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Detection of *Xanthomonas axonopodis* pv. *vignicola*, causal agent of bacterial blight of cowpea in seeds by non-serological methods

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ABSTRACT

Among the diseases infecting cowpea, bacterial blight caused by *Xanthomonas axonopodis* pv. *vignicola* (Burkholder,1944). Vauterin *et al.* (1995) is a major production constraint. First necrotic lesions are formed on leaves and later the stem is attacked and the pathogen reaches vascular bundles and the disease becomes systemic. In the present study, attempted have been made to develop the suitable methods for the detection of the pathogen in the seeds and to find out the nature of transmission using selective and semi-selective media and compare them for their efficacy. Results indicated NSCAA medium to be more efficient in recovering the colonies of seed borne bacterium *Xanthomonas axonopodis* pv. *vignicola* with 132×10^5 cfu/ml as compared to 75×10^5 cfu/ml of colonies on Nutrient agar. The next best medium was SIBU agar (126×10^5 cfu/ml) followed by XTS medium (118×10^5 cfu/ml). All the media for isolation of plant pathogenic bacterium from seeds revealed that the cowpea bacterium is seed borne in nature. Another method employed to detect pathogen in seeds is Van Vuurde *et al.*(1983) method and results revealed that unsterilized seeds of susceptible cultivar C-152 yielded more number of colonies 35×10^2 cfu/ml by direct plating the seed extract on NSCAA whereas the resistant germplasm, DCS 47-1 yielded very less number of colonies 2×10^2 from diseased unsterilized seeds confirming its seed borne nature.

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INTRODUCTION

Cowpea forms an important component of farming system, being cultivated for seeds (shelled green or dried), pods or leaves that are consumed as green vegetable or for pasture, hay, silage and green manure. It fits well in a variety of cropping systems and is grown as a cover crop, mixed crop, catch crop or green manure crop in different parts of India (Gupta, 1978).The overall grain yields of cowpea in the present traditional systems is low (Singh *et al.,* 1997) due to a complex of biotic and abiotic factors.

Among the diseases infecting cowpea, the bacterial disease popularly known as 'bacterial blight' caused by *Xanthomonas axonopodis* pv. *vignicola* (Burkholder, 1944) Vauterin *et al.* (1995) is a major production constraint. In India, it was first reported by Patel and Diwan (1950) from Pune and subsequently Chakravarti *et al.* (1972) and Gupta (1978) reported the disease from Rajasthan.

The first symptoms of disease appear on cotyledons of seedlings emerging from infected seed and look reddish and

wrinkled. First necrotic lesions are formed on leaves and later the stem is attacked. The pathogen reaches vascular bundles and the disease becomes systemic. The growing tip of the infected plant is killed and the plant ultimately dies. Cankers are often developed on the stem near the union of cotyledons and first leaves. Such stems are unable to bear the load of the plant and easily break in strong winds. For the successful management of any disease under normal conditions, sanitation, eradiation of primary source and chemical protection at initial stages are some of the measures recommended.

The seed borne inocula initiate epidemic of a number of bacterial plant diseases and play an important epidemiological role in bacterial diseases of leguminous crops. The primary damage due to the disease is through high mortality in the seedlings (Patel and Jindal, 1970). Shekhawat and Patel (1977) reported for the first time seed transmission of *Xanthomonas axonopodis* pv. *vignicola* (Burkh.) Vautrein *et al.* from India. They recorded as high as 62 per cent incidence in summer from the inoculum load of 1 per cent infested seed. The pathogen is seed-borne and secondary spread is by wind-driven rain, soil, insects and infected plant debris (Kaiser and Vakili, 1978). Since then few workers have attempted to develop suitable methods for the detection of the pathogen in the seeds.

Gitaitis and Nilakhe (1982) for the first time devised a method of detecting the bacterium in naturally infected seeds which considered of extracting the bacterium from seed coupled with plant bioassay method. Later several workers have succeeded in detecting the bacterium in the seed, their detection method suffers from a number of inherent and practical constraints.

Another major problem in the detection of bacterium from naturally infected seeds is competition from fast growing saprophytic bacteria which outnumber the pathogen and also it may be complicated by seed borne fungi that can confuse symptoms expression with bacterial infection. Complete media such as YDCA (Schaad and Stall, 1988) are used for detection of *X. axonopodis* pv. *vignicola*. No specific medium for the isolation of *X. axonopodis* pv. *vignicola* has been reported till date. Then Wydra *et al.* (2004) developed a semi-selective medium for the isolation and enumeration of *Xanthomonas axonopodis* pv. *vignicola* from cowpea plant and soil samples. So, there is a need to evaluate different selective and semi- selective media for the detection of seed borne *Xanthomonas axonopodis* pv. *vignicola* pathogen.

