction between *S. indica* and other beneficial microbes, such as mycorrhizal fungi or rhizobia, and their combined effects on plant growth and health. Overall, this protocol has the potential to advance research in the field of plant–microbe interactions and to contribute to the development of sustainable and environmentally friendly agricultural practices.

## Materials and reagents

### Biological materials

1. Maize seeds (Variety HQPM-5 from Indian Agriculture Research Institute, Pusa, New Delhi, India)
2. *Serendipita indica* fungus (strain DSM11827 gifted from Prof. Ajit Verma) (Varma, Kost, Rexer & Franken, 1997, European patent office, Muenchen, Germany. Patent No. 97121440.8-2105, Nov 1998)

### Reagents

1. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 1310-58-3)
2. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: 7647-01-0)
3. Phenol (Sigma-Aldrich, catalog number: 108-95-2)
4. Lactic acid (Sigma-Aldrich, catalog number: 50-21-5)
5. Glycerol (Sigma-Aldrich, catalog number: 56-81-5)
6. Trypan blue (Sigma-Aldrich, catalog number: 72-57-1)
7. Glucose (Sigma-Aldrich, catalog number: 50-99-7)
8. Calcium chloride (CaCl<sub>2</sub>) (Sigma-Aldrich, catalog number: 10043-52-4)
9. Ferrous chloride (FeCl<sub>2</sub>) (Sigma-Aldrich, catalog number: 7705-08-0)
10. Calcium nitrate [Ca(NO<sub>3</sub>)<sub>2</sub>] (Sigma-Aldrich, catalog number: 13477-34-4)
11. Magnesium sulfate (MgSO<sub>4</sub>·7H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 7487-88-9)
12. Potassium nitrate (KNO<sub>3</sub>) (Sigma-Aldrich, catalog number: 7757-79-1)
13. Potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>) (Sigma-Aldrich, catalog number: 7778-77-0)
14. Boric acid (H<sub>3</sub>BO<sub>3</sub>) (Sigma-Aldrich, catalog number: 10043-35-3)
15. Manganese sulfate monohydrate (MnSO<sub>4</sub>·H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 10034-96-5)
16. Copper sulfate (CuSO<sub>4</sub>·5H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 7758-98-7)
17. Zinc sulfate (ZnSO<sub>4</sub>·7H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 7733-02-0)
18. Ammonium molybdate [(NH<sub>4</sub>)<sub>2</sub>MoO<sub>4</sub>] (Sigma-Aldrich, catalog number: 13106-76-8)
19. Sodium-hypochlorite (NaClO) (Sigma-Aldrich, catalog number: 7681-52-9)
20. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 7447-40-7)
21. Manganese(II) chloride (MnCl<sub>2</sub>) (Sigma-Aldrich, catalog number: 7773-01-5)

