MOLECULAR PHYLOGENETIC ANALYSIS OF *Xanthomonas axonopodis* pv. *punicae* ISOLATES AND BIO-PROSPECTING OF SELECTED BOTANICALS AND BIO-AGENTS AGAINST BACTERIAL BLIGHT OF POMEGRANATE

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INTRODUCTION

Pomegranate, *Punica granatum* L., is regarded as the "Fruit of Paradise" which can be found throughout the Mediterranean region, Southeast Asia, California and Arizona in USA. It is commonly known as '*Anar'* in Hindi and '*Dalimbe'* in Kannada and it belongs to the family Punicaceae. In India, it is cultivated as a commercial fruit crop in Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Madhya Pradesh and Uttar Pradesh (Chadha, 2001). However, this crop is grown in patches and as a component of kitchen and homestead garden. Area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, drought tolerance, higher yield levels with excellent keeping quality and remunerative prices in domestic and export markets. It thrives well in arid tropics and sub-arid tropics and comes up very well in soils of low fertility status as well as in saline soils.

Pomegranate is a good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin-C and citric acid (Malhotra *et al*., 1983). Glucose (5.46%) and fructose (6.14%) are the main souces of sugars. The fruits of pomegranate are known to possess pharmaceutical and therapeutic properties and are used as components of many fold medical practices. Sweet varieties are mildly laxative, sour types are good for curing inflammation of stomach and heart ache. In India, there is a common adage 'Ek Anar Sau Bimar' meaning "one fruit cures hundred diseases". The flower buds are very useful in Ayurveda for managing bronchitis. The bark of the stem, root and rind of the fruit are used for slimming, control of dysentery, diarrhoea and killing tape worms. It is also a National Flower of Spain. It is used as a table fruit, juice, also in processed form, as juice or concentrate or syrup jelly. Seeds with fleshy portions of sour pomegranates are dried and marketed as 'Anardana' which is used as a condiment for souring curries. Pomegranate seeds contain about 15 per cent oil which has potential for the industrial use (Anon, 1969).

The rind of pomegranate is a rich source of a dye which gives yellowish brown to khaki shades and is being used for dying wool and silk. The flowers also yield a light red dye. The bark of the stem and roots contain a number of alkaloids belonging to pyridine group. The bark is used as tanning material especially in Mediterranean countries (Bose, 1985).

Pomegranate is one of the most adaptable subtropical minor fruit crops and its cultivation is increasing very rapidly. In India, it is regarded as a "vital cash crop", grown in an area of 1,07,000 ha with a production of 743,000 MT with an average productivity of 6.9 MT (Anon., 2011). Among different states growing pomegranate, Maharashtra is the largest producer occupying 2/3rd of total area in the country followed by Karnataka, Gujarat, Andhra Pradesh and Rajasthan. Karnataka state has the distinction of cultivating pomegranate under tropical condition in an area of 1.3 thousand ha with a production of 0.1 million tons with an average productivity of 10.5 MT (Anon., 2011). In Karnataka, this crop has spread across different districts *viz*., Bagalkot, Bangalore, Belgaum, Bellary, Bijapur, Chitradurga, Davangere, Gulbarga, Koppal and Tumkur.

Cultivation of pomegranate in recent years has met with different traumas such as pests and diseases. Major diseases of pomegranate are bacterial blight, wilt, anthracnose, leaf and fruit spots and pests like fruit and stem borer, fruit sucking moth, shot hole borer and sucking insect pests. Among the diseases infecting pomegranate, the bacterial disease popularly known as 'bacterial blight' caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh,1959; Vauterin *et al.,* 1995) is regarded as a major threat. For the first time in India, leaf spot of pomegranate was reported by Hingorani and Mehta (1952). Later on during 1959, Hingorani and Singh took a thorough investigation on the disease and causal organism and designated the pathogen as *Xanthomonas punicae* sp. Nov. Chand and Kishun (1991) reported the epidemic form of disease in Bangalore which caused 60 – 80 per cent loss during 1991 and they also isolated the causal organism and identified it as *Xanthomonas campestris* pv. *punicae* based on its pathological, cultural, biochemical and physiological characters. Later, on the basis of DNA homology, the pathogen has been renamed as *Xap* (Vauterin *et al.,* 1995).

Since 2002, the disease has reached the alarming stage and hampering the Indian economy vis-à-vis export of quality fruits. The disease accounted up to 70 – 100 per cent losses during 2006 in Karnataka, Maharashtra, Andra Pradesh and Tamil Nadu resulting in aboundoning many pomegranate orchards. During the year 2007, the total output of pomegranate production in India was down by 60 per cent (Raghavan, 2007). In Karnataka, survey report revealed 20 – 90 per cent of disease severity in Bijapur and Bagalkot districts (Ravikumar *et al*., 2006). They observed 71.14 per cent severity in Bellary district. Recent reports revealed the highest severity of disease was 74.80 per

cent in Bagalkot district and minimum severity of 6.73 per cent in Bellary districts (Anon., 2008). During 2008 – 09, the disease has reached its alarming stage bringing substantial damage to the crop and heavy loss to the farmers. However, the magnitude of the disease severity has raised several questions to researchers, farm managers, administrators, private firms and above all the farmers about how to safeguard the cultivation of this dollar earning crop. *Xap* is one of the numerous host-specific pathovars of phytopathogenic xanthomonads, a highly evolved group of bacteria. This pathovar was first reported in India on pomegranate (Hingorani and Singh, 1959). It has now emerged as a destructive pathogen in pomegranate cultivating areas of India, the biggest producer of pomegranates in the world. *X. axonopodis* pv. *punicae* has become a serious threat to pomegranate cultivation and also to the tremendous export potential. India is an area where *X. axonopodis* pv. *punicae* infections are presently endemic, the species is also a potential threat to pomegranate growing areas of the world where such infections are not endemic. In order to know if any variability exists in the organism, sequencing of the genome of *X. axonopodis* pv. *punicae* is an urgent requirement to gain deeper insights into its phylogeny and taxonomy and also to study the genomic determinants of its virulence. Disease is characterised by the appearance of small, irregular water soaked, dark coloured spots on leaves resulting in premature defoliation. Pathogen also infects stem and branches causing girdling and cracking symptoms. Spots on fruits are dark brown, irregular slightly raised with oily appearance, which splits opens with 'L'/'Y' shaped cracks at final stages. Under severe conditions it destroys the entire orchard and causes heavy economic losses upto 70-80 per cent (Yenjeerappa, 2009 and Benagi *et al*., 2011).

The antibiotics with bactericidal nature are the most effective agents being applied to manage bacterial blight of pomegranate. Several types and formulations are available commercially. However, their use is dependent on region, size of orchard and the economic situation of farmers. Baseline sensitivity of geographic isolates of *Xap* to major bactericides helps us to recommend different bactericides for different geographical region.

Residual effect of chemicals cause health hazards in human being, so there has been a major thrust on organic pomegranate production. Hence, efficient botanicals, bioagents and nutrients need to be explored to fit into the management schedule.

In bacterial plant pathogens, the 16S rRNA gene has been well established as an important locus for identification and in establishing phylogenetic distances (Ash *et al*., 1991, Nesme *et al*., 1995, Adhikari *et al*., 1995, Hauben *et al*., 1997). Though a considerable stretch of the 16S rRNA gene is conserved across bacterial genera, however a smaller variable region enables us to estimate phylogenetic distances. Among the other important housekeeping loci, *gyr*B has been demonstrated to have potential for studying the genetic relatedness among closely associated strains (Parkinson *et al*., 2009). 16S rRNA and *gyr*B are the two important genes of *Xap*. The normal PCR technique currently used for detection of this bacterium is very expensive, time consuming and involves testing independently for each gene. The multiplex PCR which enable detection of the two genes of *Xap* simultaneously is less expensive and less time consuming. This helps to trace back the origin of different isolates as well as spread of pathogen diversity to different geographical area. This also helps for successful implementation of disease management strategies. Keeping all these aspects in view the present study was proposed to assess the diversity among the different isolates collected from different agroclimatic regions and to manage this disease; the present investigation was undertaken with the following objectives.

1. Assessment of baseline sensitivity to major bactericides among *Xap* isolates.
2. Ribosomal RNA (16S rRNA) analysis and phylogeography of *Xap* isolates.
3. Prospecting the botanicals and selected nutrients for *in vitro* and *in vivo* inhibition of *Xap*.
4. To explore the usefulness of bio-agents for the management of bacterial blight of pomegranate *in vitro* and *in vivo*.