Watkins (1943) reported 60 per cent mortality in California black eye variety. Patel *et al.* (1972) reported 15 to 20 per cent seedling mortality in the certified seed production area. The use of resistant cowpea varieties remains the most practical long-term method for controlling cowpea bacterial blight because the method does not have a negative impact on the environment and economics of adoption is also minimal. The identification and evaluation of cowpea varieties for resistance have relied mainly on disease incidence and symptoms severity in the fields and greenhouse. Therefore, there is a need to screen different varieties of cowpea to identify resistant lines.

Recovery of *Xanthomonas phaseoli* from bean seeds is accomplished by soaking the seeds in liquid media. Extraction of bacteria by the liquid soak method required 24 to 48 hours and is often adversely affected by contaminant bacteria which hinder the isolation of specific pathogens. Then the problem of contaminant bacteria was avoided through the use of selective media for *Xanthomonas phaseoli and X. phaseoli* var. *fuscans* (Trigalet and Bidaud, 1978).

Gupta and Chakravarti (1982) from India demonstrated the transmission of *Xanthomonas axonopodis* pv. *vignicola* (*Xav*) through the seeds by raising the plants from artificially inoculated and uninoculated cowpea seeds under green house conditions. Thus they stated that bacterium is carried externally as well as internally along with the seed which might serve as a primary source of inoculum. Gitaitis and Nilakhe (1982) devised a method for detecting *Xanthomonas axonopodis* pv. *vignicola* in cowpea seeds.

Thind and Soni (1983) carried out studies on the seed borne nature of *Xanthomonas axonopodis* pv. *vignicola* and reported that both external infestation and internal infection play an important role in the disease development. They stated that a minimum of 10^3 cfu /ml bacterial concentration to be there in the inoculum used for soaking the seeds to get successful transmission.

Van Vuurde *et al.* (1983) extracted *Pseudomonas syringae* pv. *phaseolicola* from bean seeds by soaking the seeds in water at 5°C for 24 hours and isolated by dilution plating onto King's B medium. Two semi-selective culture media Nutrient starch cycloheximide antibiotic agar (NSCAA) and Basal starch cycloheximide antibiotic agar (BSCAA) were developed by Randhawa and Schaad (1982) which were compared with NA a non-selective media for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds.

Kaun *et al.* (1985) developed a method of detection of *Xanthomonas campestris* pv. *carotae* in carrot seeds by extracting the pathogen at a low temperature (5°C) stationary aqueous soak of carrot seed in darkness for 18 hours followed by a vigorous shaking of the seed in water containing Tween-20 and concentrating the bacterial cells by centrifugation and finally plating of dilution series of these cells onto a modified Kado and Heskett's D5 medium.

Soni and Thind (1987) detected *Xanthomonas axonopodis* pv. *vignicola* in the seeds by traditional growing-on test and they reported that the incubation growing-on test was more effective than traditional growing on test for the detection of the bacterium. Khan (1989) detected *Xanthomonas axonopodis* pv.*vignicola* using selective and

semi-selective media. He found that SIBU agar and NSCAA media were found to be efficient in the detection of pathogen in naturally infected cowpea seeds.

Anitha *et al.* (1992) reported four seed health testing techniques *viz.*, seed soaking, seed maceration, blotter test and growing-on test used for the detection of *Xanthomonas axonopodis* pv. *vignicola* (Burkh.) Dye from naturally infected seeds of cowpea, seed soaking test was found to be superior to other methods.

Shobha (1998) reported the recovery of bacterial colonies of *Xanthomonas axonopodis* pv. *glycines* on NSCAA, the semi-selective media were more (112×10^5 cfu/ml) as compared to Nutrient agar (55×10^5 cfu/ml). But, on SX and XTS media, the bacterium produced bigger colony as compared to NSCAA medium.

Wydra *et al*. (2004) developed a semi-selective diagnostic medium, Cefazoline-cellobiose-methionine (CCM) for the isolation and enumeration of *Xanthomonas campestris* pv. *vignicola* from cowpea (*Vigna unguiculata*) plant and soil samples.

MATERIAL AND METHODS

The Pathogen, *Xanthomonas axonopodis* pv. *vignicola* is reported to be seed borne (Shekhawat and Patel, 1977). Therefore, investigations were conducted to find out the nature of transmission of the pathogen through seeds.