22. Iron(II) sulfate heptahydrate (FeSO<sub>4</sub>·7H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 7782-63-0)
23. Cobalt(II) chloride (CoCl<sub>2</sub>) (Sigma-Aldrich, catalog number: 7646-79-9)
24. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 7647-14-5)
25. Sucrose (Sigma-Aldrich, catalog number: 57-50-1)
26. Ethylenediaminetetraacetic acid ferric sodium salt (NaFeEDTA) (Sigma-Aldrich, catalog number: 15708-41-5)
27. Potassium iodide (KI) (Sigma-Aldrich, catalog number: 7681-11-0)
28. Manganese(II) chloride (MnCl<sub>2</sub>) (Sigma-Aldrich, catalog number: 7773-01-5)
29. Sodium molybdate dihydrate (Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 10102-40-6)
30. Glycine (Sigma-Aldrich, catalog number: 56-40-6)
31. Ammonium phosphate dibasic [(NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>] (Sigma-Aldrich, catalog number: 7783-28-0)
32. Iron(III) chloride (FeCl<sub>3</sub>) (Sigma-Aldrich, catalog number: 7705-08-0)
33. Ethanol (Sigma-Aldrich, catalog number: 64-17-5)
34. Peptone (Sigma-Aldrich, catalog number: 73049-73-7)
35. Yeast extract (Sigma-Aldrich, catalog number: 8013-01-2)
36. Casamino acid (Sigma-Aldrich, catalog number: 65072-00-6)
37. Agar (Sigma-Aldrich, catalog number: 9002-18-0)
38. BD Difco™ Bacto™ Agar (Sigma-Aldrich, catalog number: DF0140-15-4)
39. Biotin (Sigma-Aldrich, catalog number: 7646-79-9)
40. Nicotinamide (Sigma-Aldrich, catalog number: 98-92-0)
41. Pyridoxal phosphate (Sigma-Aldrich, catalog number: 853645-22-4)
42. Aminobenzoic acid (Sigma-Aldrich, catalog number: 150-13-0)
43. Riboflavin (Sigma-Aldrich, catalog number: 83-88-5)
44. Thiamine hydrochloride (Sigma-Aldrich, catalog number: 67-03-8)
45. Pyridoxine hydrochloride (Sigma-Aldrich, catalog number: 58-56-0)
46. Nicotinic acid (Sigma-Aldrich, catalog number: 59-67-6)
47. Trypticase peptone (Sigma-Aldrich, catalog number: 91079-40-2)
48. Malt extract (Sigma-Aldrich, catalog number: 8002-48-0)
49. Myo-Inositol (Sigma-Aldrich, catalog number: 87-89-8)

## Solutions

1. Sterile water (1 L)
2. Double-distilled water (DI water) (1 L)
3. Half-strength modified Hoagland solution (1 L) (see Recipes)
4. Ethanol (1 L) 70% (w/v) (see Recipes)
5. Sodium hypochlorite (NaClO) solution (1 L) 0.75% (w/v) (see Recipes)
6. 0.8% Bacto agar (500 mL) (see Recipes)
7. Lactophenol (50%) (see Recipes)
8. Trypan blue (100 mL) (see Recipes)
9. 10% KOH (100 mL) (see Recipes)
10. Aspergillus modified medium (AMM) or Kaefer (KF) medium (1 L) (see Recipes)
11. MN-Agar medium (1L) (see Recipes)

## Recipes

### 1. Half-strength modified Hoagland solution (1 L)

Reagent	Final concentration	Quantity
<b>Macronutrients</b>		
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	4 mM	944.5 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2 mM	492.9 mg

KNO <sub>3</sub>	6 mM	606.6 mg
KH <sub>2</sub> PO <sub>4</sub>	1 mM	136.08 mg
<b>Micronutrients</b>		
H <sub>3</sub> BO <sub>3</sub>	45 μM	2.78 mg
MnSO <sub>4</sub> ·4H <sub>2</sub> O	20 μM	3.01 mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.4 μM	99.87 mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.7 μM	201.32 mg
(NH <sub>4</sub> ) <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.2 μM	41.18 mg
H <sub>2</sub> O		1,000 mL
Total		1,000 mL

**2. 70% ethanol (1 L)**

Reagent	Quantity
Ethanol (absolute)	700 mL
H <sub>2</sub> O	300 mL
Total	1,000 mL

**3. 0.75% sodium-hypochlorite (NaClO) (bleach) solution (100 mL)**

Reagent	Quantity
Sodium hypochlorite solution (6%)	12.5 mL
ddH <sub>2</sub> O	87.5 mL
Total	100 mL

**4. 0.8% Bacto agar (500 mL)**

Reagent	Quantity
Bacto agar	4g
ddH <sub>2</sub> O	500 mL
Total	500 mL

**5. Lactophenol (50%)**

Reagent	Quantity
Phenol	150 mL
ddH <sub>2</sub> O	150 mL
Lactic acid	125 mL
Glycerol	125 mL
Total	600 mL

