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A selective medium for *Xanthomonas axonopodis* pv. Betlicola, bacterial pathogen of betelvine

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***Xanthomonas axonopodis* pv. *betlicola* (Patel et al.) Vauterin et al. causes severe damage of betelvine (*Piper betle* L.) in West Bengal by producing different types of leaf spots (small to large, circular to irregular, angular), marginal leaf blight, stem lesion and wilting of vines. A selective medium was developed for isolation of this bacterium from diseased tissues and detection of this bacterium from the leaf surface, in soil and water. This bacterium grew best in Potato Sucrose Peptone Agar (PSPA) medium (Peeled potato 200 g, Sucrose 20 g, Peptone 5 g, Agar agar 20 g, and Water 1000 ml) and this medium was used as basal medium. Some fungicides and antibiotics (Carbendazim 25 mg, Copper oxychloride 25 mg, Metalaxyl 21 mg, Cycloheximide 50 mg, Pentid-200 100 mg, Nitrofurantoin 100 mg) were incorporated into the basal medium before use. Bavistin was used as source of Carbendazim, Blitox 50 as Copper oxychloride, Krilaxyl 35 WS as Metalaxyl, Furadantin capsule (human drug) as Nitrofurantoin. Pentid-200 is a human drug and it contains Penicillin-G potassium 2,00,000 units. The above fungicides and antibiotics were taken in a 25 ml sterilized conical flask plugged with cotton and 2 ml absolute alcohol was added to it. The flask was kept as such for 24 h to allow alcohol to evaporate. Then 20 ml of sterile water was added to the flask and shaken. The solution was kept in refrigerator for future use (30 days). The solution was mixed with the sterilized and melted PSPA medium prepared earlier at 4 ml/200 ml medium before use. Using this medium *X. a.* pv. *betlicola* was isolated successfully from the diseased tissue without surface sterilization.**

Key words: Bacterial disease, betelvine, selective medium, *Xanthomonas axonopodis* pv. *Betlicola.*

INTRODUCTION

Betelvine (*Piper betle* L.) is a perennial dioecious creeper cultivated in India for its leaf since time immemorial. In West Bengal it is widely cultivated as a commercial crop. Betelvine is cultivated under artificially erected structures known as '*Boroj*'. The moist and shaded conditions prevailing in *Boroj* favour vine growth and are also congenial for development of many fungal and bacterial diseases (Maiti and Sen, 1979; Maiti, 1994; Maiti and Shivashankara, 1998). Incidence of bacterial leaf spot and stem rot incited by *Xanthomonas axonopodis* pv. *betlicola* (Patel et al.) Vauterin et al. is common in all the cultivated varieties (Bhattacharya and Khatua, 2004; Bhattacharya et al., 2005). This bacterial disease causes extensive damage of betelvine in West Bengal (Bhattacharya et al., 2012).

Isolation and characterization of a pathogen are basic steps for studying a disease. Medium like PSPA (Potato Sucrose Peptone Agar), PDA (Potato Dextrose Agar), NA

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(Nutrient Agar) are common media for isolation of the bacterial plant pathogens. Contaminant growth of bacteria and /or fungus very often creates problem for isolation of this pathogen. A good selective medium can avoid such problems. In addition, selective medium could be useful in epidemiological studies like those on leaf surface microflora and detection of population of *X. a.* pv. *betlicola* directly from soil.

MATERIALS AND METHODS

Assay by fish spine method

Ten milliliter of 48 h old bacterial culture grown in potato sucrose peptone broth was poured aseptically to 200 ml melted PSPA medium (just before solidification of medium). The test bacterial culture was mixed with melted PSPA medium and then equally distributed to sterilized petri plates. For each concentration of a chemical nine fish spine were used including control. Fish spines were dipped in the sterilized petriplates filled with chemical solutions at the desired concentration. For each bacteria-seeded plate, 3 fish spines were taken with the help of sterilized forceps and put on agar medium. By the capillary action each fish spine picked up 0.05 ml of chemical solution and the chemical spread radially over bacteria seeded PSPA medium. Then the plates were incubated at 28±1°C for 48 h and then diameter of inhibition zones was measured.

Paper disc assay

In case of paper disc assay, paper disc impregnated with antibiotic was placed over solidified bacteria seeded PSPA medium. Three discs were placed per plate and plates were incubated at 28±1°C for 48 h. There were three replications (3plates) for each antibiotic. Diameter of inhibition zone, if any, was measured for each disc.

