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CHARACTERIZATION, IDENTIFICATION AND ANTIBIOGRAM STUDIES OF ENDOPHYTIC BACTERIA FROM COWPEA [*Vigna unguiculata* (L.) Walp]

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Abstract

This study included isolation, characterization and identification of endophytic bacteria from cowpea [*Vigna unguiculata* (L.) Walp] roots from Hathazari upazilla, Chittagong, Bangladesh and three specis of bacteria, *Staphylococcus intermedius, Staphylococcus caprae* and *Staphylococcus saprophyticus* were finally identified based on their morphological and biochemical characteristics according to established protocol. Results of antibiotic sensitivity pattern of those endophytic bacteria suggested that they can be utilized as a cost-effective biological control agent in future applications, such as delivery of enzymes for controlling certain plant diseases against various pathogens. The findings of this study may help to determine the potential application of those endophytic bacteria in biotechnology, medicine and agriculture.

Keywords: Endophytic bacteria, biochemical identification, *Staphylococcus intermedius*, *Staphylococcus caprae*, *Staphylococcus saprophyticus,* biocontrol agent.

Introduction

Bacteria defined as 'endophyte' are those that live within a plant for at least a part of their life without causing any apparent harm (Anderson *et al*., 2008) and could also establish a mutualistic association (Azevedo *et al*., 2000) to their host. Plants constitute vast and diverse niches for these endophytic organisms. It is worth mentioning that each plant species is a host to a number of bacteria which are living inside plant tissues, form associations ranging from pathogenic to symbiotic. Beneficial relationships include symbiosis; endophytes supply the plants with fixed nitrogen and other endophytic associations that promote plant growth by producing phytohormones, volatiles, defense compounds and enzymes (Hooper, 2001; De Matos *et al*., 2001). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, rhizomes and seeds of various plant species (Sturz *et al*., 2000). Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth (Kobayashi and Palumbo, 2000), nitrogen fixation (Sturz *et al*., 1997) and induction of resistance to plant pathogens (Liu,

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1995; Sturz and Matheson, 1996). The potential for practical applications of endophytes has led to studies addressing the bacteria's ability to control both disease and insect infestations, as well as promoting plant growth (Kavino *et al*., 2007).

Cowpea is major legumes, grown in Bangladesh which contributes a prominent portion of total pulse production of Bangladesh, providing an average yield of 871 kg/ha and net return to 11,805 BDT/ha (Salam and Kamruzzaman, 2016). It is an annual herb having a strong principal root and many spreading lateral roots in surface soil (Sheahan, 2012). They are rich in potassium, calcium, magnesium, phosphorus and also small amount of iron, sodium, zinc, copper, manganese, selenium, vitamin A, B_6 and C, as well as thiamin, riboflavin, niacin and pantothenic acid.

Cowpea suffers from its natural enemies. Various types of insects are the worst of these enemies, but nematodes, bacterial diseases and viruses also cause losses. Reliable published data are available which show evidence that insects cause devastating losses in cowpea yields. Aphids - pre harvest pest can affect cowpea at the growing season. Weevils - post harvest pest, can destroy a granary full of cowpeas within two or three months. But people need to have the grain to eat for 12 months in a year (Sheahan, 2012).

A large number of bacterial and fungal pathogens have been reported to infect crop plants which ultimately decrease their yield and hence reduce profit (Heath, 1998). Chemical treatment to prevent such incidence seems to be an easy solution but with an effect to the healthy environment and even our lives. Soil degradation and groundwater pollution are the ultimate results of over dependence on pesticides. Moreover, pesticide residues also sometimes raise food safety concerns (Gupta and Dikshit, 2010). Considering these adverse outcomes, biological controls to plant pathogens are getting immense importance day by day. Based on microorganisms, biopesticides specific to a target pest offer an ecologically sound and effective solution to pest problems. They pose less threat to eco-friendly approach to pest control (Gupta and Dikshit, 2010). Several endophytic bacteria were found to exert antimicrobial as well as antifungal effects on a number of crops (*Oryza sativa, Glycine max*), that contain several enzymes which will act as an inhibitor of their pest's digestive system and can be effective for pest control (Sunkar and Nachiyar, 2013).