REVIEW OF LITERATURE

Pomegranate (*Punica granatum* L.) is an important fruit crop of India with export potential. Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap)* is appearing in a devastating form in Southern India and became serious threat for pomegranate cultivation in recent years. The incidence of the disease has been reported as early as 1952 by Hingorani and Mehta in New Delhi. The disease assumed severe form/ status in all the pomegranate growing areas of Maharashtra, Karnataka and Andhra Pradesh resulting in severe yield loss both in terms of quality and quantity. Literature pertaining to such works on bacterial disease of pomegranate indicated in objectives is collected and are presented in the following subtopics.

2.1 Occurrence of the disease

For the first time from India, Hingorani and Mehta (1952) observed bacterial leaf spot disease in pomegranate. Later in 1959, Hingorani and Singh identified the pomegranate bacterium and reported it belonged to genus *Xanthomonas* and is host specific. They observed that this species of *Xanthomonas* is slightly different from other known species of *Xanthomonas* - in its biochemical and colony characters and designated as *Xanthomonas punicae* sp. Nov.

The disease was also observed at Annamalainagar, Madras state (Rangaswami, 1962) and Solan, Himachal Pradesh (Sohi *et al.,* 1964).

Thirumalachar and Patel (1966) identified the bacterium pathogenic on pomegranate as *X. punicae* sp. Nov. from South India and Kanwar (1976) in the orchards of Haryana.

Outbreak of the disease was noticed during 2002 (Anon., 2002) in major pomegranate areas of Bellary and Bijapur districts causing severe losses both in terms of yield and quality. Pomegranate, the boon commercial fruit crop to the farmer turned as a big bane after the outbreak of bacterial blight.

First record of the bacterial blight on pomegranate in Karnataka was by Chand and Kishun (1991). They have noticed the severe incidence of the disease causing 60 to 80 per cent losses at IIHR experimental plots in Bangalore. Later this disease was also reported by Akhtar and Bhatti (1992), Mondal and Singh (2008, 2009), Petersen *et al.* (2010). The disease alone causes 60 to 80% losses in pomegranate production in India (Mondal and Mani, 2009).

Jalaraddi (2006) conducted survey to know the disease incidence and severity which revealed that disease was spreading in five districts in an epidemic proportion depending on the agro climatic zones. Disease incidence was 81 per cent in Bijapur and 22 per cent in Raichur district.

2.2 Symptomatology

During 1952, Hingorani and Mehta described the symptoms as irregular spots varying from 2 mm to 5 mm in diameter, primarily appeared on the leaves. Adjacent spots coalesced and covered larger areas. The spots were initially light brown in colour, surrounded by water soaked margin, later turned into dark brown as the disease progressed. The formation of several spots on leaf induced shedding. Spots were not noticed on twigs, branches and fruits.

Rangaswamy (1962) described the symptoms of bacterial blight on leaves as necrotic spots surrounded by chlorotic halos with translucent water soaked appearance. Under severe infections, leaves became distorted and shed off. Fruits also infected with water soaked spots in the earlier stages, later became dark brown, slightly raised from the surface with oily appearance.

Kanwar (1976) observed small, brown, water soaked spots on leaves, flowers and fruits of pomegranate in different orchards of Haryana. In the beginning, spots on leaves were small, circular with yellowish border and brown centre. Later on, number of adjacent spots coalesced with each other and formed elongated and irregular lesions. In many cases, spots were restricted on leaves by veins, resulted into linear stripes. Tissue necrosis and defoliation occurred in advanced cases of infection. On petals of flowers, small, brown, water soaked spots were seen, which later became black and bigger in size. Symptoms were also noticed on immature fruits as small, pin head like, circular with dull green colour, which later turned to deep brown or black with yellowish zone surrounding the spots. As the disease advanced, spots on fruits coalesced together and formed irregular lesions, as a result, skin of the fruit became rough. Under heavy infection condition, fruits remained undeveloped and deformed. In 1993, Kishun reported similar kind of symptoms on leaves, but on stem the disease appeared as brown to black coloured spots around the nodes leading to girdling and cracking of

nodes. Brown to black spots also appeared on fruit pericarp, which later turned to L or Y shaped cracks.

Manjula and Khan (2002) described the symptoms as minute water soaked lesions appeared both on leaves and fruits, which later turned brown to black coloured spots surrounded by diffused water soaked margin. The lesions on fruit appear as irregular, shining brown to black spots with Y or L shaped cracking or splitting of pericarp, reducing the marketability of the fruits (Mondal *et al.,* 2012). Severely infected fruits split open, partially exposing the arils. Bacterial lesions were also noticed on twigs/branches resulting in death of the branches. Affected plants looked unthrifty, weak and died later on.

2.3 Isolation and pathogenicity

Hingorani and Mehta (1952) isolated bacterial leaf spot pathogen from the infected pomegranate leaf using dilution plate technique and proved pathogenicity by spray method of inoculation in absence of wounds. Infection readily occurred on the tender leaves of artificially inoculated young potted plants, after 7-10 days of inoculation. Isolation from the infected plants yielded an organism similar to one used in the inoculation experiments.

Isolation of the bacterial blight pathogen of pomegranate was made within 48 h after collection of samples on nutrient dextrose agar medium. All the diseased samples yielded yellow pigmented colonies after 3-5 days of inoculation at 25-30ºC temperature. Pure cultures were established by repeated single colony transfer method. For pathogenicity test, inoculation was made by spraying bacterial suspension of 48 h old culture onto the injured and uninjured leaves, flowers and fruits of healthy plants. Infection occurred more rapidly on injured leaves, flowers and fruits than uninjured plants. Organism reisolated from artificially inoculated plant was found identical with original one (Kanwar, 1976).

Chand and Kishun (1991) standardised the inoculation method for *Xap* by inoculating the bacterial suspension (2×10^8cfu/ml) on 40 days old leaves by pinprick, rubber block pressure, leaf cut and automization methods. They observed that leaf cut was found superior, where they recorded 100 per cent infection covering 70 to 90 per leaf area within 21 days. The automization of bacterial suspension was found to induce lowest infection of 6 - 7.5 per cent with maximum incubation period of 17 to 40 days.

Manjula (2002) observed characteristic water soaked lesions on leaves after 5 days of inoculation of *Xap* on to the pomegranate plants cv. Jyothi. Pathogenicity studies revealed that water soaked lesions appear after 3 days of inoculation of bacterial culture, whereas, more than 15 days are needed for the appearance of brown necrotic spots on leaves of pomegranate (Jalaraddi, 2006).

Mogle *et al.* (2009) studied the pathogenicity of *Xap* of susceptible pomegranate Cv. Ganesh grown in earthen pots in polyhouse. Forty days old healthy leaves of pomegranate were inoculated with thorny seeds of *Xanthium strumarium* and nodes by leaf cuts method. Typical symptoms of disease appeared on leaves within nine to thirteen days after inoculation. Symptoms first appeared on under surface of the leaves as irregular water soaked spots. Corresponding to these spots, on upper surface of the leaves, rough spots encircled with yellow halo were observed. Later these spots turned brown, coalesced and exhibited blighted appearance. The reisolation attempted from artificially infected /diseased plant tissue on nutrient agar consistently yielded *X. a.* pv. *punicae*.

Mondal *et al.* (2012) inoculated pomegranate plants (Cv. Bhagwa) by three different methods, namely sprays, pinprick and syringe infiltration with *Xap* inoculums adjusted at 0.1 OD (600 nm) by suspending cells in sterile distilled water. The pathogenecity of the *Xap* isolates were confirmed following Koch's postulate. PCR detection was also done after 5 days of inoculation.

2.4 Identification of the pathogen

Hingorani and Singh (1959) described the pathogen as *Xanthomonas punicae* sp. Nov., which has a short rod with rounded ends; single or in pairs; sometimes in chains; no involution forms; 1 - 2.5 × 0.5 □m in size; motile with a single polar flagellum; Gram-negative; non-endospore forming; capsulated and non-acid fast.

According to Kanwar (1976) the bacterial blight pathogen of pomegranate was seen in single pairs and also in chains. It was rod shaped with rounded ends and measured 0.75 to 3.0 □m in length and 0.45 □m in width, Gram negative, with single polar flagellum, neither capsules nor endospores were observed.