Preparation of the selective medium (11th medium)

Carbendazim 25mg (Bavistin 50 WP 50 mg; BASF India Ltd.), Copper oxychloride 25 mg (Blitox 50 WP 50 mg; Rallis India Ltd.), Metalaxyl 21 mg (Krilaxyl 35 WS 60 mg; Krishi Rasayan), Pentid-200 100 mg (Pentid-200 containing Penicillin-G potassium 2,00,000 units; Sarabhai Chemicals), Cycloheximide 50 mg (Actidion; Sisco Research Laboratory), Nitrofurantoin 100 mg (Furadantin tablet; Glaxo Smithkline Pharmaceutical Ltd.) were taken in 25 ml sterilized conical flask and 2 ml of absolute alcohol was added to it. The flask was kept as such for 24 h to allow alcohol to evaporate. Then 20 ml of sterile water was added in the flask and shaken. The solution was kept in refrigerator for future use (30 days). Now the solution was mixed with the melted PSPA medium prepared earlier at 4 ml /200 ml before use.

Leaf surface microflora study

Bacterial leaf spot affected leaves were collected at 7 am and kept separately in polypropylene packets, brought to laboratory for testing. Each leaf was cut into small pieces (0.25 cm^2) and put in conical flask containing 50 ml sterile water. After five minutes shaking, the water was directly used for plating. One milliter water from the flask was taken in a sterile *petri* plate and 15 ml of melted medium was added to this, followed by rotary shaking. After solidification of medium, the plates were incubated at 28±1°C. Three days after incubation, types of fungal and bacterial colony appeared in each plate were counted. Yellow colony forming bacteria X. a. pv. betlicola grew in majority of the plates.

RESULT AND DISCUSSION

In vitro screening of chemotoxicants against *X. a.* pv. *betlicola* by fish spine method

In the present study 3 antibacterial compounds, 18 antibacterial antibiotics and 4 antifungal antibiotics, 10 fungicides, 4 copper salts were tested against *X. a.* pv. *betlicola* (Table 1) by Fish spine method (following agar diffusion principle).

Among three antibacterial compounds, Thimerosal appeared to be a strong inhibitor of *X. a.* pv. *betlicola*. Four antifungal antibiotics did not inhibited growth of *X. a.* pv. *betlicola*. Three copper fungicides (commercial preparation) did not inhibited growth of the bacterium even at concentration of 1000 ppm whereas Bordeaux mixture showed some degree of inhibition. Three systemic fungicides viz. Bavistin (Carbendazim), Kri-Benomyl (Benomyl), Krilaxyl (Metalaxyl) were not potent inhibitors while Cosko, a combined formulation of Thiram plus Carboxin showed inhibition. Indofil M-45 (Mancozeb) and Thiram inhibited growth of *X. a.* pv. *betlicola* at higher concentration. Four copper salts did not inhibited growth of *X. a.* pv. *betlicola* at concentration of 500 ppm or below.

From this study, Pentid-200 (Penicillin G), Cycloheximide, Carbendazim, Metalaxyl, Copper oxychloride were selected to be used as component of selective medium.

Assay by paper disc method

Through assay by paper disc method 31 antibacterial antibiotics and 6 antibacterial compounds were tested (Table 2) against *X. a.* pv. *betlicola*. Penicillin G, Pipercellin and Cephalexin did not inhibited growth of the bacterium. In fish spine method of assay, Cephalexin inhibited growth of *X. a.* pv *betlicola* at 100 ppm. This disparity might be due to low concentration of antibiotic in paper disc. Nitrofurantoin was selected as a component of selective medium.

Testing of different media for their selectivity against *X. a.* pv. *betlicola*

The fungicides, antifungal and antibacterial antibiotics, which did not show inhibition against *X. a.* pv. *betlicola* (Tables 1 and 2) such as Carbendazim, Metalaxyl, Copper oxychloride, Cycloheximide, Penicillin and Nitrofurantoin were incorporated in the PSPA and/or PDA medium either individually or in combination. As this bacterium grew well in PSPA medium, it was finally

Table 1. *In vitro* screening of chemicals against *X. a.* pv. *betlicola* by fish spine method.

