A Study on Bacterial Disease of Betelvine in West Bengal, India

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Abstract

Betelvine (*Piper betle* L.) is an important cash crop in West Bengal. This crop is commonly affected by stem rot and leaf spot disease caused by two different genera of bacteria- *Xanthomonas axonopodis* pv. *betlicola* and *Pseudomonas betle*. Two bacterial pathogens enter into the host through stomata, hydathode and injury. Both the bacteria, at any portion, of the vine stem form prominent dark brown lesions. Surface of such lesion becomes sticky in humid condition. Occasionally small brown cracked lesions are found on the stem. Such lesion is formed due to infection of *X. a*. pv. *betlicola.* On the leaf, small to large, circular to irregular and/or angular brown colored spots and marginal leaf blight symptoms are produced by both the bacteria. All types of spots are surrounded by yellow halo or the halo is present in between brown and green tissue. At the under side of the leaf, the brown lesion is encircled by a water soaked zone or water soaked area, which is found in between brown lesion and green tissue in marginal blight. In addition, yellow colony forming bacterium, *X. a*. pv. *betlicola* produces very small brown spot surrounded by prominent yellow halo but without water soaked zone. Frequently both the bacteria have been detected from same leaf spot or stem lesion. Association of these bacteria increases disease severity. Initially the bacteria colonize in parenchyma tissue and later move into vascular tissue. After entry into the vascular tissue *X. a*. pv. *betlicola* becomes systemic, produces no further lesion but causes sudden wilting of vine. Such situation causes much damage of the plantation in West Bengal. Pre-inoculation treatment with streptomycin and oxy-tetracycline hydrochloride greatly reduces lesion expansion.

1. Introduction

Betelvine (*Piper betle* L.) is a perennial dioecious creeper cultivated in India for its leaf since time immemorial. The cultivated betelvine in India is usually the male plant selected from certain races which consequently does not fruit. Among the states in India, West Bengal, Assam, Karnataka and Tamil Nadu have the maximum acreage in terms of cultivation of betelvine. Other important states are Maharashtra, Kerala, Andhra Pradesh, Madhya Pradesh, Bihar and Uttar Pradesh. The most important betelvine growing districts in West Bengal are Purba Medinipur, Howrah, Hooghly, South 24 Parganas and Nadia. Besides the above districts, cultivation has now been extended to North 24 Parganas, Birbhum, Bankura, Uttar Dinajpur and Murshidabad. Common varieties cultivated in India are *Bangla*, *Mitha*, *Sanchi*, *Kapoori*, *Desawari*, *Khasi* and *Ghanagnete*. Betelvine is cultivated under artificially erected structures known as '*Boroj*'. The moist and shaded conditions prevailing in *Boroj* favor vine growth and are also congenial for development of many fungal and bacterial diseases (Maiti and Sen, 1979; Khatua et al., 1984; Maiti, 1994; Maiti and Shivashankara, 1998). Bacterial leaf spot disease caused by *Xanthomonas axonopodis* pv. *betlicola* (Patel et al., 1951; Vauterin et al., 1995) is prevalent in betelvine plantations. A little is known about the disease in changed agricultural situation, particularly in West Bengal. Present investigation relates to study of detail symptoms of the disease, nature and mode of entry of the pathogen, and identification of chemicals for its management.

2. Materials and Methods

Surveys were conducted during April, 2006 to October, 2010 to record the detail symptom of the bacterial disease of betelvine. Diseased leaf and/or stem samples were collected from 60 plantations from nine districts (Purba and Paschim Medinipur, Nadia, North and South 24 Parganas, Maldah, Uttar Dinajpur, Burdwan, and Coochbehar) of West Bengal covering all the months of a year except May and June, and also from three potted plants. Symptoms were recorded on cultivar *Mitha pan*

(sweet betelvine) and *Bangla pan* (Bengal betelvine).

2.1. Isolation of the bacterial pathogen

Photograph was taken before isolation of bacterial pathogen(s) from collected diseased leaf or stem specimen. Bacteria were isolated in potato sucrose peptone agar (PSPA) medium following standard procedure. In isolation two types of bacterial colony—yellow and white—were detected. Both types of colonies were separately maintained for further study.