DNA *gyrB* gene has been demonstrated to offer greater resolution in discriminating pathovars of bacterial species including the genus *Xanthomonas* (Motoshima *et al*., 2007; Parkinson *et al.,* 2009). Recently, *gyrB* gene based phylogeny resolved the distinctiveness of the species, *Xanthomonas citri* and *X. fuscans*, which were previously classified as pathovars of *X. axonopodis* (Schaad *et al.,* 2007). The species designation of pomegranate blight bacterium has recently been changed from *X. campestris* pv. *punicae* to *X. axonopodis* pv. *punicae* based on *gyrB* sequence (Parkinson *et al.,* 2009).

2.4.1 Growth phase

Monod (1949) reviewed the growth of bacterial culture and mentioned quantitative characteristics of the growth cycle, essentially the three growth constants *i.e*., total growth (G), exponential growth rate (R), and growth lag (L).

2.5 Physiological studies

2.5.1 Temperature

Hingorani and Mehta (1952) reported that the optimum temperature for growth of *Xanthomonas punicae* was about 30°C, maximum 40°C and minimum about 5-10°C. The thermal death point was about 52°C. Later in 1954, they reported that the pathogen needed an optimum temperature of 30°C for growth and has a thermal death point of about 52°C. However, Hingorani and Singh (1959) reported that the cardinal temperatures for growth of the pathogen was minimum 41°F (5°C), optimum 80.6°- 86.0°F (27^{O}-30°C) and maximum 104°F (40°C). The thermal death point is near 125°F (51.66°C). It can resist desiccation upto 14 days at 86°F (30°C). They also reported the maximum growth of pathogen at optimum pH 6.8 to 7.6 and no growth at pH 10.2.

Gour *et al.* (2000) got the similar results while working with *X. axonopodis* pv. *vignicola*, the causal agent of leaf blight of cowpea. They have recorded the maximum growth of the pathogen at a temperature level of 30^{0}C. The optimum temperature for the growth of bacterium was found to be 25-28^{0}C and minimum and maximum temperature was 10 and 40^{0}C, respectively. The optimum pH required for the growth was 7.0- 7.2, while, minimum and maximum was 5 and 8, respectively (Manjula, 2002).

Yenjerappa (2009) reported that the temperature of 30^{0}C was optimum for the maximum growth of *X. axonopodis* pv. *punicae,* although, it grew at a wide temperature levels ranged between 15 and 45^{0}C. No growth of the pathogen was recorded at highest and lowest extreme temperature levels of 50 and 5^{0}C, respectively. Madhu (2009) observed the growth of eleven isolates of *X. axonopodis* pv. *punicae* at temperature 20^{0}C whereas other five isolates at 30^{0}C.

Later Mondal and Kumar (2011) reported the maximum growth of *Xap* at 28°C and absence of growth at 37^{0}C. It grows well at pH 6 and above and absence of growth at pH below 5. It also grows at 1 per cent NaCl but no growth above 2 per cent NaCl.

2.5.2 Effect of different pH levels

Gour *et al.* (2000) got the similar results while working with *X. axonopodis* pv. *vignicola*, the causal agent of leaf blight of cowpea. They have recorded the maximum growth of the pathogen at pH of 7.0. Growth (number of colonies) declined considerably at pH values higher and lower than 7.0 being minimum at a pH of 5.0. Manjula (2002) recorded the highest number of colonies of *Xap* at a pH of 7.2. Madhu (2009) reported that the maximum growth of all isolates of *X. axonopodis* pv. *punicae* at pH 5.0 and the least growth at pH 8.0. Good growth of bacterium was observed between the pH range of 5.0 to 6.0.

2.5.3 NaCl sensitivity

Mondal and Kumar (2011) found that *Xap* tolerates upto 1 per cent NaCl concentration, beyond that no growth of the pathogen is observed.

2.5.4 Fuscan pigment production on LBA without NaCl

Fuscan pigment production is the characteristic feature of *Xap* which is observed after 72-96 h of growth especially beneath the lawn of bacteria in the medium or in the liquid medium (Mondal and Kumar, 2011).

2.6 Assessment of baseline sensitivity to major bactericides among *Xap* isolates representing major pomegranate growing regions

The sensitivity of different isolates to antibiotics by paper disc method against *Xanthomonas campestris* pv. *mangiferaeindicae* was studied by Venugopal (1983). Isolates obtained from varieties such as Raspuri, Bappukai and Lalbaugh exhibited sensitivity to streptomycin and paushamycin @ 100 and 250 ppm, respectively.

In vitro studies conducted by Sushma (1997) on efficacy of antibiotics and bactericides against *X. c.* pv. *dieffenbachiae* indicated that Streptocycline (500 ppm) was very effective followed by Paushamycin (500 ppm) with 23.3 and 20.8 mm inhibition zone, respectively and under *in vivo* condition Streptocycline, Streptomycin and Paushamycin were effective.

Shobha (1998) observed Streptocycline was effective followed by Streptomycin sulphate and Paushamycin at 500 ppm against *X. a.* pv. *glycines.*

Krishnan and Madhumeeta (1988) observed that plantomycin, streptocycline and paushamycin each at 500 ppm concentration were equally effective in inhibiting the growth of *Xanthomonas campestris* pv. *dieffenbachiae* causing bacterial blight of anthurium. Screening of antibiotics/ bactericides under *in vitro* conditions revealed that Streptocycline and Paushamycin was highly effective against the *X. a*. pv. *punicae* at 500 ppm with 25.60 mm and 19.0 mm inhibition zone, respectively (Manjula, 2002).

Manjula *et al.* (2002) studied the *in vitro* efficacy of bactericides against *Xap* and results indicated that paushamycin (500 ppm) and K cycline (500 ppm) produced the highest inhibition zone followed by bacterinol and bacteriomycin. Copper oxychloride @ 2000 ppm was found moderately effective and Kasugamycin @ 500 ppm concentration was ineffective.

Yenjerappa (2009) reported that bactinash-200 (15.07 mm), bronip (14.67 mm) and plantomycin (13.77 mm) exhibited significantly superior efficacy in inhibiting the growth of *Xap*. The other bactericides *viz.,* K cycline, copper oxychloride, Bordeaux mixture, bactinashak, bleaching powder and streptocycline were found moderately effective with an inhibition zone ranging from 8.40 to 10.23 mm. In respect of concentration, efficacy of each chemical was significant at relatively higher concentrations.

Raju (2010) reported that Streptocycline + COC with an inhibition zone of 3.3 cm exhibited superior efficacy against *Xap* followed by Streptocycline (2.80 cm) and COC (2.65 cm). However, both the treatments were on par with each other. All other chemicals *viz.,* Streptocycline + Copper hydroxide, Copper hydroxide were found to be moderately effective but were significantly different from each other, Bromopal and Kasugamycin were less effective and were on par with each other. In respect of concentration, efficacy of each chemical was significant at relatively higher concentrations.

2.7 Ribosomal RNA (16S rRNA) analysis and phylogeography of *Xap* isolates occurring in pomegranate growing situations

Polymerase Chain Reaction (PCR) based molecular markers are useful tools for different detecting genetic variation within population of phytopathogens. The analysis of RAPD polymorphism in single cell isolates of *X. axonopodis* pv. *punicae* from different districts across Karnataka revealed the occurrence of high level of polymorphism (89.56%) indicating wide and diverse genetic base. RAPD analysis grouped all the 16 isolates into two major clusters. The highest similarity degree was observed in Gulaganjikoppa (*Xap*-03) and Kurubararamanahally (*Xap*-16). The genetic relationship between Lokapur (*Xap*-01) and Ramatala (*Xap*-02) isolates may be correlated to their geographical location as they were grouped in same clusters. H. B. Hally (*Xap*-05) isolate of Bellary formed a separate cluster. The isolate Gulaganjikoppa (*Xap*-03) and Kurubararamanahally (*Xap*-16) as well as Shravanagere (*Xap*-09) and Bandi (*Xap*-14) were found genetically related, though from different geographical location (Madhu, 2009). Thus, in some isolates, phylogenetic grouping based on our RAPD data did not appear to be congruent with geographical locations similar results were obtained by Pooler *et al.* (1996) and Yenjerappa (2009).

