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# Endophytic Testing of *Serratia marcescens* strain NPKC3_2_21 against Inpara 3 Rice Variety

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## Abstract

*Pest management is a crucial concern, especially when dealing with insect pests that can cause extensive damage to agricultural crops and the economy. One such pest is the rice stem borer (Scirpophaga innotata), which infests rice stems and poses a significant threat. To combat these pests, various microbial agents have been developed for effective pest control. Among these, the endophytic microbe in biological plant protection plays a key role in the program of sustainable pest management. An endophytic bacterium, Serratia marcescens strain NPKC3_2_21, has been identified as a key player in sustainable pest management, particularly in rice crops. In this study, we aimed to investigate the endophytic characteristics of S. marcescens strain NPKC3_2_21 in the swamp rice plant of the Inpara 3 variety of swamp rice plants. To establish its roles. Marcescens as an endophytic bacterium in rice plants, specifically the Inpara 3 variety, we conducted tests by inoculating bacteria on the plant tissue of rice plants that have been sterilized rice plant tissues with the bacteria. We assessed the presence of S. marcescens strain NPKC3_2_21 in plant tissues by applying isolates to the surface of rock wool, which supported the growth of wet rice plants aged ten or over ten days or older after planting. Samples were collected from the underside of the stem, the bottom of the leaves, and the roots on days 2, 7, and 14 after the application of isolates to the rock wool. The samples were then washed in 70% alcohol and 4% chlorox for 30 seconds and subsequently isolated on Luria Bertani (LB) agar media. Furthermore, we conducted tests to determine the ligninolytic, cellulolytic, and proteolytic activities of S. marcescens, which helped elucidate its endophytic ability. Based on the result, the endophytic capabilities. Based on the results, we found that S. marcescens strain NPKC3_2_21 exhibited endophytic characteristics solely in the stem tissue of the Inpara 3 rice variety. However, we did not observe its presence in the root and leaf tissues.*

*Keywords: Endophytic bacterium; cellulolytic; ligninolytic; rice endophyte; rice pest; swamp rice plant*

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## 1. Introduction

Endophytic microorganisms are a diverse group of symbiotic organisms that live inside plant tissues [23]. Produce numerous bioactive compounds [22], increase disease resistance in plants, and do not harm plants [34] can be considered endophytic microorganisms. According to [32], endophytes can be classified as bacteria, fungi, or actinomycetes can be classified as endophytes that establish a symbiotic relationship with plants without causing disease symptoms [35]. The opinion of [2] further supports the notion that endophytic bacteria are beneficial to plants. They facilitate plant growth and plant resistance against diseases by producing secondary metabolites with bioactive substances [8]. In addition, endophyte bacteria serve as growth-promoting agents and biocontrol, because their presence helps plants withstand both biotic and abiotic stresses [16]. They also contribute to the plant's nutrient requirements by acting as nitrogen fixers [31] and phosphate solubilizers [15].

One of the endophyte bacteria proven to have beneficial effects on plants is *S. marcescens*. Numerous scientific journals reported the benefit of *S. marcescens*. This bacterium belongs to the Gram-negative group and falls under the genus containing 14 recognized species and two subspecies [20]. According to [1], *S. marcescens* is a gram-negative and rod-shaped Enterobacteriaceae bacterium. It possesses a unique characteristic of producing a red pigment called prodigiosin even at room temperature [12]. *Serratia*, environment, ubiquitous in the environment and commonly found in soil, water, plants, animals, humans, and foods [6].

While there have been reports suggesting that *S. marcescens* can act as an opportunistic pathogen in various plants and animals [27], it is important to note that there are non-pathogenic strains of the bacterium that serve *S. marcescens* serve as a biological control agent and promote plant growth [7];[26];[25];[10];[28]. One specific strain of *S. marcescens*, NPKC3_2_21, has been reported to promote plant growth by converting phosphate (P) from an unavailable form into an available form into an accessible form through the production of organic acid compounds [28]. Additionally, another strain, *S. marcescens* AL2-16, has been found to enhance the plant growth in *Achyranthes aspera L.* by producing indole-3-acetic acid IAA [7]. Furthermore, *S. marcescens* strain ETR17 has been evaluated for its ability to improve plant growth and promote the management of root rot disease in tea [26]. Similarly, *Serratia* spp bacteria can also significantly increase the nitrogen supply for rice plants [24];[19].