Detection of *X. axonopodis* pv. *vignicola* in the infected seeds by selective and semi-selective media :

Cowpea seeds sample comprising cv. C-152 collected from naturally infected plants showing typical symptoms of bacterial blight caused by *Xanthomonas axonopodis* pv. *vignicola* were used. Twenty five seeds were used to conduct the each test.

The relative efficiency of the various media being evaluated was determined on the basis of the recovery of *Xanthomonas axonopodis* pv. *vignicola* by dilution plating on Nutrient agar. Culture of *Xanthomonas axonopodis* pv. *vignicola*, maintained in the Department of Plant Pathology, University of Agricultural Science, Dharwad (Karnataka) was used to assess the efficacy of each medium.

Isolation media :

Eight media were tested for their efficiency in recovery of known isolate of *Xanthomonas axonopodis* pv. *vignicola*. They were SX agar (Schaad and White, 1974) XTS (Schaad and Forster, 1985) Modified D-5 medium (Kaun *et al.*, 1985), NSCAA and BSCAA medium (Randhawa and Schaad, 1982; Silva *et al.*, 1970) YDCA (Schaad and Stall, 1988), KML medium, SIBU agar. Nutrient agar medium was used as a standard check for the purpose of comparing the growth of the bacterium. All the media used in the present investigation are especially meant for the isolation of plant pathogenic bacteria from seeds.

The media were sterilized in autoclave for 15 min. at 121°C for 15 lb. pressure. All the antibiotics were filtered, sterilized and added to cooled, sterilized (50°C) media after autoclaving. The media were poured into sterilized Petriplates and dried for 2 days at room temperature to eliminate surface moisture before use.

Colony assessment :

A loopful of 48 hrs. old bacterial culture was added to 10 ml sterile water blank and one ml of this suspension was further serially diluted using 9 ml sterile water and 100 µl of 10^5 and 10^6 dilutions was spread on the surface of the medium in each plate using a sterilized spreader under aseptic conditions. The inoculated plates were incubated at 28°C for 72 hrs. Observations were recorded for number of colonies developed and the characters of colonies on each medium.

Detection of *X. axonopodis* pv. *vignicola* in the infected seeds by Van Vuurde method :

Preparation of seeds extracts :

The seed lot was divided into two sets containing of 25 seeds each of different germplasm lines *viz.*, diseased and healthy seeds of susceptible, resistant, moderately susceptible and moderately resistant germplasm lines. One set of seed samples were surface sterilized in 1 per cent sodium hypochlorite for 10 min. and the other set was not surface sterilized. The seeds were incubated in 25 ml sterilized water taken in 100 ml Erleyenmayer's flask and soaked for 24 hrs. at 5°C. After four hours incubation, the soaking solution of each sample was mixed thoroughly by shaking the flasks by hand. After 24 hrs, the soaking solution was sampled for dilution plating, just below the surface of the liquid.

Isolation :

The presence of *X. axonopodis* pv.*vignicola* in seed extracts was investigated by isolating the bacterial cells by dilution plating on Nutrient agar. The inoculated plates were incubated at 28°C for three days and observed for the production of typical colonies which are mucoid, round, convex, glistening, slimy and yellow.

The suspected colonies were isolated and purified on YDCA (Schaad and Stall, 1988). The colonies from the seeds of cv. C-152 from Dharwad were maintained in the laboratory.

Detection of *X. axonopodis* pv. *vignicola* from naturally infected cowpea seeds :

The surface sterilized seeds using 1per cent sodium hypochlorite for 10 min. and unsterilized seeds were used for extraction of the bacterium. The bacterium was extracted as described by Van Vuurde *et al.* (1983) by soaking 25 seeds of the seed sample in 25 ml sterile distilled water in 100 ml Erleyenmayer's flask for 24 hrs. The seed extracts were

serially diluted to 10^2, 10^3 and 10^4, one tenth of a milliliter of each dilution was plated in triplicate on the medium which was found effective with respect to recovery of the pathogen out of eight selective and semi-selective media tested.

The bacterial population was estimated after the inoculated plates were incubated at 28°C for 3 to 5 days.

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under the following heads :

Efficacy of selective and semi-selective media for the growth of *Xanthomonas axonopodis* pv. *vignicola* :

Of the various selective and semi-selective media tested for their efficacy in supporting the growth of *Xanthomonas axonopodis* pv. *vignicola*, the NSCAA (Nutrient Starch Cycloheximide Antibiotic Agar) medium was found to be more efficient with highest number of bacterial colonies (132×10^5 cfu/ml) when compared to nutrient agar (75×10^5 cfu/ml). The next best medium which supported the growth of *X. axonopodis* pv. *vignicola* was SIBU agar medium (126×10^5 cfu/ml colonies) followed by XTS medium (118×10^5 cfu/ml) (Table 1).