In bacterial plant pathogens, the 16S rRNA gene has been well established as an important locus for identification and in establishing phylogenetic distances (Ash *et al.,* 1991, Nesme *et al.,* 1995, Adhikari *et al*., 1995 Hauben *et al.,* 1997). Though a considerable stretch of the 16S rRNA gene is conserved across bacterial genera, a smaller variable region enables workers to estimate

phylogenetic distances. Among the other important housekeeping loci, *gyrB* has been demonstrated to have potential for studying the genetic relatedness among closely associated strains (Parkinson *et al*., 2009).

PCR targeting the 16S-23S rDNA spacer region not only is sufficiently sensitive, but is also fast, easy and large copy number and a high degree of sequence conservation with little variation among species. The nucleotide sequence of the 16S-23S spacer regions of *Xanthomonas* spp. determined high level of homology among members of genus. The sequence obtained from two strains of *Xanthomonas oryzae* pv. *oryzae* were completely identical. A single 470bp fragment was found in all strains isolated from diverse regions in Japan by PCR amplification using specific primers (XOR-F and XOR-R) and same size fragment was also obtained from *Xanthomonas campestris* pv. *citri*, pv. *incanae* and *zinnia* (Adachi and Oku, 2000).

Roland *et al.* (2006) studied the genetic diversity of *Xanthomonas translucens* pv. *graminis* (*X.t.g.),* causing bacterial wilt in forage grasses, by using 16S rDNA sequencing on three Italian ryegrass (*Lolium multiflorum*) cultivars. 16S rDNA sequencing of 29 putative *X.t.g.* isolates allowed to assign 28 isolates to the pv. *translucens* while one isolate was identified as *X. t.* pv. *arrhenatheri*.

Phylogeny derived from 16S rRNA gene revealed that two *Xanthomonas* species, *Xanthomonas citri* subsp. *malvacearum* DSM 3849 T and *X. axonopodis* pv. *manihotis* NCPPB1834 formed a single cluster along with *Xap*. Further, analysis in the *gyrB* locus indicated that *X. citri* subsp. *malvacearum* shared 99.4 per cent identity while pathovars *X. axonopodis* pv. *manihotis* shared only 95 per cent identity with the *Xap* strains (Mondal *et al*., 2012).

BLAST analysis revealed that the 16S rRNA and complete *rpoB* gene sequences of *X. axonopodis* pv. *punicae* are >99% identical to those of *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker (Sharma *et al.,* 2012).. Further phylogenomics analysis using highly conserved housekeeping genes described earlier for xanthomonads (Bogdanove *et al*., 2011) confirmed its close relationship with *X. axonopodis* pv. *citri.*

2.7.1 Multiplex PCR

Figueiredo and Torres (2007) through multiplex PCR approach detected both BSV and CMV by using BSV and CMV specific oligonucleotide primers. Multiplex PCR for parallel detection of BBrMV, BSV and CMV was also undertaken in *Musa sp*. by Ramesh (2009). However, multiplex PCR has also been developed for detection of viruses in pepper (Kim *et al*., 2006), grape (Gambino and Gribaud, 2006), legumes (Bariana *et al*., 1994) and viroids in citrus (Takao *et al*., 2002).

Rodrigues *et al.* (2003) studied the amplification of gene from *Xylella fastidiosa* in field collected plant and insect sample using primer pairs designed for the 16S rRNA and *gyr*B genes. Sizes of amplification of both the genes were 745 and 429 bp for 16S rRNA and *gyr*B respectively in a single PCR.

A multiplex PCR assay was used to simultaneously detect genes from the five major clinically relevant *Campylobacter* spp. Those genes selected were *hipO* and 23S rRNA from *Campylobacter jejuni; glyA* from each of *C. coli, C. lari,* and *C. upsaliensis*; and *sapB2* from *C. fetus* subsp. *fetus*. The assay was evaluated with 137 clinical and environmental isolates and was found to be rapid and easy to perform and had a high sensitivity and specificity for characterizing isolates, even in mixed cultures (Wang *et al.,* 2002).

Ozdemir (2005) reported that CMM-5-CMM-6 and RST_2-RST_3 primer pairs were used to amplify template DNAs of *Clavibacter michiganensis* spp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria*, respectively by multiplex PCR. Annealing temperatures 58.5-59.5℃ were optimal for RST_2-RST_3 and CMM-5-CMM-6 primer pairs for multiplex PCR.

2.7.2 Colony PCR

The alternative technique to avoid some of the problems associated with DNA extraction is the use of environmental samples directly in the PCR without prior isolation of DNA. This approach has been practiced with intact cultured bacterial cells. DNA extraction from samples is a critical initial step in PCR detection of bacteria in environmental samples. There are numerous commercially available DNA extraction kits that can be used for recovering bacterial DNA directly from colony, soil and water samples. Although the use of DNA extraction kits in research laboratories has become routine, the amount and quality of DNA recovered depend on the skill of the researcher and on the choice of the DNA extraction protocol (Kong *et al.,* 2005).

Umesha *et al*. (2012) developed colony PCR-SSCP technique to identify *Ralstonia solanacearum* by the PCR product without DNA isolation was denatured and separated on a non-denaturing polyacrylamide gel. SSCP banding patterns were detected by silver staining of nucleic acids and compaired with the other phytopathogenic bacteria. Under non-denaturing conditions, ssDNA has a secondary structure that could be determined by the nucleotide sequence. The mobility of the ssDNA depends on the secondary structure of the amplified product. The different positions of the bands of ssDNA on the gel indicate different sequences. PCR-SSCP is capable of detecting 90% of all single-base substitutions in 200 bp fragments (Hayashi, 1991).

2.8 Prospecting the botanicals and selected nutrients for *in vitro* and *in vivo* inhibition of *Xap* of pomegranate

2.8.1 Botanicals

According to Grainage *et al.* (1985) the aqueous extract from the cloves of *Allium sativum* and *Artabotrys uncinatus* (ylang ylang) when used against the pathogen *X. c.* pv*. oryzae* produced 68 mm and 64 mm inhibition zone, respectively.

Rajendra and Alankara (1987) tested the antimicrobial effect of essential oils of five species of ocimum. All the samples showed antibacterial activity against Gram positive and Gram negative bacteria.

Srinivasachary (1995) found that *Ocimum* plant extract was more effective in inhibiting the growth of *Xanthomonas campestris* pv. *mori* isolated from mulberry under *in vitro* followed by *Bursara, Citronella* and *Cinnamom*, whereas garlic extract was highly inhibitory to the growth of *Xanthomonas campestris* pv. *dieffenbachiae* followed by *Ocimum* and *Citronella* (Sushma, 1997) further she opined that garlic extract was highly inhibitory to the growth of *X. c.* pv. *dieffenbachiae* followed by ocimum and citronella with 28, 26.5 and 13.5 mm inhibitory zone, respectively under *in vitro* conditions.

Shobha (1998) observed that *Adahtoda zeylenica* was found inhibitory for the growth of *X.a.* pv *glycines* followed by garlic extract, ocimum, eucalyptus and citronella.

Thammaiah *et al.* (1998) observed the effective inhibition of *X. c.* pv. *vignicola* in *in vitro* (1.52 cm diameter inhibition zone) by the root and leaf extract of *Adahtoda zeylanica.* The suppression of the bacterial blight of cowpea was attributed to the presence of water soluble alkaloid vasicine in Adhatoda plant extract, root extract was more effective than leaf extract.

Kiran (2000) reported that alcohol extract of *Ocimum sanctum* was potent in inhibiting the growth of *Ralstonia solanacearum, Xanthomonas axonopodis* pv. *vignicola* and *X. campestris* pv. *campestris* upto dilution of 1:10. He also reported the effectiveness of alcohol extract of *Nigella sativa* seeds at 1:10 dilution on these three pathogens. Under *in vivo* conditions the alcohol extract of *O. sanctum* effectively suppresses the growth of *X. a.* pv. *vignicola* (bacterial blight of cowpea) when undiluted and the effectiveness decreased as the dilution increased.

Madhiazhagan *et al.* (2002) studied the field efficacy of five botanical extracts in controlling the bacterial bight of rice caused by *Xanthomonas oryzae* pv. *oryzae* through pot culture experiment and mentioned that among the five plant extracts, *Adahtoda vasica* significantly minimized the disease with higher grain yield levels. *Curcuma longa* and *Allium cepa* were the next best effective treatments.