**6. Trypan blue (100 mL)**

Reagent	Quantity
Trypan blue	0.1 g
Lactophenol	100 mL
Total	100 mL

**7. 10% KOH (100 mL)**

Reagent	Quantity
KOH	10 g
ddH <sub>2</sub> O	100 mL
Total	100 mL

**8. Aspergillus modified medium/Kaefer medium (1 L)**

Reagent	Quantity
Glucose	20 g
Peptone	2 g
Yeast Extract	1 g
Casamino acid	1 g
Vitamin stock solution	1 mL
Macro-element stock solution	50 mL
Micro-element stock solution	2.5 mL
CaCl <sub>2</sub> (0.1 M)	1 mL
FeCl <sub>2</sub> (0.1 M)	1 mL
Agar	10 g
pH (Adjust with 1 N HCl)	6.5
ddH <sub>2</sub> O	944.5 mL
Total	1,000 mL
<b>Macro-elements stock</b>	
KCl	10.4 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	10.4 g
KH <sub>2</sub> PO <sub>4</sub>	30.4 g
ddH <sub>2</sub> O	1,000 mL
Total	1,000 mL
<b>Micro-elements stock</b>	
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	22 g
H <sub>3</sub> BO <sub>3</sub>	11 g
MnCl <sub>2</sub> ·4H <sub>2</sub> O	5 g
FeSO <sub>4</sub> ·7H <sub>2</sub> O	5 g
CoCl <sub>2</sub> ·6H <sub>2</sub> O	1.6 g
CuSO <sub>4</sub> ·5H <sub>2</sub> O	1.6 g
ddH <sub>2</sub> O	1,000 mL
Total	1,000 mL
<b>Vitamin stock</b>	
Biotin	5 mg
Nicotinamide	50 mg
Pyridoxal Phosphate	10 mg
Aminobenzoic Acid	10 mg
Riboflavin	25 mg
ddH <sub>2</sub> O	100 mL
Total	100 mL
<b>0.1 M FeCl<sub>2</sub></b>	
FeCl <sub>2</sub>	1.62 g
ddH <sub>2</sub> O	100 mL
Total	100 mL
<b>0.1 M CaCl<sub>2</sub></b>	
CaCl <sub>2</sub>	1.11 g
ddH <sub>2</sub> O	100 mL
Total	100 mL

**9. MN-Agar medium (1 L)**

Reagent	Quantity
NaCl	23.4 mg
KNO <sub>3</sub>	80 mg

Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	288 mg
Sucrose	10 g
NaFeEDTA	8 mg
KI	0.8 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	6 mg
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.0024 mg
Glycine	3 mg
KH <sub>2</sub> PO <sub>4</sub>	272.2 mg
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	40.8 mg
CaCl <sub>2</sub>	81.6 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	731 mg
FeCl <sub>3</sub>	583.9 mg
Thiamine hydrochloride	67.4 mg
Pyridoxine hydrochloride	67.4 mg
Nicotinic acid	0.5 mg
Trypticase peptone	1 g
Glucose	10 g
Malt extract	50 g
Myo-Inositol	50 mg
Bacto agar	10 g
KCl	65 mg
H <sub>3</sub> BO <sub>3</sub>	1.5 mg
MnSO <sub>4</sub> ·H <sub>2</sub> O	6.0 mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2.7 mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.2 mg
pH	5.8
Total	1,000 mL

*Notes:*

- All the stock solutions are stored at 4 °C and the vitamin stock at -20 °C. The stock of FeSO<sub>4</sub>·7H<sub>2</sub>O is prepared separately.
- Filter sterilize the vitamin stocks.

