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Efficacy of isolated bacteria in *in vitro* inhibition of *Xanthomonas axonopodis* pv. *cyamopsidis* and prevention of bacterial leaf blight of cluster bean in the field

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ABSTRACT: Twelve isolates of plant growth promoting rhizobacteria (PGPRs) were tested for their efficacy against bacterial blight of cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.) caused by *Xanthomonas axonopodis* pv. *cyamopsidis* in laboratory and field conditions. The isolates PGPR-1, PGPR-4 (*Bacillus subtilis*), PGPR-12 and PGPR-7 (*Pseudomonas fluorescens*) showed maximum *in vitro* bacterial growth inhibition, increased the seed germination (more than 98%) and increased plant vigour. Under field conditions, PGPR-12 (*P. fluorescens*), PGPR-5 (*B. subtilis*), PGPR-4 (*B. subtilis*) and PGPR-7 (*P. fluorescens*) resulted in low of per cent disease index and in that order increased plant yield compared to other PGPR strains.

KEY WORDS: Disease management, *Cyamopsis tetragonoloba*, *Xanthomonas axonopodis* pv. *cyamopsidis,* PGPRs

INTRODUCTION

Cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.) is an important crop of Rajasthan, India, and is grown in 5.56 million ha with 0.4 million tonnes seed yield (Anonymous, 2004). The leaf blight pathogen *Xanthomonas axonopodis* pv. *cyamopsidis* causes drastic reduction in plant stand and yield as high as 58% in cultivar Nav Bahar (Gupta, 1978). It is seed borne (Shrivastava and Rao, 1963) and can survive in seeds for up to one year. Difficulties have been encountered in the management of bacterial diseases of plants because of the availability of few bactericides. In addition, available antibiotics are expensive and not efficacious against all the bacterial pathogens. Considerably great efforts have been made in the recent past to evolve non-conventional and environmentally safe approaches including biological, cultural, integrated pest management, and molecular breeding for plant disease management. This study was undertaken to screen *in vitro* 10 antibiotics, 20 plant extracts and 12 plant growth promoting rhizobacteria (PGPRs) and the best selected treatments were further evaluated under field conditions to manage the crop losses.

MATERIALS AND METHODS

Isolation and pathogencity test of pathogen

The bacterium was isolated from infected plant parts like leaves, stem and pods on nutrient agar (NA) medium at 30±2°C and yellow colonies so developed were transferred onto yeast glucose chalk agar slants for storage (Yeast extract - 3g, Peptone - 5g, Glucose - 5g, $CaCO_3$ - 20g, Agar - 15g, pH - 7.0). Pathogenicity was proved on one-month-old plants in a greenhouse (RH 78-91%, 30±2°C) by carborundum method using 48h old bacterial inoculum (Leben *et al*., 1969) and following Koch's postulates.

Management of disease

Twelve PGPRs (Table 1) were tested for their efficacy against the disease both in laboratory and field conditions (Satish *et al*., 1999).

In vitro assay of PGPRs

The putative PGPR isolated from cluster bean rhizosphere and phyllosphere (Table 3) (1-6, *Bacillus* sp. and 7-12 *Pseudomonas* sp.) were tested by disc diffusion technique on bioassay medium (peptone 10g, beef extracts

3g, yeast extracts all bacteriological type (Hi-Media) 5g, agar 20g) previously seeded with 48h old bacterial cultures (1 ml/250 ml) in Petri plates (30 ml). Paper discs (10 mm dia) of Whatman No.1 filter paper were dipped in 48h old PGPR culture suspensions of 10^6 and 10^4 cfu ml^{-1} concentration, separately by using a standard stock of 10^8 cfu ml^{-1} (OD_{540} = 0.2) and placed on medium in Petri plates (3 discs/plate) as for chemical tests. The growth inhibition (%) was recorded after 72h of incubation following the formula, I=C-T/Cx100, where, I=inhibition %, C=colony diameter in control plate (mm), T=colony diameter in treatment plate (mm) and inhibition data were analyzed by analysis of variance (ANOVA) and the treatments were separated by F-protected (P=0.05) LSD.

