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Evaluation of phenotypic characteristics and confirmatory tests of isolates from *Cajanus cajan*

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Abstract
The goal of the current study is to separate and screen soil samples from the Rajgarh district in the state of Madhya Pradesh for the morphological and biochemical traits of several isolates of *Cajanus cajan.* After three to five days of incubation on YEMA plates, the isolates generated from *Cajanus cajan* formed white colonies with a diameter of one to three millimeters. The colonies were round, raised, and produced a significant amount of mucus. Five of the 32 isolates that were examined showed gram-positive staining, whereas 27 showed gram-negative staining. Most of the isolates exhibited rod-shaped cells, except for 4 isolates that were coccus-shaped. On YEMA-Cr plates, the isolates generally showed white colonies, although 5 isolates absorbed the Congo red dye, resulting in red colonies. The ketolactose test indicated that 6 of the isolates could produce the ketolactose enzyme.

Keywords: YEMA, *Cajanus*, Congo red, gram-negative

Introduction
Pigeon pea (*Cajanus cajan*) ranks as the second most significant kharif pulse crop cultivated in India, following chickpea. In India, it is primarily cultivated under rainfed conditions. This material serves multiple purposes, including as a source of food, animal feed, and fuel wood. Soil fertility is sustained through nitrogen fixation by the micro symbiont Rhizobium, along with the contributions from leaf fall and nutrient recycling. Although often viewed as an underappreciated crop, it possesses significant untapped potential to enhance production in India, both in terms of volume and quality. Pigeon peas demonstrate a superior adaptability to the region compared to other legumes, as they provide an exceptional blend of elevated biomass productivity, significant resilience to environmental stress, optimal nutritional profiles, and substantial contributions to soil moisture and nutrients. Pigeon peas are rich in starch, protein, calcium, manganese, fat, crude fiber, trace elements, and minerals. Pigeon peas serve as a traditional folk remedy in China, India, the Philippines, and several other nations, alongside their significant nutritional benefits (Sharma, *et al*., 201). A significant challenge that constrains the economically viable agricultural production of pigeon pea globally is inadequate soil fertility. One method to address this issue is the incorporation of biofertilizers, which enhance soil fertility by providing essential nutrients required for optimal crop growth through processes such as atmospheric nitrogen fixation and mineral solubilization (Osman *et al*., 2011) [19]. Pigeon pea stands out for its agronomic and economic significance as a low-input crop, requiring no chemical fertilizers due to its natural symbiosis with rhizobia for nitrogen supply (Abaidoo, *et al*., 1999) [1]. The legume typically shows compatibility with rhizobia associated with the cowpea miscellany group, which also nodulates other legumes like cowpea (*Vigna unguiculata*), siratro (*Macropitilium atropurpureum*), lima bean (*Phaseolus lunatus*), and peanut (*Arachis hypogaea*) (Thies *et al*., 1991; Saxena, 2008; Ronemeyer & Reinhod-Huren, 2018) [27, 22, 11].

Methodology
After two to three days individual colonies were seen for their size, color, form and elevation (Vincent, 1970) [28]. The isolates were grown on YEMA plates and incubated at 28±2 0C. Streking the isolates on ketolactose agar media and incubation at 28±2 0C for 3-5 days, the ketolactose test was carried out as per Sharma *et al*., 2010 [23]. Benedict's reagent then tops the plates. Agrobacterium presence is shown by yellow coloration; rhizobial colonies are indicated by none such pigment. One single colony from a pure culture plate was selected

using a sterile loop and combined with 3% hydrogen peroxide on a glass slide to do the catalase test. Within few seconds, bubbles appearing suggest that the isolate is producing catalase enzyme (Kumari *et al*., 2010) [17]. One colony from a pure culture plate was smeared on oxidase discs, and an instantaneous blue staining of the discs shows positive oxidase activity (Kovacs, 1956) [15]. In nitrate broth, one colony from a pure culture was inoculated and incubated for three days at 28±20C adding few drops of dilute sulfuric acid and nitrate test reagent to the broth. Considered as a favorable nitrate reduction activity, development of the blue hue was regarded (Kumari *et al*., 2010) [17]. Starch hydroly the isolates were streaked on starch agar plates and cultured for two to three days at 28±2 0C. These plates were flooded with iodine solution after incubation and watched for starch breakdown surrounding the colonies (Singh *et al*., 2008) [25].