### Laboratory supplies

- Gloves (Genesee Scientific, catalog number: 44-100M)
- Lab coat (Fisher Scientific, catalog number: 19-181-570)
- Pipettes of different sizes (Fisher Scientific, catalog number: 13-690-029)
- Tip boxes (Fisher Scientific, catalog number: 01-670-713)
- 1 L Glass bottles (Fisher Scientific, catalog number: 13951L)
- 100 mL Glass bottles (Fisher Scientific, catalog number: 06-414-1A)
- Petri dishes 20mm (Fisher Scientific, catalog number: 08-757-099)
- Petri dishes 150mm (Fisher Scientific, catalog number: 50-403-868)
- Plastic trays (Fisher Scientific, catalog number: 11-394-455)
- Blotting papers (Fisher Scientific, catalog number: 09-301-199)
- Germination paper (Fisher Scientific, catalog number: NC1466201)
- Spreader (Fisher Scientific, catalog number: 14-665-230)
- Beaker (Fisher Scientific, catalog number: 07-250-056)
- 250 mL flask (Fisher Scientific, catalog number: 10-040F)
- Surgical blade (Fisher Scientific, catalog number: 22-079-774)
- Muslin cloth (Amazon.in, item model number: HAZC017639)
- Nail paint (Amazon.in, item model number: CC4407)



## Equipment

1. Laminar flow hood (Thermo Scientific™, catalog number: 1323TS)
2. Light microscope (Leica Microscope, Type 020-518.500, Germany and Nikon Eclipse Ti)
3. Autoclave (Thymol autoclave, India, product code: TAI-902)
4. Incubator shaker (Multitron Incubator Shaker, HT-Infors, Switzerland)
5. Glass house (School of Life Sciences, Jawaharlal Nehru University, New Delhi India)

## Software and datasets

1. Microsoft Office Excel 10
2. GraphPad Prism 8

## Procedure

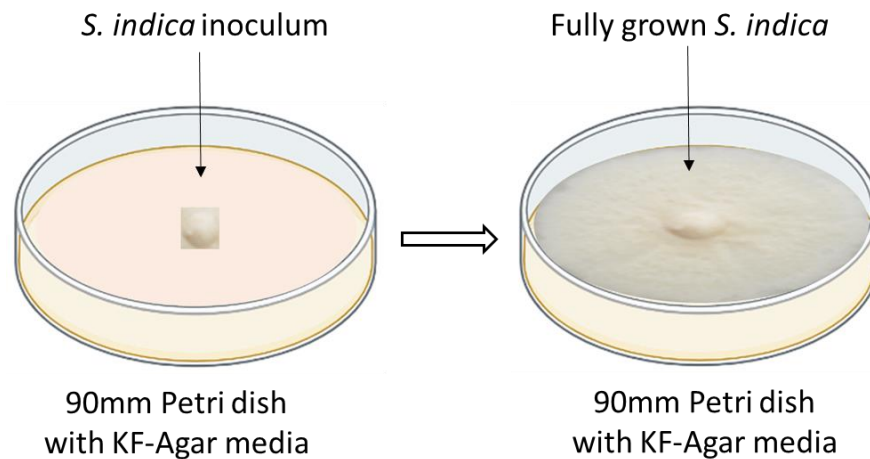
### A. Preparation of stock solutions

1. To ensure the successful execution of all procedures outlined in this protocol and the proper maintenance of the *S. indica* culture, it is necessary to prepare the listed stock solutions mentioned above, and then prepare working solutions and mixtures in sterile water and store in glass bottles at room temperature, as required during the different steps.
2. Sterilize the KF medium using the autoclave at 121.6 °C under 15 psi for 20 min before plating.

### B. Maintenance and growth of fungal species (*S. indica*)

1. Prepare 25 mL of KF-agar medium in a 100 mL glass bottle and autoclave it.
2. Add the vitamin stock into this KF-agar medium at a temperature of 40 °C (kept in a water bath) and mix gently to avoid bubble formation.
3. Pour this KF-agar medium into a 90 mm Petri dish in a laminar flow hood.
4. Allow 20–30 min to complete the solidification of the KF media in the Petri dish inside the laminar flow hood. *Note: To improve solidification and prevent water droplet accumulation inside the lids, it is recommended to keep the Petri dish lid open.*
5. Inoculate a single ball/colony of *S. indica* from the liquid KF medium (the sample has already been incubated in a shaker incubator at  $30 \pm 2$  °C for 7–9 days with shaking at 100 rpm) or cut a slice sized 5 × 5 mm of pregrown *S. indica* on a solid dish culture using a sterilized surgical blade and place this slice of KF-agar-containing *S. indica* culture on the center of the 90 mm fresh KF medium Petri dish. *Note: A fine pressure can be applied onto the slice to make sure the *S. indica* slice can make full contact with the fresh KF medium.*
6. Incubate the dishes at 28–30 °C incubator in darkness for 5–7 days for full growth.
7. After incubation, observe the growth of the *S. indica* by visualizing the grey-colored, cotton-like growth (see the right panel of Figure 1).