2.2. Leaf injection-infiltration technique

Leaves of *Bangla pan* were collected from farmer's *Boroj*. Bacterial suspension was prepared by adding sterile distilled water in the tube containing 48 h old slant culture of the bacteria. The tube was properly shaken to form uniform suspension. Then with the help of a hypodermic syringe, the bacterial suspension was injected in the veins of betel leaves. Upon injection, the leaf tissue adjacent to the injected vein becomes water soaked. The size of the water soaked area varies from 2-6 mm in diameter (usually angular) depending upon the amount of suspension pushed. The inoculated leaves were kept in polythene bags containing a moist cotton wool, blown with air and the mouth tied with a rubber band. These polythene bags were then incubated at 28±1°C in BOD incubator. Observation was taken after 5 days by measuring the diameter of the necrotic brown lesion developed (Klement, 1963).

2.3. Mode of entry of the bacterial pathogens into the host

In order to know the mode of entry of *Xanthomonas axonopodis* pv. *betlicola* (Patel et al.,1951; Vauterin et al., 1995) and *Pseudomonas betle* (Ragunathan 1928) Savulescu 1947 the bacterial pathogens were inoculated in field condition following different methods of inoculation.

2.3.1. Smearing bacteria with brush without wounding

Circular areas of approximately 1 cm diameter were marked with sketch pen on upper and lower surface of the leaves. Separate leaves were used for inoculation on upper and lower surface. Bacterial culture from PSPA slant was smeared within the marked area with the help of camel hairbrush without wounding. Inoculation was done after sunset and inoculated leaves were covered with polythene packet.

2.3.2. Wounding by inoculating needle and smearing bacteria over the wound surface

Circular areas of about 1 cm diameter were marked on upper and lower surface of leaves separately. Bacterial culture was taken on inoculating needle and smeared within marked area by rotating in a circular fashion. Such action caused wound on leaf surface.

2.3.3. Injection of bacterial suspension in leaf and stem

Bacterial culture was injected in leaf following procedure described in section 2.2.

2.3.4. Application of bacteria along the margin of leaf

Forty eight hours old bacterial culture was applied along the edge of the leaf (margin) with help of fine brush. Such leaf was covered with polythene packet for 48 h. Five days after inoculation observation was recorded on symptom development in all the methods of inoculation.

2.4. Pre-inoculation treatment with antibiotics and antibacterial compound

Ten antibiotics namely terramycin oxy-tetracycline hydrochloride, streptomycin, chloramphenicol, cephalexin, amoxycillin, ampicillin, ofloxacin, ciprofloxacin, norfloxacin, streptocycline (streptomycin+tetracycline) and one anti-bacterial compound, bacterimycin (2-bromo-2-nitro propane-1,3-diol) were selected for pre-inoculation treatment. Solutions of three concentrations, i.e. 100, 200, 300 ppm were prepared separately for each chemical. Disease free fresh leaves of *Bangla pan* were collected from farmer's *Boroj*. Petioles of 21 leaves were put in each concentration of antibiotics and antibacterial compound for 12 h with three replicates. Suitable control was maintained by putting the petioles of leaves in water. Due to transpiration pull, the antibiotic moved into the leaf tissue. The leaves were then removed from the chemical solution and inoculated with bacterial suspension of *X. a*. pv. *betlicola* and *P. betle* following leaf injection infiltration technique. All the leaves were kept in polythene packets with moistened cotton wool, blown with air and the mouth tied with rubber band. They were incubated at 28±1°C in a BOD incubator. Observations were recorded after five days of inoculation by measuring the diameter of the diseased spot developed at the place of inoculation. Percent disease reduction was then calculated to compare the performance of the chemicals.