Manjula (2002) observed the significant difference in the inhibitory effect among the eight plant extracts screened against the growth of *X. a.* pv. *punicae*. Kalangi extract (1:1) was found more effective against the growth of Bangalore fruit isolate followed by Miswak, Tulsi and Patchouli, where as miswak exhibited highest inhibitory effect followed by Kalangi and Patchouli on the growth of Bijapur isolate.

Jalaraddi (2006) found that both aqueous and alcoholic extracts of Garlic, Miswak and Citronella were inhibitory to growth of the *Xanthomonas axonopodis* pv.*punicae*, while Botanical Sufoof-E-Shoneez recorded highest inhibition zone.

Yenjerappa (2009) reported that among the different plant extracts tested against *X. a.* pv*. punicae*, garlic extract (10%) produced the maximum inhibition zone followed by parthenium, lantana leaf extract and onion bulb extract each at 10 per cent concentration in *in vitro* condition. Under field condition, garlic and onion bulb extract each at 10 per cent concentration significantly recorded the

lowest disease incidence and severity of the disease. In respect of yield, garlic extract (10%) recorded significantly highest yield than all other plant extracts in the trial.

Raju (2010) reported that tulsi leaves extract at 40% and 30% concentration was significantly greater in efficacy than all other treatments followed by neem seed oil and garlic bulb extract at 40% concentration.

Soapnuts have naturally anti-fungal and anti-microbial activity. Although time tested for safety for thousands of years, soap nuts and saponin are just beginning to be studied in depth to determine all their potential natural benefits (www.soapnuts.pro, 2011). Many biological activities like antimicrobial, antidiabetic, cytotoxic, molluscicidal, fungicidal, anti-inflammatory, *etc.* were shown by fractions of crude extracts and isolated compounds.

Thirumalesh *et al.* (2012) reported that the crude fruit extract of *Sapindus laurifolia* (soapnut) (15.0 mm) and leaf extracts of *Asclepias curassavica* (7.50 mm), *Helicteres isora* (7.0 mm), *Piper betel* (7.0 mm), *Tamarindus indica* (5.0 mm), *Tridax procumbens* (5.0 mm) and *Azadirachta indica* (7.0 mm), exhibited greater antibacterial activities in Nutrient agar medium with MIC ranging from 3.0% to 12.0% against *Xanthomonas campestris* pv. *mangiferaeindicae* causing bacterial black spot of mango

2.8.2 Nutrients

Strong correlation between boron uptake in leaf tissues, residual boron in soil with black rot incidence in cauliflower was reported by Kumar and Kotur (1989). Exogenous supply of boron (0 – 6.4 mg/kg soil) in low boron containing altisols (hot water soluble soil containing boron of 0.1 mg/kg soil) indicated that this nutrient has a definite role in susceptibility of cauliflower to black rot caused by *Xanthomonas campestris* pv. *campestris*. Susceptibility was greatly observed in boron deficient (below 0.4 mg/kg soil) and boron excess (above 1.6 mg/kg soil) plants than the plants grown with optimum level of boron (0.4 – 1.6 mg/kg soil).

Ansari and Shridhar (2001) studied the effect of iron on the virulence of *X. o.* pv. *oryzae* by pot culture experiment. Twenty five days old rice seedlings were clip inoculated with 48 h old culture grown in the medium amended with different levels of iron *i.e.*, $FeSO_4$ at 0.20, 2.00, 4.00, 8.00 mg/ l and noticed that increase in lesion length was observed upto 8.0 mg/ l in susceptible cultivar (IR-8) but restricted upto 4 mg/ l in resistant cultivar (DV-85). Pathogen grown in iron starved medium produced minimum lesion length.

Dordas (2008) reviewed the effect of nutrients, such as N, K, P, Mn, Zn, B, Cl and Si, on disease resistance and tolerance. Among the nutrients, Mn could control a number of diseases as Mn has an important role in lignin biosynthesis, phenol biosynthesis, photosynthesis and several other functions. Zn showed decreased or increased susceptibility to disease in some cases. Boron was found to reduce the severity of many diseases because it has function on cell wall structure, plant membranes and plant metabolism. Nutrients can reduce disease to an acceptable level, or at least to a level at which further control by other cultural practices or conventional organic biocides are more successful and less expensive.

2.9 To explore the usefulness of bio-agents in the management of bacterial blight of pomegranate under *in vitro* conditions

Unnamalai and Gnanamanickam (1984) reported that in two methods of *in vitro* bioassay, *P. fluorescens* siderophore inhibited the growth of *X. citri,* in another set of *in vitro* studies, *X. oryzae,* the incitant of bacterial leaf blight of rice was also inhibited.

Sakthivel *et al.* (1986) examined *in vitro* antagonism between strains of *P. fluorescens* and bacterial plant pathogens. The pathogens used were *X. o.* pv. *oryzae* (bacterial leaf blight of rice) and *Pseudomonas syringae* pv. *phaseolicola* (halo-blight of French bean), strains of *P. fluorescens* showed *in vitro* antagonism to both the bacterial pathogens tested. Selected strains of *P. fluorescens* used for bacterization of rice and cotton seeds enhanced plant growth by 2-27 per cent in rice and 8-40 per cent in cotton.

Sivamani *et al.* (1987) reported the toxicity of *P. fluorescens* towards bacterial plant pathogens of banana (*P. solanacearum)* and rice (*X. c.* pv. *oryzae).* They suggested that the native strains of *P. fluorescens* could be used as biological control agents against *P. solanacearum* on banana and the rice bacterial leaf blight pathogen.

Tzeng *et al.* (1994) reported that out of total of 151 strains of fluorescent pseudomonads isolated from various crop at different localities in Taiwan, the foliar strains tested exhibited 9.2-35.1 per cent inhibition of the growth of *X. c.* pv. *campestris, X. c.* pv. *vesicatoria, X. c.* pv. *citri* and *X. c.* pv. *mangiferaeindicae* on King's B nutrient agar and potato dextrose agar.

The biochemical studies by Valasubramanian *et al.* (1994) showed that the efficient strains of *P. fluorescens* produced the antibiotic phenazine-1-carboxylic acid (PCA) whereas, Laha *et al.* (1992) stated that fluorescent pigments produced by the pseudomonas are sequester Fe3+ and are considered siderophores which inhibit a large number of phytopathogenic bacteria and fungi.

Safiyazov *et al.* (1995) reported that *P. fluorescens* 41, *B. subtilis* 23 and *B. megatherium* 26, were tested for their ability to control major agents of major cotton diseases like *X. c. pv. malvacearum, Rhizoctonia solani, Fusarium vasinfectum* and *Verticillium dahliae.* Control was achieved in several trials and results demonstrated that the antagonism of tested isolates was in some versions active and in others passive. In laboratory tests *B. subtilis* 23 was the most effective against three of four phytopathogens. In field trials, the antagonistic metabolites inhibited development of diseases. In this case the most effective antagonists were *B. subtilis* 23 and *P. fluorescens* 41.

Kalita *et al.* (1996) examined the reduction of canker disease on citrus, when phylloplane species of antagonists *viz*., *Pseudomonas fluorescens*, *Bacillus subtilis* and *Bacillus polymixa* were applied onto the citrus crop foliage of variety Assam lemon. They recorded least canker incidence with the treatment by *Bacillus subtilis*.

Nagaraj (1997) tested the antagonistic bacteria like *P. fluorescens, B. subtilis* and *Psuedomonas aeroginosa* under *in vitro* conditions on the growth of *X. c.* pv. *moricola* and found that *P. fluorescens* was more effective (25.33 mm) in inhibiting the growth of the pathogen followed by *P. aeruginosa* (21.6 mm).

The efficacy of *Pseudomonas fluorescens* in reducing the bacterial blight intensity of rice was reported by Kaur and Thind (2002). Among the three isolates of *P. fluorescens* tested in glasshouse and field, the isolate *P. fluorescens* LR (Ludhiana rhizosphere) proved as most effective antagonist in significantly reducing the disease intensity. Among the different methods of antagonist application tried, seed bacterization followed by two foliar sprays recorded the lowest bacterial blight intensity both in glasshouse and field conditions.

Jalaraddi (2006) reported that *Pseudomonas fluorescens* was found moderately effective in inhibiting the pathogen *Xap* by producing inhibition zone of 10.8 mm, whereas, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Bacillus polymyxa* were not effective in inhibiting the pathogen.

