Population dynamics of cotton phylloplane bacteria antagonistic towards Xanthomonas campestris pv. malvacearum

S. SAHA, R.P. SINGH, J.P. VERMA and J. JAYARAMAN
Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi

ABSTRACT: A cotton phylloplane bacteria (Plb) belonging to genus Pseudomonas was selected for intensive studies on the basis of its in vitro antagonistic activity against the most predominant and virulent race of Xanthomonas campestris pv. malvacearum, the bacterial blight pathogen of cotton. Plb-29 was chosen for its multiplication ability on some Gossypium hirsutum genotypes. On G. hirsutum lines viz., Acala-44, Stoneville 2B-S9, Stoneville 20 and I-10B, Plb-29 could multiply both in presence and absence of pathogen. It was capable of reducing pathogen population drastically when co-inoculated with the pathogen by multiplying and heavily colonising the active multiplication sites of cotton leaves.

Key words: Cotton, population dynamics, phylloplane bacteria, biocontrol, Pseudomonas, Xanthomonas campestris pv. malvacearum

Bacterial blight caused by Xanthomonas campestris pv. malvacearum (Xcm) is one of the economically important diseases of cotton throughout the world. In India, the problem is more vehement due to the predominance of the highly virulent race 32 (Verma, 1986), which is capable of overcoming five major bacterial blight resistant genes (B2, B4, Bm, Bn and Bn) in cotton. Management of the disease through foliar spray of chemicals and the use of resistant varieties is possible, but the hazardous impact of agrochemicals on the environment, development of resistant mutants, escalating cost of pesticides and frequent breakdown of resistant varieties strongly demand a sustainable and an alternative management approach of the disease. Attempts to develop biological control agents of the plant pathogens have not been particularly successful, because the organisms selected on the basis of in vitro antagonism, were ecologically unsuited to the natural environment of the pathogen. In most of the cases survival and multiplication of introduced microbial agent is very much restricted which may be due to exploitation competition or interference competition operating in the natural environment (Kenerley and Andrews, 1990). Biological control through resident epiphytic bacteria against Xcm has been reported (Habish, 1968; Verma and Singh, 1976; Verma et al., 1978, 1980, 1982, 1983). To commercialise this approach and make it economically viable, more basic data on their multiplication and colonising ability need to be generated. In the present investigation cotton phylloplane bacteria were studied for their antagonistic property, multiplication and colonising ability on the phylloplane with an objective to find a potent, naturally occurring biocontrol agent which could be effectively exploited against the bacterial blight pathogen, Xcm.

MATERIALS AND METHODS

Bacterial isolates and culture media

A total of 63 cultures/isolates were established from naturally infected tetraploid cotton plants (Gossypium barbadense L. and G. hirsutum L.) from the fields of Indian Agricultural Research Institute, during August-September, 1997-98. These were maintained on YGCA agar slants (g/l: yeast extract, 10; glucose, 10; CaCO₃, 20 and agar, 20) and stored at 4°C. A batch of these cultures was stored in 15% glycerol solution at -80°C. All the isolates were streaked fresh (from glycerol stock culture) on YGCA before attempting pathogenicity studies. The race characterisation was done on the basis of their reaction on cotton differential hosts (Hunter et al., 1968) employing the scheme of Verma and Singh (1974).

Plbs were isolated from phylloplane of five week old seedlings of cotton viz. G. barbadense and G. hirsutum in the field and glasshouse of Indian Agricultural Research Institute, New Delhi. Suspensions (0.1 ml) of 10⁶ and 10⁸ dilution of leaf washed water
(1 g of leaf in 10 ml of distilled water) were spread on NSA (g/l: sucrose, 5; yeast extract, 4; beef extract, 2 and agar, 20) plates incubated at 27°C for 24 hours and observed the appearance of bacterial colonies. At least 46 bacterial colonies of different size, shape and colour were picked and further purified by single colony isolations. Subculturing (14 days interval) was done on NSA slants. A batch of Pib cultures were kept in 15% glycerol at -80°C for long-term storage.

**In vitro antagonism of cotton phylloplane bacteria**

The phylloplane bacterial isolates were screened for their antagonism against a strain belonging to race 32 of Xcm in vitro. Suspension of Xcm (10⁷ cfu/ml) was spread over NSA and left open under laminar flow for 30 minutes (to facilitate absorption of liquid into the medium). Single colony of each Pib isolate suspended in 10 μl of sterile distilled water was then spot inoculated at the center of NSA plates and then incubated at 27°C for 72 hours. Observation was taken as diameter of inhibition zone (in cm) surrounding the growth of Pib on Xcm lawn.