Results and Discussions

Phenotypic Characteristics and Confirmatory Tests of Isolates from *C. cajan*

After three to five days of incubation on YEMA plates, the isolates taken from *C. cajan* formed white colonies with diameters ranging from 1 to 3 mm (see Figure 15). Round, raised, and generating a lot of mucus, the colonies were Out of the thirty-two isolates examined, twenty-seven were gram-negative and five were gram-positive; twenty-eight showed rod-shaped cells under a microscopic view. The ketolactose test revealed 26 isolates were negative whereas the confirmatory tests revealed 27 isolates developed white colonies on YEMA-Cr medium (Table - 1).Among the thirty-two isolates under analysis, twenty-three had morphological, microscopic, and confirmatory traits matching the reference strains. In the confirmatory assays, however, 9 isolates (KCC2, MKCC1, MKCC2, SICC2, HCC1, NNCC1, TECC1, JCC2, and BCC1) showed different outcomes relative to the reference strains. Thus, 23 likely rhizobial isolates were chosen for additional study depending on the main screening findings. The morphological characteristics displayed by the isolates and the reference strains examined in the current work match the typical morphological characteristics of rhizobia. Holt *et al*., 1994 [12] also recorded same kind of findings. Moreover, several past studies separated rhizobia from physically similar legumeous plant root nodules. Agreeing with the current work, Costa *et al*. (2014) [5] revealed white color colonies with a maximum colony size of 3 mm from bacterial colonies recovered from *C. cajan* root nodules. Apart from this, the microscopic characteristics of majority of the isolates match the typical traits of rhizobia and with the microscopic characteristics of reference strains as earlier documented by Vincent, 1970 [28]: Edulamudi *et al*., 2015 [8]; Bhargava *et al*., 2016 [3].

Table 1: Phenotypic Characteristics and Confirmatory Tests of Isolates from *C. cajan*

Isolates	Sample collection Site	Morphological features		Microscopic features		Confirmatory tests	
		Colony color	Colony size (mm)	Gram staining	Cell shape	Color on YEMA-Cr	Ketolactose test
RCC1	Saredi	White	1-2	-	Rod	White	-
RCC2	Saredi	White	1-2	-	Rod	White	-
ICC1	Saredi	White	2-3	-	Rod	White	-
ICC2	Saredi	White	1-2	-	Rod	White	-
ICC3	Saredi	White	1-3	-	Rod	White	-
KCC1	Saredi	White	1-2	-	Rod	White	-
KCC2	Saredi	White	1-2	+	Coccus	Red	-
KCC3	Barkheda	White	1-3	-	Rod	White	-
MKCC1	Barkheda	White	2-3	+	Rod	Red	+
MKCC2	Barkheda	White	1-2	-	Coccus	Red	+
MKCC3	Barkheda	White	1-3	-	Rod	White	-
SICC1	Barkheda	White	1-2	-	Rod	White	-
SICC2	Barkheda	White	1-3	-	Rod	White	+
SICC3	Barkheda	White	2-3	-	Rod	White	-
HCC1	Barkheda	White	1-2	-	Rod	Red	-
HCC2	Kaalipith	White	1-3	-	Rod	White	-
HCC3	Kaalipith	White	1-2	+	Rod	White	-
DCC1	Kaalipith	White	1-3	-	Rod	White	-
DCC2	Kaalipith	White	1-2	-	Rod	White	-
NCCC1	Kaalipith	White	1-3	-	Rod	Red	+
NCCC2	Kaalipith	White	1-2	-	Rod	White	-
NCCC3	Kaalipith	White	1-2	-	Rod	White	-
TECC1	Kaalipith	White	1-2	+	Rod	White	-
TECC2	Kaalipith	White	1-3	-	Rod	White	-
TECC3	Karedi	White	1-2	-	Rod	White	-
SCC1	Karedi	White	1-2	-	Rod	White	-
JCC1	Karedi	White	1-2	-	Rod	White	-
JCC2	Karedi	White	1-2	-	Coccus	White	+
KOCC1	Karedi	White	1-2	-	Rod	White	-
KOCC2	Karedi	White	1-2	-	Rod	White	-
BCC1	Karedi	White	1-2	+	Coccus	White	+
BCC2	Karedi	White	1-2	-	Rod	White	-