Furthermore, *S. marcescens* has been studied as a potential biological agent for controlling various insect pests. According to [28] found that bacteria of the *S. marcescens* strain NPKC3_2_21 exhibit entomopathogenic properties, specifically targeting larvae of *Spodoptera litura* and *Scirpophaga innotata* in rice plants. Additionally, other studies have demonstrated that *S. marcescens* bacteria can provide protection to rice plants against the rice pest *Nilaparvata lugens* [25].

However, it is important to note that the diversity of endophytic colonies can vary significantly depending on various factors, including the specific bacterial species, the host plant, and the environmental conditions [33]. Contrary to the findings mentioned earlier, a study by [28] reported the presence of endophytic characteristics in *S. marcescens* strain NPKC3_2_21 in the root, stem, and leaf tissues of the IPB 3S rice plant variety. Additionally, other studies have documented the presence of *S. marcescens* in the tissues of both roots and stems across different rice varieties, including IR387, IR26578, Palawan, Kinandang Patong, Moroberekan, Oking Seroni, and IR72 [11]. To further investigate the endophytic properties of *S. marcescens* strain NPKC3_2_21 in a specific host, this study focused on swamp rice plants of the Inpara 3 variety. The objective was to determine whether *S. marcescens* strain NPKC3_2_21 bacteria has also exhibited endophytic characteristics in this particular type of swamp rice plant variety. A series of experiments were conducted to evaluate the presence of *S. marcescens* strain NPKC3_2_21 in the root, stem, and leaf tissues of swamp rice plants belonging to the Inpara 3.

## 2. Materials and Methods

### 2.1 Assessment of the presence of endophytic isolates in plant tissues

The experiment utilized the following materials: *S. marcescens* strain NPKC3_2_21 bacteria, rice seed of Inpara 3 variety rice seeds, Urea, SP36, NPK-16-16-16 fertilizer, 70% alcohol, and chlorox. The necessary tools included petri dishes, test tubes, a Bunsen burner, rock wool, and a sprayer. To evaluate the endophytic ability of the endophyte of *S. marcescens* strain NPKC3_2_21, we carried out two types of treatments were conducted: one with the application of *S. marcescens* strain NPKC3_2_21 and the other serving as a control (without the application of the bacterium. For each *S. marcescens* treatment, two samples of rice plants were randomly collected from rock wool, with two repetitions for each tissue type (stem underside, leaf underside, and root). In the treatment involving the application of *S. marcescens* strain NPKC3_2_21, a solution was prepared by diluting 50 ml of the *S. marcescens* NPKC3_2_21 isolate in 1 liter of water. This solution was then sprayed onto the rock wool surface until it was adequately wet. The samples were subsequently collected from the stem underside, leaf underside, and root areas on days 2, 7, and 14 after the application of the isolate on the rock wool. The rice plant tissues were washed with 70% alcohol and 4% chlorox for 30 seconds and then isolated it on *Luria Bertani* (LB) agar media. In summary, the experiment involved the use of specific materials and tools. Two treatment groups, one with *S. marcescens* strain NPKC3_2_21 and one control, were established. Samples were collected at different time points, and the rice plant tissues were processed and isolated on LB agar media after being washed with alcohol and chlorox.

The observations were conducted daily to identify cream/red bacterial colonies originating from the red pigment of prodigiosin secreted by *S. marcescens* strain NPKC3_2_21 within the plant tissues grown on the agar media. Additionally, microscopic examination confirmed the characteristics of *S. marcescens* strain NPKC3_2_21 as a Gram-negative, rod-shaped bacterium.

### 2.2 Assessment of ligninolytic, cellulolytic, and proteolytic activities

1. Lignolytic Test;

Perform a qualitative test using the spot inoculation method on Nutrient Agar media supplemented with 1% tannic acid. Incubate the samples at room temperature (24-

27°C) and 37°C for six days, repeating the process twice for each temperature treatment. A positive result is indicated by the presence of a brown zone after the incubation period. The brown zone index is calculated by dividing the diameter of the brown zone by the colony's diameter of the colony.