Chemicals	Concentration in ppm			
	1000	500	200	100
	Diameter of inhibition zone in cm			
Bacterimycin	1.33	1.00	0.80	0.56
Thiomerosal	Complete inhibition up to 100 ppm			
Kribac (Dichlorophen)	1.09	0.90	NI	NI
Polymyxin B-Sulphate	1.13	1.05	0.96	NI
Streptomycin	3.82	3.43	3.15	2.34
Spiramycin	3.53	3.01	1.82	1.43
Netromycin	0.80	0.50	0.0	0.0
Roxithromycin	3.75	3.13	2.96	2.52
Cephalexin	3.82	3.43	3.15	2.34
Norfloxacin	3.35	3.25	3.11	2.89
Sparfloxacin	3.22	3.04	2.80	2.66
Tetracycline	3.11	2.66	1.98	1.44
Doxycycline	2.55	2.12	1.63	1.20
Streptocycline	2.69	2.34	1.99	1.76
Lomfloxacin	3.22	2.97	2.64	2.27
Ampicillin	2.48	2.14	1.65	1.23
Amoxycillin	2.89	2.19	1.68	1.23
Ciprofloxacin	3.45	3.25	2.98	2.70
Ofloxacin	1.70	1.40	1.26	0.82
Chloramphenicol	3.22	3.02	2.77	2.46
Pentids 200 (Pencillin G Potassium 200000 units)	NI	NI	NI	NI
Cycloheximide	NI	NI	NI	NI
Griseofulvin	NI	NI	NI	NI
Fluconazole	NI	NI	NI	NI
Sheathmar 3 (Validamycin 3%, L)	NI	NI	NI	NI
Thiram	1.78	1.26	NI	NI
Indofil M 45 (Mancozeb 75% WP	1.38	1.18	NI	NI
Blitox (copper oxychloride 50% WP)	NI	NI	NI	NI
Kocide 101 (Copper hydroxide 77% WP)	NI	NI	NI	Ni
Shield (Copper sulphate based fungicide)	NI	NI	NI	NI
Cosko (carboxin 37.5%+ thiram 37.5%)	3.16	2.7	2.13	0.90
Bavistin (Carbendazim 50% WP)	NI	NI	NI	NI
Kri-benomyl 50 WP (Benomyl 50%, WP	NI	NI	NI	NI
Krilaxyl 35 WS (metalaxyl 35% WS)	NI	NI	NI	NI
Copper chloride	1.47	NI	NI	NI
Copper acetate	1.38	NI	NI	NI
Copper sulphate	NI	NI	NI	NI
Copper carbonate	NI	NI	NI	NI
	Concentration in ppm			
	Normal	0.5 strength	0.25 strength	-
	Diameter of inhibition zone in cm			
Bordeaux mixture	1.56	1.35	1.03	-

NI, Not inhibited, Bordeaux mixture (normal) - lime: copper sulphate: water: 10 g : 10 g : 1000 ml.

selected as basal medium. Direct streaking of bacterial culture on eleven different agar medium (Table 3) growth of this bacterium was first tested. All the eleven media supported growth of *X. a.* pv. *betlicola*.

Table 2. *In vitro* screening of chemicals against *X. a.* pv. *betlicola* by paper disc assay.

Antibiotics/ other drugs	Chemical content in paper disc (mcg)	Diameter of inhibition zone (in cm)			
		X. a. pv. *betlicola*			
		Replications			Average
		I	II	II	
Penicillin G	10 u	NI	NI	NI	-
Ampicillin	10	2.26	2.56	2.56	2.46
Amoxycillin	10	2.13	2.33	2.40	2.28
Pipercillin	100	NI	NI	NI	-
Carbenicillin	100	2.1	2.1	2.3	2.16
Tetracycline	30	3.03	3.1	2.9	3.01
Doxycline	30	1.53	1.53	2.06	1.7
Oxyteracycline	30	1.76	2.20	2.06	2.0
Ciprofloxacin	5	4.20	4.30	4.00	4.16
Norfloxacin	10	3.41	3.40	3.56	3.45
Ofloxacin	5	4.16	4.20	4.17	4.18
Novobiocin	30	1.4	1.1	1.43	1.31
Bacitracin	10 u	1.63	1.2	1.56	1.46
Pefloxacin	10	4.16	4.12	4.10	4.12
Lomefloxacin	10	3.41	3.43	3.61	3.48
Sparfloxacin	10	4.05	4.03	4.47	4.18
Cephalexin	30	NI	NI	NI	-
Cefotaxime	30	2.16	2.4	2.33	2.3
Cefoperazone	75	2.7	1.96	3.0	2.55
Ceftriaxone	30	2.0	2.4	2.3	2.33
Cephaloridine	30	2.0	1.53	1.23	1.59
Streptomycin	10	3.13	3.13	3.06	3.10
Kanamycin	30	3.37	2.96	3.56	3.20
Gentamicin	10	2.20	2.13	1.76	2.03
Vancomycin	30	3.00	3.23	3.36	3.19
Nitilmicin	30	3.16	3.36	3.24	3.25
Tobramycin	10	1.96	2.05	2.1	2.03
Erythromycin	15	3.96	3.63	3.76	3.78
Roxythromycin	15	3.90	4.37	4.5	4.25
Chloramphenicol	30	3.60	3.28	3.44	3.44
Methylmandalate	3	2.05	2.15	2.3	2.16
Nitrofurantoin	300	NI	NI	NI	-
Amoxy clavulanic acid	30	1.53	1.8	1.7	1.68
Nalidixic acid	30	3.46	3.30	3.17	3.31
Colistin	10	1.00	1.26	1.1	1.12
Co-trimoxazole	25	3.65	3.36	3.81	3.60
Neomycin	30	1.76	1.83	1.73	1.77

NI, Not inhibited, - = No inhibition.