Therefore, the aim of this study is to detect and isolate the eco-friendly endophytic bacteria from the roots of *Vigna unguiculata*, (L.) Walp to study their morphological and biochemical characteristics to identify those bacteria up to species level; and finally antibiotic susceptibility were tested in order to use them as a biocontrol agent (spray) (Nandakumar *et al.*, 2001, Damodaran *et al*., 2017) in bioremediation scheme.

Materials and Methods

Sampling area and collection of samples

Twenty (20) root samples from cowpea [*Vigna unguiculata* (L.) Walp] plants were collected from various agricultural fields of Hathazari Upazila, Chittagong, Bangladesh (Figure 1). Collected root samples were sealed in sterile zipper bags and labeled. All samples were brought to the Molecular Biology Laboratory of the Department of Genetic Engineering and Biotechnology, University of Chittagong, Bangladesh, by maintaining cold chain and stored them in the refrigerator (4°C) for further analysis.

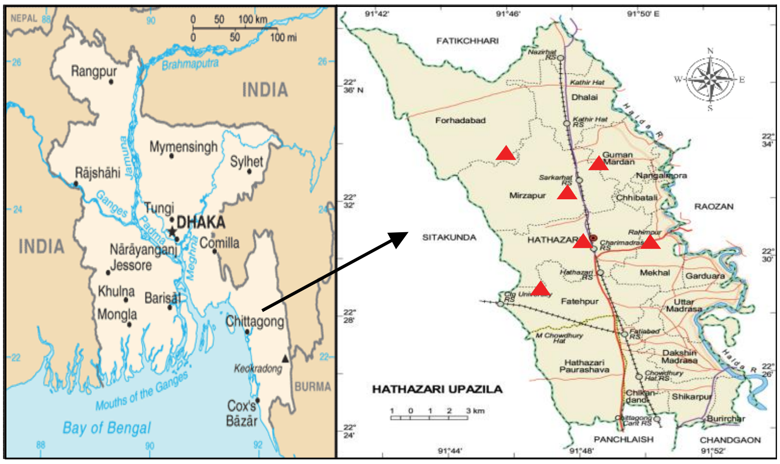

Fig. 1. Sampling sites (▲) of Hathazari Upazila

Isolation of Endophytic Bacteria from cowpea roots

The total procedure were done according to Anderson *et al*. (2008), where samples [healthy fresh roots of *Vigna unguiculata* (L.) Walp] were collected and cleaned under running tap water to remove debris and then air dried. About 25.0 gm of 2-3 cm length of roots were cut and surface sterilization was carried out by rinsing them in Tween-20 for 10 minutes, followed by further washing with dH_2O for at least 7 times. After that, root samples were dipped into 70% alcohol for 30 seconds, and then the samples were washed with dH_2O. Twenty (20.0) ml of 0.2% Hg_2Cl_2 solution was added to the samples and the beaker was put on a shaker at 240 rpm for 5 minutes at 27°C. Then the samples were washed again with dH_2O for at least 7 times. The final root rinsed water, was used as control and spread onto nutrient agar plate (Addisu and Kiros, 2016), which contained (g/L) - peptone 5.00, beef extract 2.00, yeast extract 3.00, NaCl 5.00 and agar 18.00, where pH was adjusted to 7.0. For the isolation of endophytic bacteria, root pieces were further triturated in sterile Phosphate buffer saline (PBS) (Anderson *et al*., 2008) containing (g/L) - NaCl 8.00, KCl 0.20, Na_2HPO_4 1.44 and KH_2PO_4 0.24, where pH was adjusted to 7.4 and maintained at 28°C under

150 rpm agitation. All plates including control were incubated at 37°C for 5 days and the number of CFU was determined to estimate bacterial population density according to Addisu and Kiros (2016).

Following purification, morphologically distinct colonies were identified by observing colony characteristics such as gram nature, color, shape using a binocular biological microscope (XSZ-107BN), where colonies of similar morphological features were grouped into the same species (Castillo *et al*., 2003; Beiranvand *et al*., 2017). Then isolates were selected, cultured, purified and stored in the laboratory at -80°C in glycerol stock (50%) solution for further studies.

Phenotypic and biochemical characterization of endophytic bacterial isolates

Standard morphological and biochemical tests were performed for the identification of endophytic bacteria. They were characterized by gram staining and biochemical tests as described in the Cowan and Steel's Manual for the identification of Medical Bacteria (Barrow and Feltham, 1993). For the activities of oxidase, catalase, coagulase, citrate and carbohydrate (Maltose, Sucrose, Mannitol, D-Xylose and L-Rhamnose) fermentation, isolates were biochemically analyzed (Barrow and Feltham, 1993). Then according to Bergey's Manual of systemic Bacteriology the isolates were provisionally identified up to species level (Claus and Berkeley, 1986).