2. Cellulolytic Test;

Perform a qualitative test using the spot inoculation method on Nutrient Agar media supplemented with 1% CMC (carboxymethyl cellulose). Incubate the samples at room temperature (24-27°C) and 37°C for six days, repeating the process twice for each temperature treatment. After incubation, color the media by treating it with a 0.1% Congo red solution for 15-30 minutes, followed by rinsing it with NaCl 1 M NaCl. A clear zone presence positive result is indicated by the presence of a clear zone, which signifies cellulose degradation caused by the cellulase enzyme. The precise zone index is calculated by dividing the diameter of the clear zone by the diameter of the colony.

3. Proteolytic Test;

Perform a qualitative test using the spot inoculation method on Nutrient Agar media supplemented with 1% CMC. Incubate the samples at room temperature (24-27°C) and at 37°C for six days, repeating the process twice for each temperature treatment. Assess the protease enzyme's presence of protease activity by observing the formation of clear zones and calculate the proteolytic index, which is the ratio between the diameter of the clear zone and the bacterial colony diameter.

## 3. Results and Discussion

To validate the endophytic characteristics of the *S. marcescens* bacterial strain NPKC3_2_21 in swamp rice of the Inpara 3 variety, an experiment was conducted. The bacteria were inoculated onto sterilized rice plant roots, as shown in Figure 1. The results indicated that after two days post-inoculation, *S. marcescens* bacteria were observed in the rice stem underside tissue of the rice stem. However, no red pigment of prodigiosin pigment secretion was observed (b). Notably, there was no growth of *S. marcescens* bacteria detected in the underside of the root tissue (a) and leaf (c), as depicted in *S. marcescens* Figure 2. Additionally, Figure 3 demonstrates that no bacterial growth was observed in the medium, when compared to the control, no bacterial growth is detected in the medium, as Figure 3.

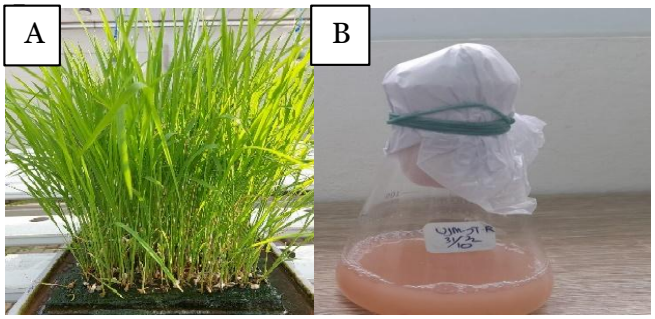


Figure 1 – These figures are (a) a sterile grown rice plant; (b) *S. marcescens* pure isolate culture.

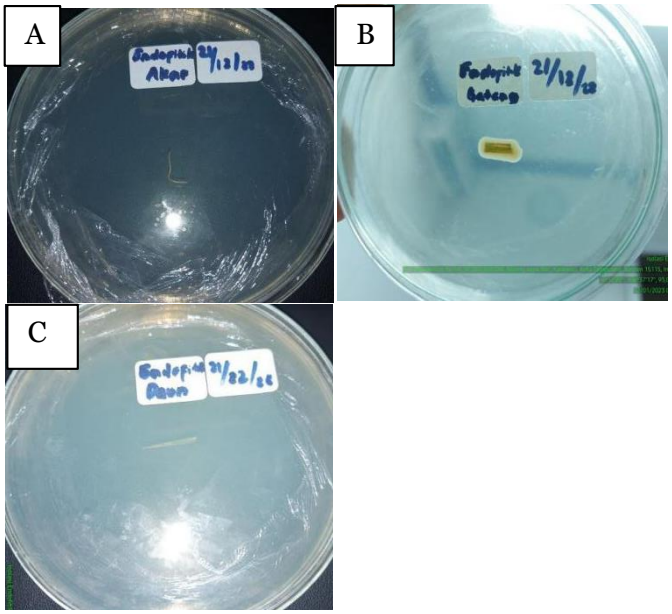


Figure 2 – These figures illustrate different tissues of the rice plants inoculated with the endophytic bacterium S. marcescens: (a) Root, (b) Stem, and (c) Leaf. The bacterium was observed specifically in the stem underside tissue of rice stems, two days after application (D+2).