3. Results and Discussion

3.1. Bacterial pathogens associated with the disease

Two types of bacterial colony- yellow and white- were isolated from diseased leaf and stem samples of betelvine collected from 60 plantations (Table 1). All the yellow colony forming bacterial isolates were found pathogenic on artificial inoculation. In many diseased samples collected from different locations, white colony forming bacteria were detected. Some of the white colony forming isolates were non pathogenic. These were contaminant bacteria having colony morphology similar to that of pathogenic white isolates. It was interesting that both pathogenic yellow and white colony forming bacteria were detected from same leaf spot or stem lesion in some cases. Comparing with the characters described in Bergey's Manual of Determinative Bacteriology (9th edition) (Holt,1994), the yellow colony forming bacterium was identified as *Xanthomonas campestris* pv. *betlicola* (Patel et al., 1951; Dye et al., 1978). But subsequently the bacterium has been renamed as *Xanthomonas axonopodis*

Table 1: Summary of isolation of bacteria from diseased stem and leaf of betelvine

Place of collection: Nine districts of West Bengal

Time of collection: January-December (except May and June)

Number of leaf samples 47	Nature of bacterial isolates	Types of symptoms recorded	Pathogenicity test	
			Positive reaction	Negative reaction
	Yellow colony forming bacteria from 17 samples	Spots: Circular to irregular, angular, very small with halo, Blight: Marginal blight	17	Nil
	White colony forming bacteria from 15 samples	Spots: Circular to irregular, angular. Blight: Marginal blight	9	6
	Both yellow and white colony forming	Spot: Circular to irregular, angular, necrotic spot with diffuse halo	Yellow 15	Nil
	bacteria from 15 samples	Blight: Marginal blight	White 10	White 5
Number of stem samples 33	Yellow colony forming bacteria from 5 samples	Dark brown or black lesion, cracked stem lesion	5	Nil
	White colony forming bacteria from 20 samples	Dark brown or black lesion, soft yellow stem	16	White 4
	Both yellow and white colony forming bacteria from 8 samples	Dark brown or black lesion, small restricted stem lesion	Yellow 8	Nil
			White 7	White 1

pv. *betlicola* (Vauterin et al., 1995; Young et al., 1996). So far from the characters studied, the white colony forming bacterium showed similarity with that of genus *Pseudomonas*. In the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), *Pseudomonas betle* has been recorded as pathogen of betelvine. However, in 9th edition the name *Pseudomonas betle* was not included due to the lack of further information but Young et al. (1996) reviewed again *P. betle* as a plant pathogenic bacterium. Characteristics recorded in Bargey's Manual of Determinative Bacteriology (8th edition) for *Pseudomonas betle* were similar to those of white colony forming bacterium isolated from different diseased leaf and stem samples of betelvine. The white colony forming pathogenic bacterium is identified as *Pseudomonas betle*. The existence of leaf spot disease (*Pseudomonas betle*) reported earlier has been confirmed again from this study.

3.2. Disease symptoms

Before isolation of bacterial pathogens, symptom of each disease sample was recorded through photography. Based on these photographs and symptoms recorded in the field and results of pathogenicity test, the symptoms of the disease are summarized as follows.

3.2.1. Leaf spot

Distinct brown colored spot develops at any part of the leaf lamina, the size of the spot vary from 0.5-1.5 cm in diameter, more or less circular (Figure 1) or irregular (Figure 2) in shape. The spot area may cover the major vein. Individual spot is surrounded by prominent yellow halo on the upper surface of the leaf, on the lower surface surrounded by water soaked zone. In moist condition sticky bacterial mass is found on the water soaked zone. More than one spot may develop in one leaf that results in defoliation. These types of leaf spots are caused by both yellow colony and white colony forming bacteria (Table 1). In some cases, many (5-20) small, circular, brown spots (1 mm or below in diameter) are found distributed all over the leaf lamina (Figure 3). Such spots are surrounded with prominent yellow halo. Water soaked zone on the lower surface which is the characteristic symptom of the disease is usually absent in this case. This type of spot is formed due to infection by yellow colony forming bacterium only.

3.2.2. Angular leaf spot

Small water soaked spot develops at any portion of the leaf, usually more than one spot in a leaf, 1-3 mm in size, limited by the small veins giving an angular appearance (Figure 4 and 5). All the spots are surrounded by yellow halo and later turned brown. Water soaked zone around the spot on lower surface is present but not so prominent. The affected leaves remain with the plant for a longer period. Both yellow and white colony forming bacteria caused angular leaf spot (Table 1).