Table 1. ***In vitro*** **efficacy of different PGPRs against** ***X. axonopodis*** **pv.** ***cyamopsidis***

PGPR strain	Inhibition (mm)[a]		
	104 c.f.u ml^{-1}	106 c.f.u ml^{-1}	108 c.f.u ml^{-1}
PGPR-1	5.23[a]	9.12[cl]	14.13[eh]
PGPR-2	2.73[b]	5.42[a]	8.24[cl]
PGPR-3	10.92[d]	22.13[f]	32.45[k]
PGPR-4	8.34[cl]	20.16[g]	30.92[k]
PGPR-5	7.54[c]	15.49[h]	22.72[f]
PGPR-6	7.61[c]	14.13[h]	21.65[fg]
PGPR-7	7.84[c]	16.15[h]	24.16[j]
PGPR-8	8.52[c]	18.25[i]	27.61
PGPR-9	3.26[b]	4.50[a]	9.35[cdl]
PGPR-10	12.58d[e]	23.62[fj]	34.35
PGPR-11	7.91[c]	16.67[hi]	22.71f
PGPR-12	9.17[cl]	21.14[fg]	30.74[k]
Control	0	0	0

[a] Mean of three replications. Values in a column superscribed by the same letter(s) are not significantly different (P = 0.05) by LSD test.

Effect of seed treatment on seed germination and plant vigor index

The seeds of cluster bean which were previously inoculated with the pathogen (6h seed soaking) were treated with PGPRs separately (1h seed soaking) and plated on three layers of moistened sterilized blotter paper in Petri pates (10-20 seeds / Petri plates) at 28±2°C. Untreated seeds served as control. On the 10th day after incubation, seed germination % was recorded and seedling/plant vigor index (PVI) was calculated by the following formula: PVI = (mean epicotyl length+ mean hypocotyl length) x % germination (Abdul Baki and Anderson, 1973). The experiment was repeated thrice. ANOVA was used to determine the effects of treatments. Angular transformation was used to stabilize variances. Complete randomized block design test was used for least significant difference to separate the means after ANOVA at P = 0.05.

Efficacy of antibiotics, plant extracts and PGPRs under field conditions

Field experiments were carried out at Rajasthan College of Agriculture Farm, Udaipur, during two consecutive crop seasons (2000 and 2001) in a randomized block design with three replications. Three antibiotics, five plant extracts and five antibacterial biocontrol agents which showed better performance *in vitro* with respect to seed germination and PVI tests at different concentrations were used to treat seeds (pathogen-inoculated) and sown in 2x2 meter plots in four replications, separately. Two weeks after germination, the crop was spray inoculated thrice at an interval of 12h with bacterial inoculum (10^6 cfu ml^{-1}) under maintained moist conditions (by frequent water spraying during daytime) for 3-4 days to create artificial epiphytotics. The three sprays at the interval of 10 days of chemicals (300 ppm), plant extracts (50%) and antibacterial PGPRs (10^8 cfu ml^{-1}) were given 3 days after the last inoculation coinciding with the time of first appearance of the symptoms. The disease intensity/index was recorded after 15-20 days of last spray. Per cent disease control (PDC) was calculated by the following formula: PDC = (infection index in control plots – infection index in treatment plots/ infection index in control plots) x 100. Per cent increase in yield was recorded by the following formula: Per cent

increase in yield = (yield in treatment – yield in control/ yield in control) x 100.

The average data of three replications of both the years 2000 and 2001 were pooled and analysis of variance (ANOVA) was used to determine the effects of the treatments on the percentage of disease incidence, PDC and total yield. Angular transformation was used to stabilize variances. Randomized block design test was used for least significant difference to separate the means after ANOVA at P = 0.05.