To investigate further, the rice plants' root, stem, and leaf tissues were re-inoculated in Petri dishes on days D+7 and D+14 after initial inoculation with *S. marcescens*. The results revealed that *S. marcescens* is consistently appeared only in the tissue of rice underside stems. Notably, these stem tissues displayed red colonies, indicating the presence of prodigiosin pigment produced by *S. marcescens* bacteria, as shown in Figure 4 and Figure 5. However, no growth of *S. marcescens* was observed in the underside of root and leaf tissues.

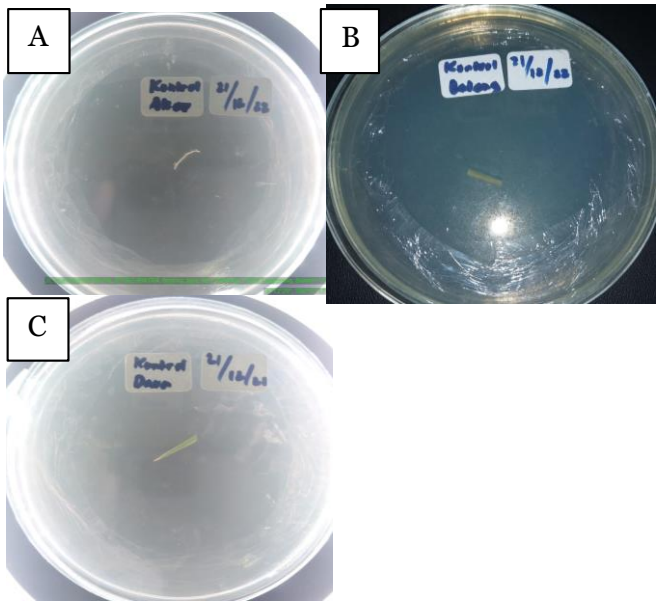


Figure 3 – These figures represent different tissues of the rice plants without the inoculation of endophytic bacteria, S. marcescens: (a) root, (b) stem, and (c) leaf.

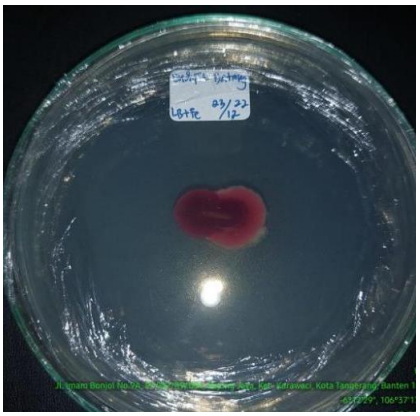

Figure 4 – This figure illustrates the presence of red colonies of *S. marcescens* found in the underside tissue of rice stems, observed on day D+7 after application.

Figure 5 - This figure illustrates the red colonies of *S. marcescens* found in the underside tissue of rice stems on day D+14 after application.

To confirm the shape and color of bacterial cells from S. marcescens, isolated colonies were observed under a microscope in subsequent observations. The microscopic examination confirmed that the bacterial colonies present in the swamp rice plants of the Inpara 3 variety were indeed *S. marcescens* bacteria. The cells were observed to be rod-shaped and gram-negative, as depicted in Figure 6.

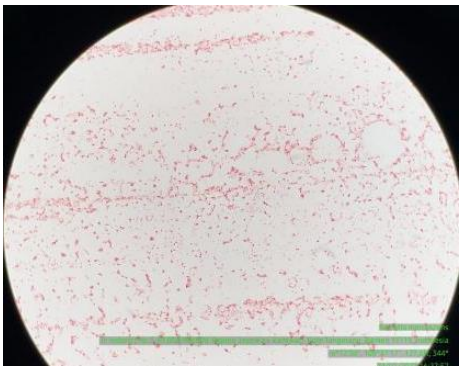

Figure 6– This figure presents the microscopic observation confirming the characteristics of *S. marcescens* colonies. The observation verified that the colonies consist of gram-negative, rod-shaped cells.

Several factors influence the observation of an endophytic character in *S. marcescens* strains NPKC3_2_21 within the stem tissue of Inpara 3 variety rice plants. According to [5], endophytic bacteria can inhabit various parts of a plant, such as aboveground and underground sections, and even seeds, ultimately benefiting plant growth. While these bacteria typically enter the plants through the root zone, they can also utilize the aerial parts of the plant, including leaves, flowers, stems, and cotyledons may also be used [36]. In a similar vein, [33] highlighted that the majority of bacterial endophytic groups originate from colonization processes initiated in the root zone, known as the rhizosphere. However, it's worth noting that these bacteria can also come from alternative sources, such as the phyllosphere, anthroposphere, or spermosphere.