Determination of antibiotic sensitivity

Susceptibility of three (3) finally identified isolates to different antibacterial agents was measured *in vitro* by employing the modified Kirby-Bauer (Bauer *et al*., 1966) method. This method allows for the rapid determination of the efficiency of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc (Wayne, 2009). Commercially available eight (8) antibiotic discs (Himedia, India) were used for the tests. The antibiotics that were tested against three isolates of this study have been listed in Table 2.

Statistical analysis

Triplicate experiments were done in all the cases during isolation, biochemical analysis and antibiotic sensitivity tests of the selected isolates. The results were measured as the mean value ± standard deviation (SD) in triplicate. Data were captured into Microsoft Excel Software, version 2010 to calculate means and standard deviations.

Results

Screening and Isolation of endophytic bacteria

After preliminary screening of the collected samples, surface sterilization was done as described previously (Anderson *et al*., 2008), then root extraction was prepared to isolate distinct bacterial colonies and plated them on NA (Nutrient agar) medium. Then three different morphologies and colored isolates were finally selected for further studies named as W (white), PW (pale-white) and Y (yellow) (Figure 2).

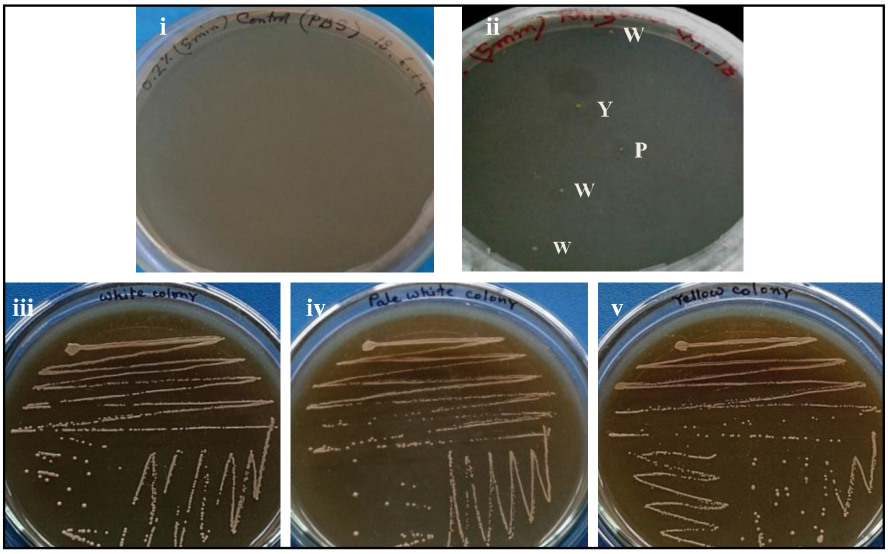

Fig. 2. Control (i) and mixed culture plate (ii); Subculture of bacterial isolate from white, pale white and yellow colony (iii, iv, v).

Characterization and identification

Three selected potential endophytic isolates (W, PW and Y) were characterized on the basis of their morphological and biochemical characteristics (Table 1). They were compared with standard description of Bergey's Manual of determinative bacteriology 9th edition (Bergey *et al.*, 1974; Bergey and Holt, 1994), the isolates were provisionally identified up to species level and are consistent with past field studies (Claus and Berkeley, 1986).

Table 1. Morphological and Biochemical Characteristics of Endophytic Bacteria

Bacterial isolates	W	PW	Y
Morphological characteristics			
Colony color	White	Pale-white	Yellow
Gram Nature	Positive	Positive	Positive
Cell Shape	Cocci	Cocci	Cocci
Biochemical Test Results			
Catalase	+	+	+

Coagulase	+	-	-
Oxidase	-	-	-
Citrate	+	+	+
Utilization of Carbohydrates			
Maltose	+	-	-
Sucrose	+	-	+
Mannitol	-	-	+
D-Xylose	-	-	-
L-Rhamnose	-	-	-
Provisionally Identified Bacteria	*Staphylococcus intermedius*	*Staphylococcus caprae*	*Staphylococcus saprophyticus*
Claus and Berkeley (1986)			