However, the colony diameter was found to be more in XTS (2-4 mm) as compared to NSCAA medium (2-3 mm). Typical colonies appeared within 48 hrs. on NSCAA whereas, it took 72 hrs. to produce them on XTS and SIBU agar media. Characters of *X. axonopodis* pv. *vignicola* colonies on various selective and semi-selective media are given in Table 1. On NSCAA medium, the colonies of *X. axonopodis* pv. *vignicola* were round, shiny, raised, yellow coloured with dark centre and translucent hallow.

Detection of *Xanthomonas axonopodis* pv. *vignicola* from the seeds of naturally infected cowpea plants by Van Vuurde method :

The pathogen was isolated from seed extracts obtained by soaking of 25 unsterilized and sterilized cowpea seeds each of cultivars, C-152 (S), DCS 47-1(R), C-152 × IC202709 (MS) and V-118 × IC257422 (MR) in 25 ml sterile distilled water for 24 hrs. at 5°C as explained by Van Vuurde *et al.* (1983) followed by direct plating the seed extract on the surface of nutrient agar and NSCCA media which was found efficient in recovering the maximum number of bacterial colonies (132×10^5 cfu/ml) in the previous test and also it is specially meant for the isolation of bacteria from seeds.

In this investigation, unsterilized diseased seeds of susceptible variety, C-152 yielded 35×10^2 cfu/ml on NSCAA medium whereas, sterilized seeds yielded 18×10^2 cfu/ml and apparently healthy seeds of same susceptible variety C-152 yielded only 12×10^2 cfu/ml. from unsterilized seeds and sterilized seeds yielded 3×10^2 cfu/ml. Healthy seeds of resistant variety DCS 47-1 not yielded any colonies whereas unsterilized diseased seeds of DCS 47-1 yielded 2×10^2 colonies (Table 2).

Ever since, Shekhawat and Patel (1977) reported the transmission of *Xanthomonas axonopodis* pv. *vignicola* through the seeds, few reports have appeared on the detection of the pathogen in seed. Due to cumbersome and time consuming nature of work, detection of seed borne pathogen was limited. Hence, efforts have been made in the present investigation to apply, modify and develop the most suitable, rapid, sensitive and accurate techniques in the detection of the pathogen in naturally infected seeds.

Seed samples :

Cowpea seed samples of cultivars C-152 (S), DCS 47-1(R), C-152 × IC202709 (MS) and V-118 × IC257422 (MR) were used in the detection of the pathogen by two different techniques.

Detection of pathogen from seeds using selective and semi-selective media :

Few attempts have been made in the past to detect the presence of *Xanthomonas axonopodis* pv. *vignicola* in cowpea

Table 1 : Detection of pathogen in seeds by selective and semi-selective media

Type of the media	Media	Population of bacterial colony (cfu/ml)*		Size of the colony (Range - mm)	Colony character
		105	106		
Standard check	NA	75	38	2.0 - 3.0	Convex, yellow, round, glistening mucoid
	BSCAA	56	12	1.0 - 2.5	Shiny, convex, white colony
Semi-selective	NSCAA	132	71	2.0 - 3.0	Yellow, glistening, slimy, convex, circular to irregular
media	XTS agar	118	60	2.0 - 4.0	Yellow, shiny round colony
	Modified D-5 medium	61	11	1.0 - 2.0	Convex, shiny, slimy, small whitish colony
	SX agar	43	14	0.5 - 1.0	Minute, convex, blue coloured centre, white colony
Selective media	KML agar	72	26	2.0 - 4.0	Highly fluidal, round to irregular, shiny white colony
	SIBU agar	126	63	2.0 - 3.0	Highly fluidal, shiny, raised yellow colonies
	YDCA	98	45	2.0 - 4.0	Deep yellow, glistening, highly fluidal, bigger, irregular colony

*-Average of three replications

seeds on agar medium. Gupta and Chakravarti (1982) failed to isolate *X. axonopodis* pv. *vignicola* by direct plating the surface sterilized and non-sterilized seeds on Nutrient agar and Yeast extract glucose chalk agar and they also failed to isolate the bacteria by directly planting the seeds on plain agar medium till they germinated. Later, Thind and Soni (1983) were able to recover the bacterium from individual seeds collected from artificially inoculated and naturally infected seeds on Potato sucrose-peptone rifampin aureofungin agar.

In the present investigation, NSCAA (Nutrient Starch Cycloheximide Antibiotic Agar) medium was found more efficient with recovery of 132×10^5 cfu/ml followed by SIBU agar medium with 126×10^5 cfu/ml of *X. axonopodis* pv. *vignicola* cells whereas SX and BSCAA media were found to be less efficient (Table 1).