Jones Nirmalnath (2010) studied the inhibitory activity of Pink Pigmented Facultative Methylobacterium (PPFM) strains against *Sclerotium rolfsii* and reported that among ten isolates of PPFM strains PPFM 155 produced the highest zone of inhibition (11 mm dia) followed by PPFM 169 which produced 10 mm dia.

Jones Nirmalnath *et al.* (2011)a reported that out of 62 fluorescent pseudomonads evaluated, following the dual culture assay, 22 isolates exhibited antagonistic activity against *Xap* with inhibition zone varied from 2.0 to 5.0 mm.

Jones Nirmalnath *et al.* (2011)b reported that PPFM H5 strain, isolated from Bagalkot, has shown maximum inhibition zone (15.00 mm) followed by PPFM 42U (14.60 mm), PPFM 71 (14.40 mm) and PPFM 75L (14.00 mm) against *Xap* causing bacterial blight of pomegranate.

2.10 Field evaluation of selected botanical, bioagents and nutrient for the control of bacterial blight of pomegranate

Jadeja *et al.* (2000) achieved the successful control of citrus canker (*Xanthomonas axonopodis* pv. *citri*) with the adoption of integrated measures involving scraping of diseased portion on the main trunk, followed by pasting with Bordeaux paste and foliar application of streptomycin sulphate (100 ppm) + copper oxychloride (0.2%) three times a year.

Das and Shyam Singh (2003) recommended the integration of chemicals and cultural practices for the management of bacterial canker of acid lime. According to them, pruning of infected twigs and combined application of streptocycline (100 ppm), copper oxychloride (0.3) and neem cake (5%) for four times at monthly interval with first spray before the onset of monsoon reduced the disease significantly to the extent of 53.2 per cent to control.

From the results of five years pilot demonstration on the management of bacterial canker in acid lime, Gopal *et al.* (2004) emphasized the need of integrated approach involving cultural and

chemical treatments. The technology demonstrated was pruning dried and cankerous twigs with immediate spray of copper oxychloride (0.3%) followed by two sprays of streptocycline (100 ppm) + copper oxychloride (0.3%) at monthly interval starting from June effectively reduced the canker on twig, foliage and fruit with high yield of cankerless, good marketable fruits, which benefited the farmer with cost benefit ratio of 1:1.59.

Method demonstration in farmers field comprising practices like clean sanitation, removing water shoots, spraying streptocycline, pasting of stem and branches with copper oxychloride + carbaryl + DDVP + sticker *etc.* were carried out in a campaign organized against oily spot disease of pomegranate, which benefitted growers at large (Anon., 2005).

David (2005) reported the successful management of leaf blight of onion (*Xanthomonas axonopodis* pv. *alli*) with the integration of different strategies such as use of plant activator, biological and chemical control. He opined that integration of acibenzolar-S methyl (plant activator), biological control agents (commercial formulations of both *Pantoea agglomerans* strain C9-1 and *Pseudomonas fluorescens* (strain A 506) with copper hydroxide in a carefully timed spray program can eliminate the leaf blight disease of onion effectively.

Yenjeerappa (2009) reported that lowest disease index of 22.3 per cent in streptocycline (0.05%) + copper oxychloride (0.2%) treated plots followed by bacterinol 100 (0.05%) + copper oxychloride (0.2%) and bacteriomycin (0.05%) + copper oxychloride (0.2%), which recorded 27.2 per cent disease index to two years experimentation against bacterial blight of pomegranate. All these three bactericides exhibited on par efficacy with each other and significantly superior efficacy to *Pseudomonas fluorescens* at 0.5% (bioagent) and garlic extract at 2% (botanical). Highest disease severity of 63.1 per cent was rcorded in untreated check plot.

Raju (2010) reported that two bio agents *Pseudomonas fluorescens* at 1.00 per cent and *Bacillus subtilis* at 1.00 per cent were moderately effective and they are significantly different from each other. Disease incidence of 56.65 per cent was recorded in case of *Pseudomonas flourescens* followed by *Bacillus subtilis* 57.26 per cent on stem but they were significantly different from each other with 36.14 per cent PDI and 38.69 per cent PDI, respectively.

MATERIAL AND METHODS

Laboratory experiments on isolation, pathogenicity, biochemical and molecular variability were carried out at Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Dharwad. Two field experiments were conducted in farmer's fields at Talkal (Yelburga taluk, Koppal district,) and Muddaballi (Koppal, taluk, Koppal district,) villages. The details of materials used and methodology followed during the present course of investigation are described here under.

General procedure

Cleaning and sterilization of material and reagents

All the plastics and glasswares were soaked overnight in potassium dichromate sulphuric acid solution and the washing solution was drained off on next day. Later the materials were washed thoroughly in running tap water and rinsed with distilled water. The plasticware were and the glasswares air-dried dried in hot air oven at 160°C for one h. The cleaned and dried materials were covered with polythene covered or paper and sterilized at 121°C at 1.1 kg/ cm^2 for 20 minutes.

Collection of isolates of *Xanthomonas axonopodis* pv. *punicae* (*Xap*) from different parts of Karnataka and other states

Infected plant parts with typical symptoms of bacterial blight of pomegranate were collected from different districts of Northern Karnataka (Bellary, Bagalkot, Bijapur, Koppal), three from Maharashtra (Sollapur, Sangli, Nasik) and two from Andhra Pradesh (Anantapur, Mehabubnagar). Nine collections from different localities were isolated by following the method given by Schaad (1992) (Table 1).

3.1 Isolation of pathogen

Infected plant parts such as leaf, bark of the stem and fruit pericarp showing typical symptoms of bacterial blight were subjected to isolation of *Xap*. The diseased samples were washed thoroughly with tap water and dried under shade. The infected portion along with healthy part was cut into small pieces and was surface sterilized with 1.0% Sodium hypochlorite solution for 60 seconds and washed three times serially in sterile distilled water to remove the traces of Sodium hypochlorite. The diseased bits were then suspended in a Petri Plate containing 1 ml of sterilized distilled water and crushed with sterilized scalpel and allowed for 2-3 minutes. When the sufficient oozing of bacterial masses were released from the crushed tissues, a loop-full of sap was taken and streaked on to Petri Plate containing Nutrient Glucose Agar medium (NGA). The inoculated plates were incubated at 28±1°C for 72-96 h. After the 72 h of incubation, *Xap* usually appeared as pinhead sized colonies. The young and fast growing colonies were saprophytic ones. Thus observations were made for the development of well separated, typical, light yellow coloured bacterial colonies resembling that of typical *Xanthomonas* sp. at 96 h.

3.1.1 Identification of the pathogen

It is identified based on shape, size, flagellation, gram reaction, endospore forming nature, acid fast nature as described by Schaad (1992).

3.1.2 Purification and preservation of bacterial culture

The suspected bacterial colonies were picked up with the help of sterilized inoculation loop and streaked onto the surface of sterilized NGA Petri Plates. The inoculated plates were incubated at 28±1°C for 72-96 h. Observations were made for the development of well separated, typical, bright yellow, mucoid colonies. Such pure colonies were further streaked onto the agar slants containing the NGA medium and incubated at 28±1°C for 72 h. Then the cultures were stored in the refrigerator at 4°C. A loop full of pure cultures were stored in sterile propylene culture tubes and stored at 4°C in refrigerator for further use as stock culture. For long term preservation, a loopful of bacterium was suspended in 600µl of nutrient glucose broth in 1.5 or 2 ml eppendorf tube and added with 600µl of glycerol (50%) and stored at –80°C for further use.