Additionally, endophytic microbes have the ability to colonize plants either actively or passively, both locally and systemically. They can establish themselves intercellularly, and intracellularly within the plant. This aligns with the findings of [14], who observed that endophytic microorganisms can enter the plant through various means such as stomata, lenticels, wounds, the emergence of lateral roots, and germinating radicals. Notably, endophyte microbes can produce cell wall degrading enzymes [33] that enable them to penetrate stem tissues directly without relying on root entry.

According to previous studies, *S. marcescens* bacteria are known to produce several cell-degrading enzymes, including cellulase and ligninase [30];[29], proteases [13], chitinase [21];[13], and lipase [18] to degrade the cell wall. In qualitative laboratory tests measuring the activity of cell-

degrading enzyme synthesis by endophytic bacteria of *S. marcescens* strain NPKC3_2_21, the results indicated the production of ligninolytic activity. At room temperature, the average diameter of the brown zone formed was 2.2 cm (Table 1, Figure 7a1 and a2), while at 37°C, the average diameter was 2.4 cm (Table 1, Figure 8a1 and a2). The bacteria also exhibited cellulolytic activity, with an average apparent zone diameter average of 1.2 cm at room temperature (Table 1, Figure 7b1 and b2) and an average of 0.4 cm at 37°C (Table 1, Figure 8b1 and 8b2). Additionally, proteolytic activity was observed, with an average apparent zone diameter average of 4 cm at room temperature (Table 1, Figure 7c1 and c2) and an average of 3.95 cm at 37°C (Table 1, Figure 8c1 and 8c2). Please refer to the attached Table 1 and Figures 7 and 8 for a visual representation of these finding

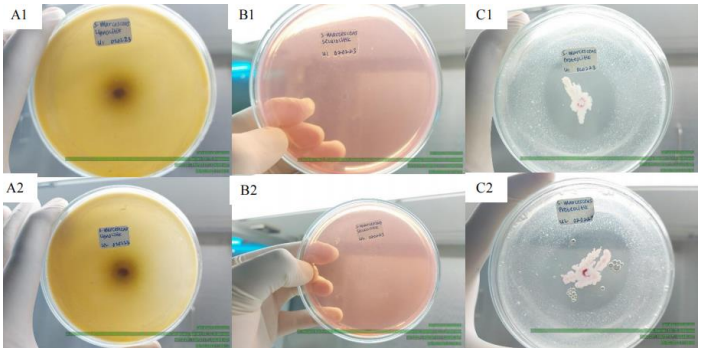


Figure 8 – These figures depict the activity test results of *S. marcescens* strain NPKC3_2_21 at a temperature of 37°C. The activities tested include (a) ligninolytic, (b) cellulolytic, and (c) proteolytic of *S.marcescens* strain NPKC3_2_21 at $37^0$C temperature.

Table 1. This is a table of ligninolytic, cellulolytic, and proteolytic activities test of S. marcescens strain NPKC3_2_21.

| Type of Test | Incubation Time | Incubation Temperature | Repetition | Zone Diameter | Colony Diameter | Description |
|---|---|---|---|---|---|---|
| Ligninolytic | 4 days | Room Temperature | 1 | 2.2 cm | 0..6 cm | Brown Zone |
| | | | 2 | 2.2 cm | 0..6 cm | |
| | | | Average | 2.2 cm | 0..6 cm | |
| Ligninolytic | 4 days | $37^0$C | 1 | 2.5 cm | 0..6 cm | Brown Zone |
| | | | 2 | 2.3 cm | 0..7 cm | |
| | | | Average | 2.4 cm | 0..65 cm | |
| Cellulolytic | 6 days | Room Temperature | 1 | 1.1 cm | 0..4 cm | Clear Zone |
| | | | 2 | 1.3 cm | 0..3 cm | |
| | | | Average | 1.2 cm | 0..35 cm | |
| Cellulolytic | 4 days | $37^0$C | 1 | 0.4 cm | 0..2 cm | Clear Zone |
| | | | 2 | 0.4 cm | 0..2 cm | |
| | | | Average | 0.4 cm | 0..2 cm | |
| Proteolytic | 5 days | Room Temperature | 1 | 4 cm | 1..3 cm | Clear Zone |
| | | | 2 | 4 cm | 1..1 cm | |
| | | | Average | 4 cm | 1..2 cm | |
| Proteolytic | 4 days | $37^0$C | 1 | 4.2 cm | 1..5 cm | Clear Zone |
| | | | 2 | 3.7 cm | 1..2 cm | |
| | | | Average | 3.95 cm | 1..35 cm | |