3.2.3. Marginal leaf blight

On leaf lamina brown lesion appears along the margin of the leaves. These lesions covers the entire length or a portion of the leaf margin (Figure 6). Breadth of these lesions is 0.5 mm to 1.0 cm. Yellow halo is present between brown lesion and green tissue. Water soaked zone is present in between yellow halo and brown lesion. These symptoms appears individually or may be associated with leaf spot on the same leaf. Marginal blight is caused by both the bacteria. More than one type of

symptoms may be present in one leaf.

3.2.4. Stem rot

Prominent brown lesion 1-5 cm in length is found at internodal area (Figure 7) at any height of vine, excepting top few internodes. In moist condition surface of the affected area becomes sticky. The plant may appear normal with presence of the lesion. When the lesion touches the node, the vine may break at the node. Nodal lesion leads to breaking of vine at the nodal region. Death of vines above the stem lesion also occurs in some cases or the vine grows as usual with the stem lesion. When the vine with stem lesion is lowered down as a routine cultural practice for the crop, stem became rotten and the entire vine wilts and dies. Both yellow and white colony forming bacteria were isolated from such stem lesions. Sometimes the condition becomes severe when the infection (originated from distinct brown lesion) spreads covering more than one inter-node, without developing prominent browning over the stem, particularly in cultivar *Mitha pan*. Such vine suddenly dries up or the plant dies after lowering of vine on the soil surface. Only yellow bacterium was detected from such vines. In some plantations, the affected stem cracks along the length of small lesion giving a pitted appearance (Figure 8 and 9). Cracking is associated only with yellow bacteria. Sometimes, in moist condition, bacterial mass exudes from the cracks giving a glistening appearance and the affected part becomes sticky.

3.3. Mode of entry of the bacterial pathogens into the host

In case of inoculation by smearing injured surface and injection, typical necrotic spots, dark brown in color surrounded by distinct yellow halo appeared on leaf (Table 2). The nature of necrosis was spreading type, though in few cases they were restricted within the inoculated area. Water soaked area around the spot developed in spreading type lesions. Symptom also developed when bacterial cultures were smeared on the lower surface without wounding but not on the upper surface of leaf. This indicated entry of the bacterium through stomata. Earlier studies by Chaurasia and Nayak (1986) confirmed this finding. When the bacteria were applied along the edge of the leaf (margin), marginal blight symptom developed. Development of such symptom indicated entry of the bacterium through hydathodes in leaf (Table 2). The bacterium *X. a*. pv. *betlicola* could enter into the host through stomata and wounds. *X. a*. pv. *betlicola* could also enter the host through hydathodes producing marginal blight symptoms. In case of black rot of crucifers, *Xanthomonas campestris* pv. *campestris* enter through hydathodes producing marginal blight symptoms (Onsando, 1992). *Pseudomonas betle*, the other bacterial pathogen of betelvine, followed the same path for entry.

3.4. Effect of pre-inoculation treatment with antibiotics and antibacterial compound on development of bacterial leaf spot

In all treatments (ten antibiotics and an antibacterial compound), there were reduction in average diameter of the spots and rate of reduction in general was increased with the increase in concentration of antibiotic (Table 3). Out of ten antibiotics and one antibacterial compound used as pre-inoculation treatment, only four (oxytetracycline hydrochloride, streptomycin, streptocycline and chloramphenicol) performed better than others. Reduction in size of the spot was more with the increase in concentration. Performance of chloramphenicol was better in case *Pseudomonas betle* than *X. a*. pv. *betlicola*. Chloramphenicol also gave better control of *Ralstonia* (*Pseudomonas*) *solanacearum* (Mondal et al., 2004, 2005). Streptomycin has been found to be effective against other *Xanthomonas* disease like *X. c*. pv. *citri* causing citrus canker (Vekateswarlu and Ramapandu, 1992), *X. c*. pv. *campestris* causing black rot of crucifers (Lenka and Ram, 1997), *X. c*. pv. *malvacearum* causing angular leaf spot in cotton (*X. c*. pv. *malvacearum*), *X. c*. pv. *vesicatoria* causing bacterial spot in pepper (Piccirilo et al., 1988). Streptocycline has been recommended against *X. c*. pv. *citri* (Kale and Peshney, 1996), *X. c.* pv. *phaseoli* causing bacterial pustules of soybean (Kawale et al., 1989), *X. c*. pv. *vesicatoria* in pepper (Jindal et al., 1995), etc. Samaddar et al. (1998) suggested use of agrimycin, plantomycin, and streptomycin for control of *Pseudomonas solanacearum*, but Gunawan (1988) suggested spraying of streptomycin or oxy-tetracycline 4-7 days interval. Dubey et al. (1996) got good result in controlling bacterial wilt of sesame spraying of streptocycline. Present findings have similarity with earlier works on *Pseudomonas* sp.