**Figure 1. Schematics showing how to inoculate the *S. indica* on a solid KF-agar 90 mm Petri dish**

- Now, *S. indica* is ready for the preparation of chlamydospores and further colonization study with maize seedlings.

*Note: For liquid broth medium growth, S. indica was maintained routinely on solidified Aspergillus (Aspergillus niger) modified medium (Hill and Kafer, 2001). Growth of S. indica was studied in 250 mL culture flasks with constant shaking at 100 rpm, and at  $30 \pm 2$  °C for 7–9 days in a metabolic shaker (Multitron Incubator Shaker, HT-Infors, Switzerland).*

### C. Maize seed surface sterilization and germination

- Take the required quantity of desired functional maize seeds (we used the HQPM5 variety from IARI, New Delhi, India).
- Take approximately 120 seeds (in our case) and dip them into 100 mL of autoclaved DD water for 1 h using a 250 mL beaker.
- Wash the maize seeds with detergent (teepol or Triton X-100) using 3–5 drops in 50 mL of water.
- Repeat washing of the maize seeds with autoclaved DD water by changing the water approximately 5 times for a total of 10 min.
- Sterilize the maize seeds by soaking them in a 250 mL beaker containing 70% ethanol for 40 s, manually swirling it.
- Remove the ethanol and wash five times with autoclaved DD water for a total of 10 min.
- Again, sterilize the maize seeds by soaking them in a 100 mL beaker containing 50 mL of 6% NaOCl (final concentration 0.75%) for 1 min, manually swirling it.
- Wash the maize seeds with autoclaved DD water six times for 10 min.
- Transfer the maize seeds into a new beaker and heat it in a water bath at 60 °C for 5 min.
- Place the maize seeds in water agar dishes/germination sheets using sterile forceps, spacing them 1 inch apart for germination at 28 °C in the dark for 3 days (10–15 seeds per dish).

*Note: Use bi size (150 mm) glass or plastic Petri dishes for making 0.8% water agar dishes (0.8% Bacto Agar, Difco, Detroit, MI, USA).*

### D. Preparation of chlamydospores of *S. indica*

- Take the fully grown *S. indica* KF dishes (6–10 days old) to prepare the chlamydospores.
- Take approximately 2–3 mL of 2% filter-sterilized glucose solution prepared in DD water and pour on the top surface of the fully grown *S. indica* KF dishes.

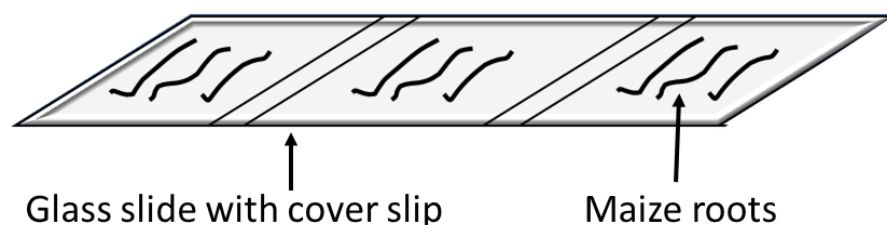
3. Spread the 2% glucose solution gently using a sterile plastic spreader and aim to just collect the chlamydo spores in the solution phase.  
*Note: Try not to disturb the bottom part of the *S. indica*-containing hyphae.*
4. Collect the solution containing chlamydo spores in 2 mL Eppendorf tubes.
5. Filter this solution using a sterile muslin cloth to remove the fungal hyphae and any part of solid medium, transferring the chlamydo spores to another tube.
6. Centrifuge the *S. indica* spores (in Eppendorf tubes) at  $3 \times g$  at  $30^\circ\text{C}$  for 3–4 h.
7. The quality of the spores can be checked by observation under a light microscope using glass slides.
8. The number of *S. indica* chlamydo spores can be maintained at  $5 \times 10^5/\text{mL}$  using a hemocytometer for further colonization with maize seedlings.