Initially these media were used to detect *X. a.* pv. *betlicola* as leaf surface microflora in an infested betelvine plantation and result of the study is given in Table 3.

When the Fungicides and Penicillin were incorporated individually, 3 or 4 types of fungus and two different types of bacteria were found along with the yellow bacteria, *X. a.* pv. *betlicola* as per variation in colony morphology, in the study of leaf surface microflora. When four chemicals viz. Bavistin (Carbendazim 50% W.P.), Krilaxyl (Metalaxyl 35% WS), Blitox (Copper oxychloride 50% WP) and Pentid-200 (Potassium penicillin) were incorporated in PSPA medium growth of two types of fungi and one type of bacterium were seen along with *X. a.* pv. *betlicola* as

Table 3. Growth of microorganism in fungicide and antibiotic incorporated media.

Serial number	Basal medium	Addition to the basal medium	Dose per liter (mg)	Growth habit of *X. a.* pv. *betlicola* by direct streaking	Colony growth of yellow bacteria	Contaminants in leaf surface microflora study	
						Fungal growth	Growth of other bacteria
1	PDA	Carbendazim	25	Normal	Presence of yellow growth, Counting of colony number was difficult	+++	+++
2	PDA	Metalaxyl	20	Normal		+++	+++
3	PSPA	Carbendazim	25	Normal		+++	+++
4	PSPA	Metalaxyl	20	Normal		+++	+++
5	PSPA	Cycloheximide	50	Normal		+++	++
6	PSPA	Pentid 200 (K-Penicillin)	200	Normal		++++	+
7	PSPA	Pentid 200 (K-Penicillin) +Carbendazim	200 25	Normal		++/+++	+/-
8	PSPA	Copper oxychloride	50	Normal		+++	++
9	PSPA	Carbendazim +Metalaxyl +Copper oxychloride + Pentid 200	25 20 50 200	Normal	Distinct yellow colony	++	+/-
10	PSPA	Carbendazim + Metalaxyl + Copper oxychloride + Cycloheximide + Pentid 200	25 20 50 50 200	Normal	Distinct yellow colony	nil	+/-
11	PSPA	Carbendazim + Metalaxyl + Copper oxychloride + Cycloheximide + Pentid 200* +Nitrofurantoin	25 20 50 50 200 100	Normal	Distinct yellow colony	nil	Nil

*Penicillin-G potassium 2,00,000 unit, (+) Types of fungi/other bacteria, (-) No growth.

per colony morphology. But the presence of these contaminants did not cause any problem in counting the number of colonies of *X. a*. pv. *betlicola.* When five chemicals, that is Carbendazim, Metalaxyl, Copper oxychloride, Potassium penicillin (Pentid 200) and Cycloheximide were incorporated in PSPA medium, occasionally growth of one type of bacterium was seen along with *X. a*. pv. *betlicola*. When six chemicals, that is, Carbendazim, Metalaxyl, Copper oxychloride and Potassium penicillin (Pentid-200), Cycloheximide and Nitrofurantoin were incorporated in PSPA medium, *X. a.* pv. *betlicola* grew without any contamination. *X. a.* pv. *betlicola* was isolated, using the last media (Table 3), from the diseased leaf tissue without surface sterilization of the diseased plant tissue. The last medium (11th medium in Table 3) was successfully used for detection of *X. a*. pv. *betlicola* from soil. The above medium did not support growth of *X. campestris* pv. *campestris*, X. *axonopodis* pv. *citri*, *X. campestris* pv. *punicae*.

Tripathi et al. (2007) has followed similar method to develop semi-selective medium for *X. campestris* pv. *musacearum*. Earlier attempts have also been made to develop selective medium for *Xanthomonas* sp. This include KSM- Kritzman's selective medium (Kritzman and Ben-yephet, 1990) for *X. c.* pv. *campestris*. Efficacy of four semi-selective media for *X. c.* pv. *campestris* were tested by Fukui et al. (1994). These were CS20ABN, aesculin trehalose (ET), Field house-Sasser (FS) and Starch methionine (SM) media. FS media was found most suitable for soil studies in terms of consistent recovery of the pathogen. A semi-selective medium containing maltose, methyl green, and antibiotics (MMG) was developed for the isolation of *X. campestris* pv. *vitians* by Toussaint et al. (2001). These media were not suitable for *X. a.* pv. *betlicola*. Medium presently developed is not costly, easy to prepare and selective for *Xanthomnas axonopodis* pv. *betlicola*. This selective medium can be used for isolation and detection of *X.*

axonopodis pv. *betlicola.* This is the first report of a selective medium developed for isolation and detection of *X. axonopodis* pv. *betlicola.*

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