RESULTS AND DISCUSSION

Pathogen and pathogenicity test

The isolated bacterium produced straw yellow, round, smooth colonies, Gram-negative rods, 0.8 x 1.74 µm in size, single polar flagellum, capsulate and KOH soluble, growing at 10-37°C, pH 5.7- 8.0 and 2.5 to 5.0 % NaCl conc. Positive in oxidase, catalase, H_2S, levan production, citrate utilization, and gelatin liquefaction, casein and starch hydrolysis. Negative in methyl red and Vogous Proskeur test, indole production, nitrate, and nitrite reduction, Tween 80, urea, arginine, and tyrosine hydrolysis. Produced acid from arabinose, cellobiose, glucose, dextrose, fructose, galactose, mannitol, sucrose and trehalose but slow from sorbitol, salicin, and xylose. The cultural, morphological, and physio-biochemical characteristics well facilitate with that of genus *Xanthomonas,* species *axonopodis* and distinct host specificity justifies classifying species into pathovar *cyamopsidis* (Schaad and Stall, 1988).

In vitro assays

PGPRs

Among the 10 putative PGPR isolated from cluster bean rhizosphere and phyllosphere (1-6, *Bacillus* sp. and 7-12 *Pseudomonas* sp.), PGPR-10 showed significantly (LSD < P 0.05) maximum inhibition diameter at 10^8 cfu ml^{-1} concentration (Table 1), followed by PGPR-3, PGPR-4, and PGPR-12. However, PGPR-8, PGPR-7, PGPR-5, and PGPR-11 also inhibited the bacterial growth to a certain extent.

Effect of seed treatment on seed germination and plant vigor index

Almost all the PGPRs studied improved the overall growth and % germination and at the same time reduced the pathogenic effects of the bacterial strain. A significant (LSD < P 0.05) increase in % germination (> 98%) was recorded in seeds treated with PGPR-1, PGPR-4, PGPR-12 and PGPR-7 as compared to both the controls. Average $plant^{-1}$ dry weight was significantly higher in treatments of PGPR-3, PGPR-2, PGPR-12, PGPR-5, PGPR-4 and PGPR-11, but there was no significant difference amongst these treatments (Table 2). However, the PVI was significantly higher in the treatment PGPR-12 followed by PGPR-7, PGPR-5, PGPR-11 and PGPR –3 as compared to other treatments and controls.

Table 2. Effect of seed inoculation of different PGPRs on germination percentage and growth parameters of seedlings

Treatment	Seed germination[W] (%)[X]	Average shoot length[W] (cm)	Average root length[W] (cm)	Average fresh weight[W] (g)	Average dry weight[W] (mg)	Vigor index[W]
PGPR-1	100(90.00)^{a}	5.40^{d}	6.25^{de}	2.68^{e}	320	1165.00
PGPR-2	93.3(75.00)^{bc}	6.15^{c}	8.25^{c}	3.21^{b}	375^{a}	1343.52
PGPR-3	93.3(75.00)^{bc}	6.40^{bc}	9.35^{b}	3.47^{a}	380^{a}	1469.47
PGPR-4	100(90.00)^{a}	2.83	6.85	3.12^{b}	365^{b}	968.00
PGPR-5	95.5(77.75)^{b}	6.75^{a}	9.35^{b}	2.91^{e}	365^{b}	1537.55
PGPR-6	91.2(72.74)^{c}	4.55	6.15^{e}	2.53^{ef}	325^{d}	975.84
PGPR-7	98.4(82.73)^{b}	6.75^{a}	9.25^{b}	3.09^{c}	321^{d}	1574.4
PGPR-8	93.4(75.11)^{bc}	5.65$_{d}$	8.35^{c}	3.25^{bc}	355^{c}	1307.6
PGPR-9	91.4(72.95)^{c}	4.95-^{e}	6.12^{e}	2.61^{ef}	325^{d}	1011.79
PGPR-10	92.3(73.89)^{bc}	4.87^{e}	5.91^{d}	2.45^{f}	312	994.99
PGPR-11	94.2(76.06)^{bc}	6.55^{ab}	9.43^{ab}	2.95^{cd}	365^{b}	1510.11
PGPR-12	100(90.00)^{a}	6.80^{a}	9.85^{a}	2.85^{d}	375^{a}	1665.00
Uninoculated control	84.2(68.7)	8.15	9.20^{b}	3.50^{a}	350^{c}	1460.87
Inoculated control	74.2(59.47)	4.85^{e}	6.25^{d}	2.14	212	823.62