## E. Colonization of *S. indica* with maize plants

1. Take out the germinated maize seedlings from the water agar dishes or germination sheets (3–4 days old) and wash the roots with sterilized water, then dip the roots in *S. indica* chlamydo spore suspensions (maintained at  $5 \times 10^5$  spores per mL) to start the inoculation.  
*Note: Forceps can be used to dip the roots into the *S. indica* chlamydo spore suspensions.*
2. Transfer the inoculated maize seedlings with the chlamydo spores of *S. indica* to the sterile MN-agar medium.
3. Grow the inoculated maize seedlings in the MN medium for a minimum of 7 days for colonization.
4. Alternatively, grow the inoculated maize seedlings in pots filled with a mixture of sterile sand and soil in the ratio of 3:1.
5. Grow the inoculated plants under controlled conditions in a glasshouse with an 8 h light (1,000 Lux)/16 h dark period at a temperature of  $28^\circ\text{C}$  with a relative humidity of 60%–70%.
6. Supplement the plants weekly with half-strength modified Hoagland solution.
7. For control plants (without *S. indica* inoculated), mock inoculate with autoclaved DD water–mixed sand. Check the *S. indica* colonization at different time points under the light microscope. In our case, plant roots were harvested at different dpi (5, 10, 15, and 20 days) and were assessed for colonization by trypan blue staining as shown below.

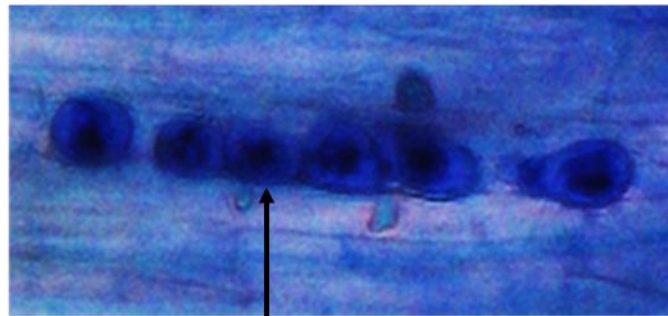
## F. Histochemical analysis

1. Harvest the plant roots at 5, 10, 15, and 20 days after inoculation, and choose approximately 10–20 random samples of the root system for colonization estimation.  
*Note: Use small surgical scissors to cut the root sample from the maize plants.*
2. To soften the root samples, treat them with 10% KOH solution for 15 min and then acidify them with 1 N HCl for 10 min. Finally, stain them with 0.02% trypan blue overnight (Narayan et al., 2021).
3. Samples were destained with 50% Lactophenol for 1–2 h. Keep the samples on a rotary shaker.
4. Cut the root samples into 1 cm small fragments using the surgical blade and mount them on glass slides.  
*Note: Label the glass slides with sample number and root number (see Figure 2).*



**Figure 2.** Schematics showing a glass slide mounted with small fragments of the *S. indica*-colonized maize roots after the trypan blue staining, for microscopic study

5. Cover the root samples with the glass coverslips, pressing the coverslips gently to crush the roots, and seal slides using nail paint (see Figure 2)
6. Observe these slides under a light microscope to see the blue-stained and pear-shaped chlamydo spores of *S. indica* under the different magnifications. Please see Figure 3 to see how chlamydo spores of *S. indica* look like in a colonized state inside the root cortical cells, under light microscope observation.