The results are in confirmation with the findings of Khan (1989) who reported that, SIBU agar a semi-selective medium as most efficient medium with 95 per cent recovery of *Xanthomonas axonopodis* pv. *vignicola* cells followed by KML agar medium with 82.25 per cent and NSCAA with 59.28 per cent whereas, XTS and SX were found to be less efficient. Shobha (1998) also reported the maximum recovery of bacterial colonies of *Xanthomonas axonopodis* pv. *glycines* on NSCAA medium with 112×10^5 cfu/ml as compared to the Nutrient agar with only 55×10^5 cfu/ml.

Detection of *Xanthomonas axonopodis* pv. *vignicola* by Van Vuurde method :

Both unsterilized and sterilized seeds of different germplasm lines which were screened in the present investigation against *X. axonopodis* pv. *vignicola* and found resistant, moderately resistant, susceptible and moderately susceptible in their reaction for bacterial blight disease were used to know the efficiency of Van Vuurde *et al.* (1983) method and confirm the seed borne nature of the pathogen. The results indicated that, diseased unsterilized seeds of susceptible variety C-152 yielded 35×10^2 cfu/ml on NSCAA medium which was found effective in recovering maximum number of bacterial colonies and specially meant for isolation of bacteria from seeds whereas, seeds from resistant and moderately resistant line DCS 47-1 and V-118×IC257422, respectively not yielded any colonies on NSCAA medium indicating that the pathogen is not carried internally in these lines. Also number of cfu/ml of the extract varied from cultivar to cultivar. This variation may be due to the difference in the degree of susceptibility of cultivars tested against *Xanthomoans axonopodis* pv. *vignicola* as evident from the results of screening of varieties/lines for their resistance to bacterial blight (Table 2). Further, the colonies obtained from Van Vuurde *et al.* (1983) method were confirmed as *Xanthomonas axonopodis* pv. *vignicola* on the basis of morphological, cultural and biochemical studies. Gupta and Chakravarti (1982) failed to isolate the pathogen from the surface sterilized and unsterilized seeds by direct plating the seeds on Nutrient agar while Khan (1989) isolated the pathogen both from surface sterilized and unsterilized seeds collected from naturally infected plants by soaking the seeds in sterile water for 24 h at 5°C as explained by Van vuurde *et al.* (1983). Similarly, Anitha *et al.* (1992) also reported that, of the four seed health testing techniques *viz.*, seed soaking, seed maceration, blotter test and growing-on test used for the detection of *Xanthomonas axonopodis* pv. *vignicola* from naturally infected seeds of cowpea and seed soaking test was superior than other methods.

Summary and conclusion :

The detection of *Xanthomonas axonopodis* pv. *vignicola* in seeds by using different selective and semi-selective medium revealed that NSCAA medium was more efficient in recovering the colonies of seed borne bacterium *Xanthomonas axonopodis* pv. *vignicola* with 132×10^5 cfu/ml as compared to 75×10^5 cfu/ml of colonies on nutrient agar. The next best medium was SIBU agar medium with 126×10^5 cfu/ml colonies followed by XTS medium (118×10^5 cfu/ml). All these media used are specially meant for isolation of plant pathogenic bacteria from seeds. Hence, this revealed that the cowpea bacterial blight organism, *Xanthomonas axonopodis* pv. *vignicola* is seed borne in nature.

Another method employed to detect pathogen in seeds

Table 2 : Detection of *Xanthomonas axonopodis* pv. *vignicola* in naturally infected seeds by Van Vuurde *et al.* (1983) method

Treatments		Population of bacterial colony (cfu/ml)*	
		Unsterilized seeds	Sterilized seeds
Apparently healthy seeds	C-152	12 × 102	3 × 102
	C-152 × IC202709	6 × 102	1 × 102
	V-118 × IC257422	2 × 102	-
	DCS 47-1	-	-
Diseased seeds	C-152	35 × 102	18 × 102
	C-152 × IC202709	21 × 102	7 × 102
	V-118 × IC257422	5 × 102	1 × 102
	DCS 47-1	2 × 102	1 × 102

* indicates of significance of values at P=0.05, respectively

has been developed by Van Vuurde *et al.* (1983). The results of this method revealed that unsterilized seeds of susceptible cultivar C-152 yielded more number of colonies 35×10^2 cfu/ml by direct plating the seed extract on NSCAA, whereas, the resistant germplasm, DCS 47-1 yielded very less number of colonies (2×10^2) from diseased unsterilized seeds.

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