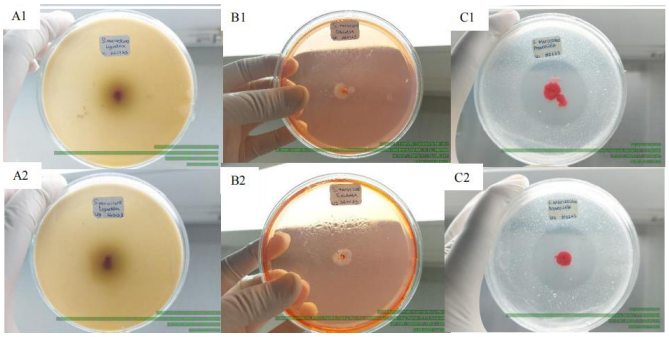


Figure 7 – These figures illustrate the activity test results of *S. marcescens* strain NPKC3_2_21 at room temperature. The activities tested include (a) ligninolytic, (b) cellulolytic, and (c) proteolytic.

Plant cell wall degrading enzymes, including ligninolytic and cellulolytic enzymes, have been extensively reported in various microorganisms. Ligninolytic enzymes are crucial in deleting lignocellulosic and other wood-containing fibers [3]. Ligninolytic enzymes play a crucial role in the breakdown of lignocellulosic and other wood-containing fibers. Lignocellulosic material is composed of three main components: cellulose (40%–50%), hemicelluloses (25%–30%), and lignin (15%–20%) [4];[30]. Cellulose, the most abundant form of fixed carbon, has the potential to serve as a renewable source of renewable organic energy in the eco-

system. It has a fibrous, rugged, and water-insoluble structure that provides mechanical strength and chemical stability to plants, enabling them to maintain their cell wall structure. Hemicellulose, the second most prevalent polysaccharide present in plants, consists of various five and six-carbon monosaccharide units. Additionally, lignin acts as a binder, connecting cellulose and hemicellulose [30].

The ligninolytic enzyme has promising applications in biotechnology, particularly for the biodegradation of lignin and other organic compounds found in plant cell walls [17]. Additionally, the cellulase enzyme, which is cellulolytic in nature, plays a vital role in breaking down cellulose, the primary and widely present polysaccharide component of the plant cell walls. Cellulose is characterized by its fibrous, astringent, crystalline, and water-insoluble properties, and serves as an organic carbon source [30].

While *S. marcescens* bacteria have several advantages, their *S. marcescens* pathogenicity has sparked controversy. Numerous reports indicate that *S. marcescens* can act as an opportunistic human pathogen, leading to nosocomial infections that may even reach epidemic proportions [9];[1]. However, [10] explained that *S. marcescens* with nonpathogenic strains of *S. marcescens* can be beneficial as biological control agents or as bacteria that promote plant growth. According to [28], examined the toxicological effects of *S. marcescens* strain NPKC3_2_21 bacteria were examined using hemolysis tests, acute dermal tests, and acute oral tests conducted on white rats. The results of these toxicity tests classified *S. marcescens* strain NPKC3_2_21 as nonpathogenic bacteria. Similarly, *S. marcescens* strain S-JS1 [7], *S. marcescens* strain AL2-16 [14], *and S. marcescens strain ETR17* [26] have been evaluated and deemed nonpathogenic bacteria.

## 4. Conclusion

Based on the observations conducted to assess the endophytic characteristics of *S. marcescens* strain NPKC3_2_21 on Inpara 3 variety swamp rice plants of the Inpara 3 variety, it has been determined that the *S. marcescens* bacteria exhibit endophytic properties specifically in the stem tissue and the lower part of the rice plant. Conversely, in the root and leaf tissue, *S. marcescens* strain NPKC3_2_21 did not demonstrate endophytic properties.

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