*S. indica* chlamydo spores

**Figure 3. Blue-colored chlamydo spores of *S. indica* inside the maize root cortical cells, viewed under a light microscope.**

7. Count the colonized root segments for further calculation.
8. The distribution of chlamydo spores within the root was taken as an index for studying colonization. Percent colonization was calculated for the inoculated plants according to the method described previously (Narayan et al., 2017 and 2021). Specifically, it was calculated using the following formula. The calculation involved the total number of randomly selected roots, the number of colonized root segments observed under a microscope, and the total number of root segments taken from a random sample to observe chlamydo spores under a microscope. Both biological and technical repeats were combined to calculate the standard deviation and determine the significance of the data. Table 1 provides an example of percent colonization.

$$\text{Percent colonization} = 100 \times \frac{\text{No. of colonized root segments}}{\text{Total number of segments}}$$

**Table 1. Percent colonization of *S. Indica* with maize plant root**

Time after inoculation (days)	Percent colonization (%)
5	7.5 ± 5
10	27.5 ± 9.6
15	45 ± 12.9
20	70 ± 11.5

## Data analysis

Data can be analyzed using Excel. In our case, we used three biological and three technical repeats. Each time we used 120 seeds of maize. Statistical tests like standard deviation and standard error analysis can be done by using sigma plot or GraphPad Prism 8.

## Validation of protocol

This protocol or parts of it has been used and validated in the following research article(s):

- Kundu et al. (2022). *Piriformospora indica* recruits host-derived putrescine for growth promotion in plants. *Plant Physiology*. (Figure 6, panel h).
- Narayan et al. (2021). [Sulfur transfer from the endophytic fungus \*Serendipita indica\* improves maize growth and requires the sulfate transporter SiSulT](#). *Plant Cell*. (Figure 1, panel c).
- Verma et al. (2022). Functional characterization of a high-affinity iron transporter ( PiFTR ) from the endophytic fungus *Piriformospora indica* and its role in plant growth and development. *Environmental Microbiology*. (Figure 6, panel c).
- Narayan et al. (2017). Antioxidant enzymes in chickpea colonized by *Piriformospora indica* participate in defense against the pathogen *Botrytis cinerea*. *Scientific Reports*. (Figure 1).
- Jogawat et al. (2013). *Piriformospora indica* rescues growth diminution of rice seedlings during high salt stress. *Plant signaling & behavior*. (Figure 1, panel b).
- Kumar et al. (2009). Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiology*. (Figure 1).

## General notes and troubleshooting

### General notes

1. The colonization efficiency of *S. indica* may vary depending on the maize cultivar, growth conditions, and chlamyospore quality.
2. Overstaining can hide the chlamydo spores with the root tissues.
3. To improve colonization efficiency, use a fresh culture of *S. indica*.
4. Limitations associated with this protocol:
  - a. The colonization efficiency of *S. indica* may vary depending on the maize cultivar, growth conditions, and inoculation methods, which may affect the reproducibility of the results.
  - b. The use of high-throughput molecular techniques, such as RNA sequencing or metabolomics, may require a large amount of plant tissue, which may limit the application of this protocol to small-scale experiments.
  - c. This protocol only focuses on the early stages of maize growth, and the long-term effects of *S. indica* on plant growth and stress tolerance are still unclear.

### Troubleshooting (Table 2)

**Table 2. Troubleshooting**

Common problems	Troubleshooting
Low colonization efficiency	Check the viability of the chlamydo spores by growing on a liquid broth or solid KF-agar dishes and compare with normal <i>S. indica</i> growth.
Low germination efficiency	Check the quality of seeds. Avoid using very old, dormant seeds.
Chlamydo spores are not visible in the microscope	Stain chlamydo spores with trypan blue for a longer time.
Blue-colored chlamydo spores are hidden in root tissues	Try to destain the roots for a longer time using Lactophenol.
Contamination of other fungal species	Ensure all equipment and reagents are in sterile conditions.

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## Competing interests

All the authors declare no conflict of interests.

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