[W] Mean percentage of seeds germinated after 10 days of treatments with three replications having 10 seeds per replication; [Xf]or percentages, the analysis done on angular transformations; values in a column superscribed by same letter(s) are not significantly different (P = 0.05) by LSD test; [Y] PVI = (mean epicotyl length+ mean hypocotyl length) x % germination

Table 3. Efficacy of bio-agents (PGPRs) against *X. axonopodis* pv. *cyamopsidis* under field condition

Treatment	PDI[W](%)[X]	PDC[W](%)[X]	Yield (q/ha) [W]	Per cent increase in yield[W]
Tetracycline	21.52 (27.63)[a]	70.87	9.62[a]	43.36
PGPR-1	49.38 (44.64)	33.04	8.57[b]	27.77
PGPR-4	33.34 (35.26)	54.79	9.10[ab]	35.61
PGPR-5	30.25 (33.37)	58.98	9.16[ab]	36.51
PGPR-7	39.21 (38.76)	46.83	9.05[ab]	34.87
PGPR-12	26.84 (27.16)[a]	63.60	9.27[ab]	38.15
Un treated control	73.75 (59.84)	00.00	6.71	00.00

[W] Pooled mean percentage of two-year field trial (2000 & 2001). Each trial has three replications for each treatment with 50 plants per replication; [X]for percentages, the analysis was based on the angular transformation; values in a column superscribed by the same letter(s) are not significantly different (P = 0.05) by LDS test.

These results obviously indicated that PGPRs possess the plant growth promoting activity, which not only acts as antibacterial, but also enhances PVI and seed germination. *B. subtilis*, *Pseudomonas* spp. and *Bacillus* sp. have been reported to be highly efficacious to *X. campestris* pv. *malvacearum* (Assis *et al*., 1995; Mondal *et al.,* 1999), *B. subtilis* BO34 isolated from rice leaves against *X. oryzae* pv. *oryzae* (Tong *et al*., 1999), *B. polymyxa* BP1 isolated from cauliflower seeds against *X. campestris* pv. *campestris* (Assis *et al*., 1995; Pichard and Thouvenot, 1999) similar to kasugamycin and *B. subtilis* isolates *in vitro* as well as *in vivo*.

Efficacy of PGPRs under field conditions

Field experiments revealed that PGPR-12 showed significantly (LSD <P 0.05) higher PDC and higher yield (Q/ha) than all other treatments (Table 3). PGPR-5, PGPR-4 and PGPR-7 also showed considerably higher decrease in per cent disease index and increase in yield as compared to control and the rest of the treatments. However, these treatments are statistically (LSD < P 0.05) on par with regard to increase in yield (at 5 and 1% degree of freedom). Good control of black rot of cabbage by applying *Pseudomonas* sp. (rhizoplane) as seed treatment and two sprays (Dzhalilov *et al*., 1994) and bacterial blight of cluster bean by seed treatment with *B. subtilis* and spray of antibiotics streptomycin at 35 and 49 DAS (Lodha, 2001) in the field have been reported.

The results obtained with the treatments of PGPR with regard to seed germination, dry weight and PVI were different to the trends observed *in vitro*. This study showed that not only bacterial growth inhibition tests in the laboratory, but seed germination and seedling vigor index tests in field trials should also be conducted for successful disease management in field conditions. Thus the best performing plant extracts and PGPRs, in this case the most successful treatment combination of PGPR 12, PGPR-7 and PGPR-5 which showed considerable decrease in % disease index and increase in yield against bacterial blight caused by *X. axonopodis* pv. *cyamopsidis,* could be used in successful integrated disease management.

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