Table 2: Different method of inoculation and development of disease symptom in field condition

Method of inoculation		Development of symptoms	
		PB	XAB
Smearing of bacteria with brush without wounding	On upper leaf surface	-	-
	On lower leaf surface	+/-	+
Wounding by needle and then smearing of bacteria	On upper leaf surface	+	+
	On lower leaf surface	+	+
Injection of leaf with bacterial suspension	Injection on sub-vein	+	+
	Injection at inter-veinal area	+	+
Application of bacteria along the margin of leaf		+	+
Injection of bacteria in stem		+	+
Control: Injection with distilled water		-	-

XAB=*Xanthomonas axonopodis* pv. *betlicola*; PB=*Pseudomonas betle*; (+)=Symptom developed; (-)=No symptom developed

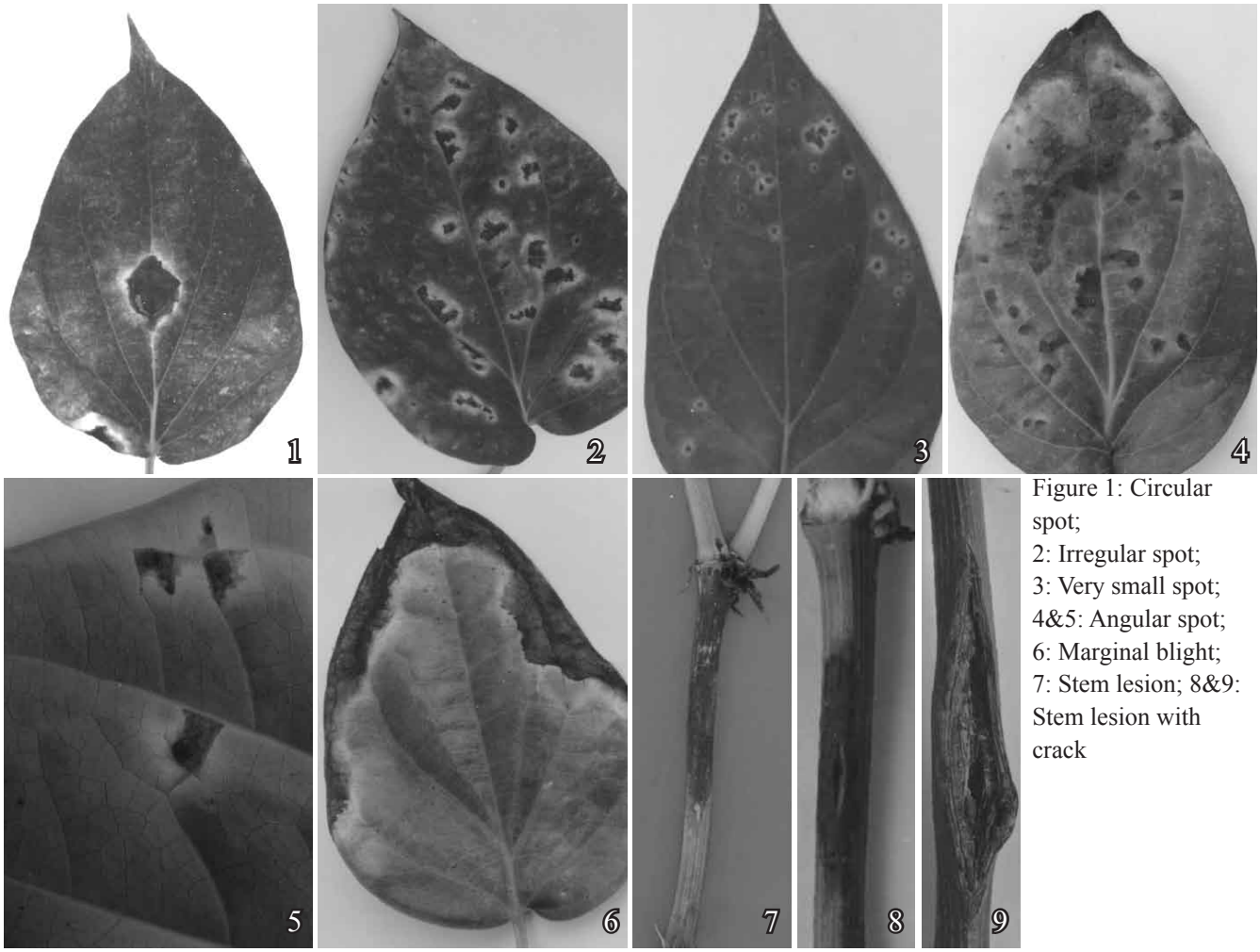

Figure 1: Circular spot; 2: Irregular spot; 3: Very small spot; 4&5: Angular spot; 6: Marginal blight; 7: Stem lesion; 8&9: Stem lesion with crack

Table 3: Extent of bacterial leaf spot control in betelvine with pre-inoculation treatment by antibiotics and anti-bacterial compound

Antibiotics and anti-bacterial compound	*Xanthomonas axonopodis* pv. *betlicola*			*Pseudomonas betle*		
	Concentration in ppm					
	100	200	300	100	200	300
	% disease control (Reduction in size of lesion over control)					
Terramycin	73.0 (58.69)*	80.9 (64.09)	82.0 (64.90)	63.7 (52.95)	78.8 (62.52)	85.5 (67.62)
Streptomycin	56.8 (48.91)	74.1 (59.41)	81.3 (64.38)	54.5 (47.58)	70.9 (61.61)	75.5 (64.53)
Streptocycline	75.0 (60.00)	79.2 (62.87)	83.4 (65.96)	64.3 (53.31)	73.0 (58.69)	76.5 (61.00)
Chloramphenicol	48.4 (44.08)	55.9 (48.39)	62.4 (52.18)	74.2 (59.47)	77.4 (61.61)	81.5 (64.53)