Note: (+) and (-) indicates positive and negative results respectively

Antibiotic sensitivity test

All three different isolates were tested for their antibiotic susceptibility against the eight (8) commonly prescribed antibiotics: Ampicillin (AMP), Bacitracin (B), Kanamycin (K), Penicillin G (P), Rifampicin (RIF), Streptomycin (S), Trimethoprim (TR) and Vancomycin (VA) according to the protocol mentioned previously. The antibiotic response was measured by inhibition zone creating by selected endophytes (sample and control), which revealed that all (n=3) were susceptible to 7 (seven) antibiotics (Wayne, 2009; CLSI M100-S21), whereas they (n=3) were resistant to Ampicillin (AMP) and only PW isolate were resistant to Bacitracin (B) (Table 2; Figure 3).

Table 2. Antibiotic Susceptibility Tests

Antimicrobial Agent	Disc code	Disc potency (µg)	Bacterial isolates		
			W	PW	Y
			Zone ranges (mm)		
Ampicillin	AMP	10	12 (R)	17 (R)	10 (R)
Bacitracin	B	10 units	24 (S)	15 (R)	21 (S)
Kanamycin	K	30 µg	29 (S)	28 (S)	35 (S)
Penicillin G	P	10	34 (S)	33 (S)	28 (S)
Rifampicin	RIF	5 µg	34 (S)	35 (S)	39 (S)
Streptomycin	S	10	21 (S)	25 (S)	25 (S)
Trimethoprim	TR	25	17 (S)	26 (S)	32 (S)
Vancomycin	VA	30	22 (S)	22 (S)	23 (S)

Note: R= Resistance, S= Susceptible

Ref.: Wayne, (2009)

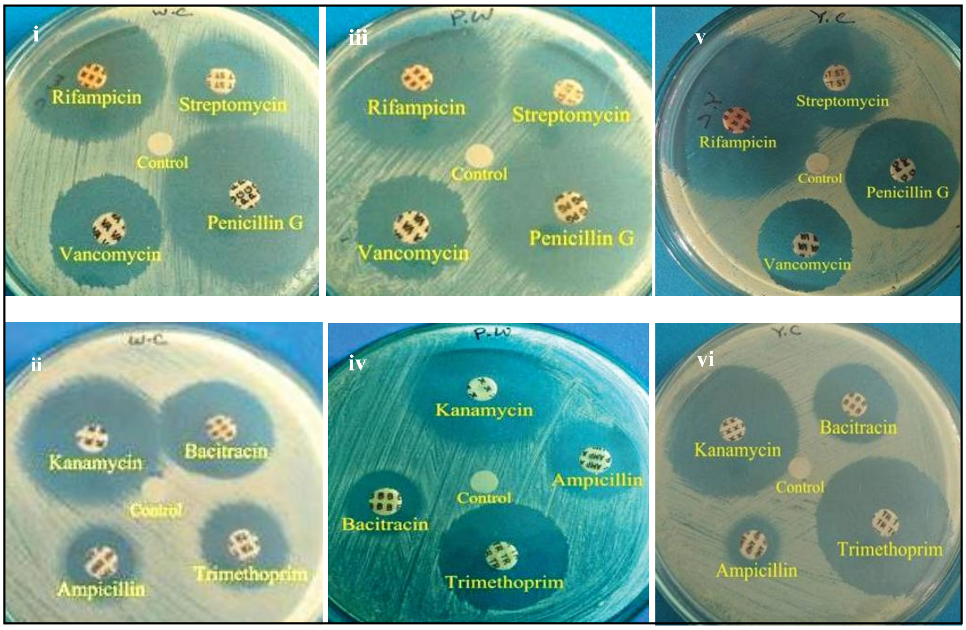

Fig. 3. Antibiotic sensitivity tests; WC= i and ii; PW= iii and iv; YC= v and vi

Discussion

Plants harbor a number of endophytic bacteria and fungus species. Of the nearly 300,000 plant species on earth, each is likely to be the host to at least one endophyte (Strobel, 2003); but relatively few of their endophytes have been characterized so far. So, the major focus of this study was to isolate, characterize and identify the endophytic bacteria from healthy cowpea [*Vigna unguiculata* (L.) Walp] roots which can be used as bio-control agent as an alternative to pesticides that may mitigate environmental pollution as well.