3.1.3 Proving pathogenicity by Koch's postulates

Susceptible pomegranate variety (Bhagwa) to bacterial blight was raised first in earthen pots by planting apparently healthy layered cuttings. One month old saplings were then transplanted into

Table 1: Details of sampling locations

Isolate Code	Place of collection	Taluk	District	State
*Xap*1	Matrangi	Koppal	Koppal	Karnataka
*Xap*2	Kanamadi	Bijapur	Bijapur	-do-
*Xap*3	Kannur	Bijapur	- do -	-do-
*Xap*4	Kaladagi	Bagalakot	Bagalakot	-do-
*Xap*5	Chikkasamshi	Bagalakot	- do -	-do-
*Xap*6	Ankalagi	Bagalakot	- do -	-do-
*Xap*7	Hiraj	Sollapur	Sollapur	Maharashtra
*Xap*8	Jumnal	Bijapur	Bijapur	Karnataka
*Xap*9	Malegaon	Malegaon	Nasik	Maharashtra
*Xap*10	Sholapur	Solapur	Solapur	Maharashtra
*Xap*11	Anantpur	Anantpur	Anantpur	Andhra Pradesh
*Xap*12	Mandarna	Chalisgaon	Jalgaon	Maharashtra
*Xap*13	Govinkoppa	Bagalkot	- do -	Karnataka
*Xap*14	Lakmapur	Satara	Nasik	Maharashtra
*Xap*15	Mehboobnagar	Mehboobnagar	Mehboobnagar	Andhra Pradesh
*Xap*16	Kerur	Badami	- do -	Karnataka
*Xap*17	Laxmipur	H.B.Halli	Bellary	Karnataka
*Xap*18	Talkal	Yalaburga	Koppal	Karnataka

the pots containing sterilized soil amended with necessary nutrients. The seedlings were regularly watered and exposed to sufficient sunlight.

To prove the pathogenicity of each isolate collected from nine regions, eighteen isolates were separately multiplied in nutrient glucose broth (200 ml) in Erlenmayer flasks by inoculating a loopful of bacterial culture. The inoculated flasks were incubated for three days at 28±1^{0}C.

Two months old pomegranate susceptible variety (Bhagwa) plants were first sprayed with water and then covered with a polythene sheet for 24 h before inoculation. The pre-incubated pomegranate plants were sprayed with 4x10^7 cfu/ml of bacterial suspension (200ml: 800ml; bacterial suspension: sterilized distilled water) with hand sprayer on three branches.

The sprayed plants were covered with polythene sheets and kept in humid chamber for the next 48 h, in which humidity (>95%) and temperature (28±1^{0}C) was maintained. Plants sprayed with sterilized distilled water served as control. Inoculated plants were sprayed twice a day with distilled water to maintain humidity (Plate 1).

Observations were recorded every day for the appearance and development of symptoms. When artificially inoculated leaves expressed symptoms, reisolation of the pathogen was done and compared with the original culture. The experiment was repeated twice.

3.1.4 Hypersensitive reaction on tobacco

The hypersensitive reaction test was carried out to know the pathogenic nature of the eighteen isolates collected from nine regions.

A loopful of the bacterial culture from the stock culture stored at 4^{o}C was streaked onto Petri Plates with sterilized NGA medium. The plates were incubated at 28±1^{0}C for 72 h. Individual bacterial colonies were picked and inoculated into conical flasks containing 100 ml of NG broth and incubated at 28±1^{0}C for 72 h in a rotary shaker incubator at 120 rpm.

The bacterial suspension (4x10^7 cfu) was infiltered into the intercellular spaces of the intact tobacco leaves (*Nicotiana tabacum*) by holding the barrel of the hypodermic syringe against the bottom of the leaf. A water soaked area developed as the suspension infiltrated the mesophyll. The leaves were then washed with sterile distilled water and tagged.

Observations were made for development of chlorotic lesions followed by confluent necrosis in the injected regions after 24 h of incubation.

3.1.5 Morphological variability

Shape and size

Smear of the isolates from the 72 h old cultures grown on NGA was stained with methylene blue and examined under oil immersion objective for shape of the bacterial cells (Salle, 1961). For measuring the size of bacterial cells, the method suggested by Salle (1961) was followed. Fifty cells of each isolate were measured at 1000X (Carl Zeiss Primo Star microscope) and the average size was worked out.

3.1.6 Growth phase

Nutrient glucose broth (NGB) was used to study the growth phase of *Xap*$_{10}$ isolate. As this isolate has less incubation period, it was selected for further study. Five ml of NGB was dispensed in individual test tubes and sterilized at 1.1 kg/cm^2 for 15 min. The inoculum consisted of 96 h old culture; suspended in sterile distilled water containing 4x10^7 cfu/ml used at the rate of 50µl per test tube. Three replications were maintained. The test tubes were incubated at temperature 28±1^{0}C. The growth was recorded turbidometrically at 8 hrs interval upto 168 hrs using Systronics PC based double beam spectrophotometer 2202 at 660 nm. The increase in the turbidity of culture broth indicates increase of microbial cell mass. It was statistically analysed by Completely Randomized Design.

3.2 Physiological variability

3.2.1 Temperature sensitivity

LBA (Luria Bertani's Agar) is also one of the best medium for growth of *Xap* (Mondal and Kumar, 2011). Hence for further studies LBA was used. Fifteen ml of LBA was poured into sterilized

Plant covered with polythene cover

Plant after inoculation

Plate 1: Proving pathogenicity by Koch's postulates

Petri Plates. These plates were inoculated with loopfull of 96 hrs old *Xap* culture and incubated at 28±1^{0}C and 37±1^{0}C temperature to identify the *Xap* which grows well at 28±1^{0}C but absent at 37^{0}C. Three replications were maintained. The growth was recorded by visual observations at 24 h, 48 h, 72 h, 96 h and 120 h at both temperatures.

3.2.2 Effect of pH on the growth of *Xap*

pH of Luria Bertani's broth was adjusted by adding 0.1 N alkali (NaOH) or acid (HCl) to the broth at different pH like 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. Luria Bertani's broth (5.0 ml) was dispensed in individual test tubes and sterilized at 1.1 kg/cm^2 for 15 min. The 96 h old culture; suspended in sterile distilled water containing 4x10^7 cfu/ml was used at the rate of 50µl per test tube. Two replications were maintained. The test tubes were incubated at 28±1^{0}C. The growth of eighteen isolates of *Xap* with three replications were recorded turbidometrically after 96 h of incubation using Systronics PC based double beam spectrophotometer 2202 at 660 nm.

3.2.3 NaCl sensitivity

Luria Bertani's broth was used for the NaCl sensitivity (0-8 per cent) test against eighteen isolates of *Xap*. Five ml of this medium was dispensed in individual test tubes and sterilized at 1.1 kg/cm^2 pressure for 15 min. The inoculum consisted of 96 h old culture; suspended in sterile distilled water containing 4x10^7 cfu/ml used at the rate of 50µl per test tube. The test tubes were incubated at 28±1^{0}C. The growth was recorded turbidometrically after 96 h of incubation using Systronics PC based double beam spectrophotometer 2202 at 660 nm.

3.2.4 Fuscan pigment production

Luria Bertani's agar was used for the production of fuscan pigment by eighteen isolates of *Xap*. Fifteen ml of this medium was poured into sterilized Petri Plates. These plates were streaked with a loopfull of 96 h old *Xap* culture and were incubated at 28±1^{0}C. The growth was recorded by the production of brown colour fuscan pigment in the medium by visual observation.

3.3 Assessment of baseline sensitivity to major bactericides among *Xap* isolates representing major pomegranate growing regions

Infected plant parts showing typical symptoms of bacterial blight of pomegranate were collected from Karnataka, Maharashtra and Andhra Pradesh states in Bhagwa variety based on agroclimatic regions and area under the crop. Such collections from different locations were subjected to the isolation of *Xap* by following the above mentioned method.

A set of five bactericides/ antibiotics were selected to assess the baseline sensitivity of eighteen isolates of *Xap.* 100, 250, 500, 750 and 1000 ppm concentrations pertaining to Bronip, K-cycline, COC, Streptocycline and Plantamycine.

Eighteen isolates of *Xap* were multiplied on NGB. From this 300µl of *Xap* suspension was spread by sterilized spreader on to sterilized Petri Plates containing NGA medium, so as to get a thick lawn of bacterium on the surface of the medium. Sensitivity of eighteen *Xap* isolates to these bactericides were analysed by modified paper disc inhibition zone assay method.

Sterilized filter paper (Whatman No.1) discs (6 mm diameter) were impregnated with required concentrations of five bactericides for 10 minutes. Each set of bactericides impregnated discs were placed on the NGA containing the bacterium in Petri dishes and incubated at 28±1^{o}C for 96 h. Inhibitin zones in cm around the discs were recorded.

Experiment was conducted with three replications. Filter paper discs dipped in sterile distilled water served as check. Data recorded on the inhibition zones was statistically analyzed by three factorial Completely Randomized Design (CRD).