**Characterisation of antagonistic Pib**

The most effective antagonistic Pib isolate was characterised on the basis of gram reaction, cultural [colony type, pigment production on King’s A and King’s B (King et al., 1954) media], spore production and biochemical (gelatin liquefaction, arginine dihydrolase, oxidase tests, levan production, catalase tests etc.) characters using the identification scheme of Stolp and Gadkari (1981). The guanine-cytosine (GC) content of the antagonist Pib was also determined (Ramaswamy et al., 1992).

**Population dynamics of Xcm and Pib in cotton**

The cotton differentials i.e. differentials I (Acala-44), II (Stoneville 2B-S9), III (Stoneville-20), IV (Mebane B1), V (1-10B) , VI (20-3) and VII (101-102B) were grown in the glasshouse and 4-6 leaf stage of the host plant was used. The multiplication ability of Pib was studied with the use of ampicillin resistant mutant of Pib (Pib-amp) which were developed by plating Pib on NSA medium containing 125 μg/ml of ampicillin. 0.1 ml of both Xcm and Pib suspensions (10⁷ cfu/ml) were inoculated at various points of the leaf following the injection and infiltration method of Klement (1963). The surface was washed with water to remove excess bacterial cells. Leaf sample (1g) was collected at 0, 24, 48 and 72 h after inoculation; it was macerated in 10 ml of sterile distilled water and allowed to stand for an hour with intermittent shaking. The suspension was serially diluted in sterile distilled water. The population of Pib-amp and Xcm R-32 was ascertained by streaking NSA plates with and without ampicillin (100 μg/ml). Colony counts were made after incubation at 27°C for 24h.

**RESULTS AND DISCUSSION**

**Identification of races of Xcm**

Sixty-three isolates of Xcm were grouped into three races. Race-32 was most predominant and comprised 65 per cent of the total isolates, followed by race-27 (25 per cent) and race-23 (10 per cent). Predominance of race-32 was reported earlier; 49.53 per cent of total isolates before 1981 (Singh and Verma, 1981), 53.62 per cent in 1983, 82.23 per cent in 1984 and 61.61 per cent in 1985 (Duttamajumder and Verma, 1994). The continuous cultivation of resistant cotton cultivars exerted tremendous selection pressure on Xcm population (Verma, 1986) which resulted in the predominance of race-32, the only race which is able to overcome five bacterial blight resistant genes including B₂, B₄, B₇, B₉ and B₉. Hence race-32 of Xcm was selected for further studies.

**Selection of antagonistic Pib**

Out of 46 isolates of PIBs tested, only 3 isolates namely Pib-7, Pib-29 and Pib-33 were antagonistic towards Xcm R-32 in vitro. These 3 antagonistic Pibs were tested for their multiplication on Acala-44 (susceptible cotton differential with no gene for resistance); Pib-29 multiplied at least 100 times faster than the other two Pibs (Table 1). Further, Pib-29 was the strongest antagonist of Xcm (with an average diameter of inhibition zone of 1.2 cm) followed by

<table>
<thead>
<tr>
<th>Isolate Zone of inhibition</th>
<th>Population of Pib in Acala-44 in cfu / g of fresh weight of leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate Zone of inhibition</td>
<td>0 h</td>
</tr>
<tr>
<td>Xcm (cm)</td>
<td></td>
</tr>
<tr>
<td>Pib-7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(2.20)**</td>
</tr>
<tr>
<td>Pib-29</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(2.36)</td>
</tr>
<tr>
<td>Pib-33</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>(2.26)</td>
</tr>
</tbody>
</table>

*Mean of 3 replications
**Figures in parenthesis denote transformed log value
Plb-33 and Plb-7. Thus, Plb-29 was taken up for further characterisation and population dynamics studies.

Characterisation of Plb-29

Plb-29 was gram-negative and exhibited typical pseudomonad morphology of being rod-shaped with polar flagella. It neither produced yellowish-green fluorescent pigment on King’s B medium nor blue-green pigment on King’s A medium; so it might not belong to either *Pseudomonas aeruginosa* or *P. fluorescens* group. As Plb-29 can liquefy gelatin it might not belong to *P. putida* group too, because members of *P. putida* produce yellowish-green fluorescent pigment on King’s B medium and cannot liquefy gelatin (Stolp and Gadkari, 1981). Plb-29 exhibited positive catalase, oxidase and arginine dihydrolase activities, but was unable to produce levan from sucrose and also unable to utilize lactose as carbon source, which are the characteristics of most pseudomonads (Holt et al., 1994). Finally, the GC content of Plb-29 was determined to be 64.9 per cent, which is in accordance with the observation of De Ley and Van Muylem (1963) that the GC content of the genus *Pseudomonas* ranged from 57-70 per cent. Plb-29 was, therefore, identified as a strain of *Pseudomonas* species.