After sample collection, following purification and visual observation of growth, three different types and colors of colonies were selected initially from culture plate as: W (white), PY (pale-white) and Y (yellow) (Figure 2).

The bacterial isolates were then characterized by morphological and biochemical characteristics (Table 1). Identification of bacterial isolates was done according to Bergey's Manual of determinative bacteriology 9th edition (Bergey *et al*., 1974; Bergey and Holt, 1994; Barrow and Feltham, 1993).

Depending on gram staining, three isolates (W, PW and Y) were identified as gram positive bacteria (Table 1) by detecting peptidoglycan which is present in a thick layer in bacteria (Marzan *et al*., 2017).

During oxidase test all three isolates showed negative results (Thelwell *et al*., 1998). Catalase positive results indicate the three bacterial isolates (W, PW and

Y) may be either *Micrococcus* or *Staphylococcus.* Besides, isolates PW and Y were found coagulase negative, where W was found coagulase positive. Beside this, negative result in mannitol salt fermentation test for W, PW found similarities to *Micrococcus* spp. or *Staphylococcus* spp. respectively. To confirm, two isolates were observed for their pigmentation properties on mannitol salt agar media, where lack of yellow pigmentation identified both isolates as *Staphylococcus* spp. During citrate utilization all three isolates showed positive results.

Five carbohydrate utilization tests (Maltose, Sucrose, Mannitol, D-Xylose and L-Rhamnose) were done, where PW showed negative result in all cases, which is strongly indicative of *Staphylococcus caprae* (Tille, 2017). On the other hand, the positive result for maltose and sucrose, as well as negative for the other three carbohydrates found for the isolate W, strongly match with *Staphylococcus intermedius*. Besides, the isolate Y showed positive result for sucrose and mannitol, but negative for the other three carbohydrates, strongly match with *Staphylococcus saprophyticus* (Tille, 2017; Bergey *et al*., 1974).

It was observed that W, PW and Y colony have a close resemblance with *Staphylococcus intermedius*, *Staphylococcus caprae* and *Staphylococcus saprophyticus,* respectively. Bacterial genus *Staphylococcus*, were found previously as endophyte in various agronomic crops (Costa *et al*., 2012; Jasim *et al*., 2013); *Staphylococcus caprae* and *Staphylococcus saprophyticus* were found as endophyte in *Phaseolus vulgaris* (Tille, 2017). The genera isolated in the present study that have been previously reported as endophytes are *Staphylococcus caprae* and *Staphylococcus saprophyticus*, where *Staphylococcus intermedius* is found to be new in our research.

The multidrug resistance problems in microbes increase the demand for further research on novel metabolites obtained from endophytes (Rathod *et al*., 2012). Eight (8) antibiotic discs were used to assess antibiotic sensitivity, where those endophytes can be a good candidate (inhibition zone against antibiotic in maximum cases) to act as a biocontrol agents; where there is a suitable chance to apply directly (spray) in the agricultural environment (Damodaran *et al*., 2017). Here all three isolates were showed antibiotic sensitivity against seven (7), and are harmless to environment for suitable application (Alström and Vuurde, 2001) and their antimicrobial activity could be exploited in biotechnology, medicine and agriculture (Gashgari *et al*., 2016) to control pathogen attack in cowpea and possibly in other plants. A number of endophytic bacteria were reported to prevent the deleterious effects of certain pathogens by producing antimicrobial compunds or inducing systemic resistance in the host plant (Kandel *et al*., 2017). Hence, discovery of novel and effective antibiotics are necessary (O'Donnell *et al*., 2010). Some of the pathogenic microorganisms now a day become resistant to the effective antibiotics (Bisht *et al.*, 2009). Recent data showed that endophytes have a novel source to development of highly effective antibiotics

(Shukla *et al.*, 2015). Hence, invention of effective bio-controlling techniques by using eco-friendly endophytic bacteria, will open a new platform for further studies to fulfill the future demand of cost effective agro-based bio-control agents for plants (Egamberdieva *et al.*, 2017) as well as agricultural systems.

Conclusion

Depending on morphological and biochemical characteristics three isolates collected from cowpea were provisionally identified as *Staphylococcus intermedius*, *Staphylococcus caprae* and *Staphylococcus saprophyticus*. All the results presented in this study support the concept that three endophytic bacteria have significant antibiotic sensitivity which might be used to formulate them as biocontrol agent against pest, insect or pathogen in bioremediation scheme for agricultural environment.

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