Cephalexin	26.8 (31.18)	28.2 (32.08)	40.3 (39.41)	54.0 (47.29)	55.0 (47.29)	66.0 (54.33)
Ampicillin	29.7 (33.02)	31.9 (34.39)	44.9 (42.07)	28.10 (32.02)	47.10 (43.34)	56.8 (48.91)
Amoxycillin	15.4 (23.11)	39.6 (39.01)	60.7 (51.18)	28.1 (32.01)	49.2 (44.54)	56.84 (48.93)
Ofloxacin	23.22 (28.79)	46.85 (43.19)	53.93 (47.24)	36.18 (36.97)	47.65 (43.65)	65.10 (53.79)
Ciprofloxacin	24.40 (29.60)	38.16 (38.15)	47.20 (43.39)	30.07 (33.25)	55.51 (48.16)	63.10 (52.59)
Norfloxacin	40.78 (39.68)	45.88 (42.63)	52.40 (46.38)	30.16 (33.31)	34.16 (35.77)	55.83 (48.35)
Bacteriomycin	17.74 (24.89)	32.35 (34.66)	46.21 (42.82)	11.70 (20.00)	24.60 (29.73)	44.70 (41.99)
CD (p=0.01)	7.00	5.67	5.19	4.992	5.716	3.248

Figures in parenthesis are angular transformed values

4. Conclusion

The present investigation suggested that more emphasis should be given to study the pathogenic nature of betelvine diseases and their detailed symptomatology. The mode of entry of the pathogens and their association has also enormous importance to undertake appropriate integrated disease management (AIDM) practices. Furthermore, other area under betelvine cultivation of West Bengal should be included for the study to know the spreading behaviour of the diseases, their economic damage and interactions of the groups of pathogens (or other organisms if any) in diverse agro-climatic situations which may help to generate future research and location specific management strategies for the benefit of the farming community.

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