Biochemical Characteristics of Isolates from *C. cajan*

While producing negative findings for the starch and gelatin hydrolysis tests, all 23 isolates derived from *C. cajan* showed positive results for both the catalase and oxidase assays (Table -2). Among the isolates, the urease, nitrate, and citrate test findings varied somewhat. Fifteen of the

twenty-three isolates tested exhibited favorable findings for urease production as well as nitrate lowering. The citrate test revealed out that five isolates could use citrate. Catalase enzyme has been observed to be quite important as it breaks down harmful hydrogen peroxide into water and oxygen. The present work found that every one of the twenty-three (100%) isolates that were chosen exhibited good findings of the catalase test, therefore proving that the isolates could generate catalase enzymes. Oxygen bubbles developed on the slides of the isolates when hydrogen peroxide was applied, indicating the synthesis of the catalase enzyme. Comparable to the present study, several previous studies have shown strong catalase activity of rhizobia isolated from different legume plants (Kumari *et al*., 2010; Patil *et al*., 2014) [17, 20]. Because of their cytochrome oxidase enzyme, oxidase positive bacteria are crucial in the movement of electrons from electron donors such NADH to electron acceptor such oxygen. Like the findings of the catalase test, every one of the twenty-three chosen isolates (100%) revealed positive oxidase test results. Blue disc coloring of the oxidase test spreading isolates on oxidase disc indicates the synthesis of oxidase enzyme. In line with the current work, Dubey *et al*., 2010 [7] revealed the positive oxidase activity of rhizobia.

Furthermore disclosed in the present work were negative findings for starch hydrolysis tests among all 23 (100%) isolates. Many earlier investigations clearly support the detrimental outcomes of starch hydrolysis by rhizobial isolates (Kumar *et al*., 2012) [16]. Like the present results, several other research shown that rhizobia produces urease enzyme (Sadowsky *et al*., 1983; Deshwal & Chaubey, 2014) [21, 6]. Another typical property of rhizobia is negative gelatinase activity (Singh *et al*., 2008) [25]. Hunter *et al*. (2007) [13] revealed negative gelatinase activity by Rhizobium isolates, same as the outcome of current investigation.

Table 2: Biochemical Characteristics of Isolates from *C. cajan*

Isolates	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
RCC1	+	+	+	-	+	+	-
RCC2	+	+	+	-	+	-	-
ICC 1	+	+	-	-	+	-	-
ICC 2	+	+	-	-	-	-	-
ICC3	+	+	+	-	+	-	-
KCC 1	+	+	-	-	+	-	-
KCC3	+	+	-	-	+	-	-
MKCC3	+	+	+	-	+	-	-
SICC1	+	+	+	-	+	+	-
SICC3	+	+	+	-	+	+	-
HCC 2	+	+	-	-	+	-	-
HCC3	+	+	+	-	+	-	-
DCC 1	+	+	+	-	-	-	-
DCC 2	+	+	+	-	+	-	-
NCCC2	+	+	+	-	+	-	-
NCCC3	+	+	-	-	+	-	-
TECC2	+	+	+	-	+	+	-
TECC3	+	+	-	-	+	-	-
SCC1	+	+	+	-	+	-	-
JCC 1	+	+	+	-	+	-	-
KOCC1	+	+	-	-	-	-	-
GCC1	+	+	+	-	+	+	-
JCC 2	+	+	+	-	+	-	-