Colonisation and multiplication of Plb-29

The multiplication of Plb-29 was investigated both in the presence and absence of *Xcm* R-32; Plb-33, another antagonist of *Xcm* R-32, was also studied to demonstrate a comparative analysis with particular reference to multiplication of Plbs in the absence of *Xcm*. When Plb-29 was inoculated in the absence of *Xcm* (i.e. monoinoculation) on differential-I Acala-44), it multiplied approximately 525 times; in contrast Plb-33, within the same time span of 72 h, multiplied (Table 1) only about 5.2 times. Populations of Plb-29 and Plb-33 maintained similar trend on most of the differentials (used for race identification) where the former (Plb-29) multiplied 80-675 times while Plb-33 showed only slight increase in its population. Plb-29 did not multiply so rapidly on two differentials (IV and VI), data not shown), the reasons for which are not clear at present. It was concluded that Plb-29 multiplied rapidly on most cotton differentials even in the absence of *Xcm*. This is in contrast to the earlier reports where most of the Plbs, which gave protection to the host plant, did not show any significant multiplication in monoinoculation, and either maintained their population at the inoculated level or declined slowly with time (Young and Paton, 1972; Chowdhury and Verma, 1980).

Plb-29 recorded higher multiplication in presence of *Xcm* than when it was inoculated alone. On
differential I (Acala-44, susceptible to *Xcm*) Plb-29 continued its multiplication in presence of *Xcm* (Fig. 1) and reached a population of \(10^{5.6}\), whereas the multiplication of *Xcm* declined after 24 h in presence of Plb29, and the *Xcm* population remained below \(10^6\) at which symptoms were not produced. On the resistant differential (Fig. 2) the population of *Xcm* declined rapidly and there was no HR (hypersensitive reaction); Plb-29, however, reached a population of \(10^4\) to \(10^5\) indicating net multiplication. In monoinoculation the *Xcm* multiplied rapidly in a susceptible reaction and reached a population of \(10^8 - 10^{10}\) (Chowdhury and Verma, 1980) to produce symptoms; symptoms were not produced at an *Xcm* population of \(10^7\) or lower (Verma et al., 1983). Similar trend of limited multiplication of *Xcm* (to a level which was insufficient to produce full symptoms) was observed in presence of Plb-29 on other differentials.

Chowdhury and Verma (1980) showed rapid multiplication of Plb only in presence of the pathogen,
Xcm, and concluded that the membrane permeability was affected by Xcm infection, due to which nutrients were released, which were more actively (fast) utilised by the Plb, which multiplied rapidly; at the same time, this, resulted in the restricted multiplication and population of Xcm (due to limited availability of nutrients) to levels at which full potential of symptom was not produced. Young (1974) demonstrated that the population of Pseudomonas syringae (the non-pathogenic isolate or the Plb) increased considerably when it was coinoculated with P. phaseolicola (the pathogen) in bean. This was probably connected with the commonly observed phenomenon that pathogenic organisms increased the host-cell membrane permeability to make the nutrients available to the saprophyte, the Plb in this case (Wheeler and Hanchey, 1968; Sinclair et al., 1970). However, in the present studies the Plb-29 was demonstrated to multiply even in the absence of Xcm.

Beattie and Lindow (1999) showed that bacterial multiplication and colonisation depended on modification of leaf habitat, ingress and egression. It appears that the Plb-29 multiplied on cotton leaves and suppressed Xcm population because it (Plb-29) modified the leaf habitat by depleting the nutrients of the leaf and thereby depriving the pathogen of it, and in the process it (Plb-29) multiplied at such a fast rate that it occupied the active multiplication sites on the leaf and did no allow Xcm R-32 to multiply. Preinoculation of Plb in cotton is known to produce both protection factor as well as protection factor inducible principles against Xcm (Verma et al., 1978).

In conclusion, the present investigations suggested that Plb-29 which belongs to the genus Pseudomonas and is a resident phylloplane bacterium, could be used as a potential biocontrol agent for the management of bacterial blight of cotton particularly because it also inhibited the growth of Xcm in vitro, was capable of independent multiplication (i.e. in the absence of Xcm) on several cotton differentials and caused a drastic reduction of Xcm population which was insufficient to produce full symptoms.

ACKNOWLEDGEMENTS

The Senior author (SS) acknowledges gratefully the Director, IARI, New Delhi for providing the Senior Research Fellowship during the course of investigation, and to the Head, Division of Plant Pathology, IARI, New Delhi for necessary facilities and active help.

REFERENCES


Received for publication January 11, 2000