Acidic pH and salt tolerance test of isolates obtained from *C. cajan*: Like the isolates from L. purpureus, those from *C. cajan* showed growth on YEMA medium adjusted to pH 5 and pH 6 but did not survive at pH 4 (Table-3). Of the 23 isolates looked at, 14 grew at pH 5 and all grew at pH 6. The isolates in the salinity tolerance test were able to clearly grow on YEMA media with 1% and 2% NaCl. All 23 isolates specifically developed well on conditions with 1% NaCl, but 18 exhibited growth at 2% NaCl. On YEMA media with 3% NaCl, none of the isolates, nevertheless, survived. According to earlier research, strains of Rhizobium legumes are usually sensitive to low pH and flourish in almost neutral to basic pH environments (Graham *et al*., 1994; Jordan, 1984) [10, 14]. Many studies have shown that many legume plants produce acidic pH resistant rhizobia separately (Costa *et al*., 2014; Amel *et al*., 2013) [5, 2]. Different studies from different countries, including Kenya, Egypt, and South Africa, have recorded the isolation of rhizobia adept of surviving acidic pH ranges between 3.0 and 5.0 (Zahran 1999; Bordeleau *et al*., 1994) [18, 4]. The present results match other studies showing that the ideal pH for rhizobial development falls between pH 6.0 and 7.0 (Singh *et al*., 2008; Gauri *et al*., 2011) [25, 9].

Table 3: Acidic pH and salt tolerance test of isolates obtained from *C. cajan*

Isolates	pH 4	pH 5	pH 6	1%	2%	3%
RCC 1	-	+	+	+	+	-
RCC 2	-	-	+	+	+	-
ICC 1	-	-	+	+	+	-
ICC 2	-	+	+	+	-	-
ICC3	-	+	+	+	+	-
KCC 1	-	+	+	+	+	-
KCC3	-	+	+	+	+	-
MKCC3	-	+	+	+	+	-
SICC1	-	-	+	+	+	-
SICC3	-	+	+	+	-	-
HCC 2	-	+	+	+	+	-
HCC3	-	+	+	+	-	-
DCC 1	-	+	+	+	+	-
DCC 2	-	-	+	+	+	-
NCCC2	-	+	+	+	-	-
NCCC3	-	+	+	+	+	-
TECC2	-	+	+	+	+	-
TECC3	-	-	+	+	-	-
SCC1	-	+	+	+	+	-
JCC 1	-	-	+	+	+	-
KOCC1	-	-	+	+	+	-
GCC 1	-	-	+	+	+	-
GCC 2	-	-	+	+	+	-

Conclusion

This study focused on the rhizobia that nodulate *Cajanus cajan* cultivated across various regions of Rajgarh. A set of 20 strains isolated from fresh nodules has been established. The authentication of these isolates was achieved through the inoculation of *C. cajan* seedlings, which were cultivated in jars filled with autoclaved sand. Additionally, we have evaluated the tolerance of all strains to the primary stress factors, specifically salinity and, more importantly, NaCl and pH levels. The strains have demonstrated the ability to thrive across a relatively broad pH spectrum, specifically between 5 and 6. The current investigation indicated the existence of various rhizobial strains within the root nodules of *C. cajan*. The isolates derived from the legumes demonstrated capabilities for promoting plant growth and exhibited varying degrees of tolerance to environmental stressors. The current investigation indicates a pressing

necessity for the large-scale cultivation of efficient, indigenous, and environmentally friendly rhizobia to deliver its advantageous properties to small-scale farmers.

Conflict of Interest: Not available.

Financial Support: Not available.

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