3.4 Ribosomal RNA (16S rRNA) analysis and phylogeography of *Xap* isolates

Genetic diversity of *Xap* from different pomegranate growing areas

Xap isolates were grouped based on morphological, biochemical, bactericide sensitivity and geographical distribution. Based on grouping, the isolates which show differences were subjected for 16S rDNA sequence analysis.

Collection, isolation and storage

Xap isolates were collected from different districts of Karnataka, Maharashtra and Andhra Pradesh. Eighteen strains of *Xap* from distinct geographical origins used for this study were established in pure culture from pomegranate affected samples. Glycerol stocks for each strain were stored in 50 per cent glycerol at -80ºC. The strains were revived from stored culture by streaking on NGA and incubated at 28±1ºC for 96 h.

3.4.1 Isolation of genomic DNA from *Xap* isolates

The extraction of total genomic DNA was carried out following a modified protocol as described by Mondal and Kumar (2011).

Protocol

1. Well separated fluidal colonies of *Xap* isolates were used for genomic DNA extraction after growing them for 96 h at 28±1ºC on NGA.
2. A loop full of *Xap* culture was mixed in 1.0 ml distilled sterilized water in 1.5 ml eppendorf tube and centrifuged at 14,000 rpm (room temperature) for 5 min.
3. The supernatant was discarded and the pellet was washed three times with sterile water.
4. About 550µl of TE buffer was added to the tubes containing pellet and resuspended then added with 76 µl of 10% SDS and proteinase K and incubated at 65ºC for 15 min.
5. Then 100 µl of 5 M NaCl was added and mixed by flipping tubes and 80 µl of CTAB was mixed by flipping and incubated at 65ºC for 10 min.
6. About 700 µl of chloroform: isoamyl alcohol (24:1) was added, mixed gently by inverting the tubes, and centrifuged at 14,000 rpm for 5 min.
7. The upper aqueous phase was transferred to a new tube without disturbing the middle layer.
8. Equal volume of isopropanol was added and inverted several times to mix properly and centrifuged at 10,000 rpm at 4^0C for 15 min.
9. Gently the supernatant was drained off and 1.0 ml of 70 per cent ice cold ethanol was carefully added.
10. DNA was collected by centrifugation for 5 min at 14000 rpm.
11. Supernatant was carefully removed and the remaining ethanol was evaporated in laminar air flow cabinet.
12. DNA was dissolved in 40-50 µl of 10mM Tris (pH 8) and incubated overnight at 4ºC
13. Six µl of RNase was added to DNA solution to remove RNA contamination and incubated for 30 min at 37ºC.
14. DNA was stored at -20ºC for further use.

Agarose gel electrophoresis

Requirements

1. Electrophoretic unit : gel casting tray, gel combs, power pack, UV transilluminator
2. Agarose (0.8%)
3. Bromophenol blue (loading dye)
4. Ethidium bromide
5. The TAE buffer (1X): 20 ml of 50X TAE was added to the 980 ml of distilled water and total volume was made upto 1000 ml.

Composition of 50X TAE buffer

Tris base	: 242 g
Glacial acetic acid	: 57.1 ml
EDTA (0.5 M, pH 8.0)	: 100 ml

Volume was made upto 1000 ml using distilled water.

Procedure

- Gel casting plate and comb was washed with sterile distilled water and wiped with 70 per cent alcohol.
- The two open ends of the gel casting plate were sealed with cellotape and placed on perfectly horizontal leveled platform with comb inside the gel casting plate.
- Agarose (0.8 g) was weighed and added to a conical flask containing 100 ml of 1X TAE buffer.
- The contents were melted by heating in an oven, the solution was stirred for proper mixing and complete dissolution of agarose.
- The agarose gel solution was cooled to about 40 to 45^{0}C and 4.0 µl of ethidium bromide (10 mg/ml) was added.
- Cast agarose gel in a gel casting tray with properly placed comb. While pouring, sufficient care was taken to prevent the formation of air bubbles.
- The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1x TAE), so as to cover the wells completely.
- About 2 µl of loading dye was added to each tube containing amplified DNA.
- The amplified products (20 µl) were carefully loaded into the sample wells. Electrophoresis was carried out at 70 volts for 60min
- The desired bands of 16S rRNA and *gyr*B genes were recovered in a Gel Doc™ system under UV (302 nm).

3.4.2 Molecular characterization of *Xap* isolates by the amplification of 16S rRNA and *gyr*B loci

The 16S rRNA sequences are highly conserved and their variation if any is a function of time component. Based on conserved regions, primer sets have been designed and used successfully (Mondal *et al.,* 2012). The following primer pairs were used to amplify the target 16S rRNA and *gyr*B loci using PCR.

Sl. No.	Primers (5′-3′)	Annealing (^{0}C)	Product size (bp)	Target genes
1	16S rRNA F- AGTGAAGAGTTTGATCCTGGC	60	1537	16S rRNA
	16S rRNA R- AGGAGGTGATCCAGCCGC			
2	*gyr*B F- GTTGATGCTGTTCACCAGCG	55	491	*gyr*B
	*gyr*B R- CATTCATTTCGCCCAAGCCC			

PCR reaction mixture (20 µl)

Reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing the following components. The total volume of each reaction mixture was 20 µl.

1. Template DNA (25 ng/µl) : 1.0 µl
2. 10X assay buffer with 15 mM $MgCl_2$: 2.0 µl
3. dNTPs mix (10 mM) : 1.0 µl
4. Primer Forward (5 pM/µl) : 1.0 µl
5. Primer Reverse (5 pM/µl) : 1.0 µl
6. Sterile distilled water : 13.50 µl
7. *Taq* DNA polymerase (3.0 U/µl) : 0.5 µl

Except template DNA, the master mix was distributed to PCR tubes (19 µl/tube) and later 1 µl of template DNA of each isolate was added separately to each of the master mix tube. Final volume was made upto 20 µl. PCR was carried out in thermal cycler with specifications as described.

Optimum PCR conditions

The PCR amplifications for 16S rRNA and *gyr*B loci analysis was performed according to Mondal and Kumar (2011) with certain modifications. The optimum specifications followed for DNA amplifications were as follows.

Stage	Steps	Temperature (^{0}C)		Duration	Number of cycles
		16S rRNA	*gyr*B		
I	Initial denaturation	94	94	7 min	1
II	Denaturation	94	94	30 sec	30
	Annealing	60	55	1 min	
	Extension	72	72	1 min	
III	Final extension	72	72	3 min	1
	Hold temperature	4	4	30 min	-

3.4.3 Separation of amplified products by agarose gel electrophoresis

Same procedure was followed as mentioned in 3.4.1 except agarose 1.2%.

3.4.4 Sequencing of PCR product

The fragments of the *Xap* 16S rRNA gene of about 1537 bp amplicon were sequenced by using forward primer by commercial sequencing centre, Bangalore Genei (India) Pvt. Ltd., Bangalore using the ABI 3130 XL sequencer.

Sequence analysis

The sequences obtained using forward and reverse primers were assembled using Vector NTI software. The sequences were subjected to BLAST analysis at http://www.ncbi.nlm.nih.gov. Also, the DNA sequences were aligned using MEGA 5.1 for further comparisons.

Phylogenetic analysis

Dendrogram was constructed using unweighted pair-group arithmetic average (UPGMA) method available in MEGA 5.1 software for the nucleotide sequences of *Xap* 16S rRNA region of all isolates. Published sequence of *Xap* 16S rRNA gene (JN109175.1) was downloaded from NCBI and used in the phylogenetic analysis. *Pantoea aglummerans* (FJ611839) nucleotide sequence was used as out group in the construction of dendrogram.

3.4.5 Multiplex PCR assay

Six isolates of *Xap* were randomly selected and colonies were taken from 4 days old cultures and suspended in 100µl of sterile distilled water. Primers developed for the 16S rRNA and *gyr*B genes were combined for the development of a multiplex PCR protocol. PCR reactions were performed in thermocycler.

PCR reaction mixtures contained

1. Template DNA : 3.0 µl
2. 10X assay buffer with 15 mM $MgCl_2$: 2.0 µl
3. dNTPs mix (10 mM) : 1.0 µl
4. Primer F(5 pM/µl) : 1.0 µl
5. Primer R(5 pM/µl) : 1.0 µl
6. Sterile distilled water : 11.5 µl
7. *Taq* DNA polymerase (3.0 U/µl) : 0.5µl

Thermocycler conditions for primer concentration tests were 30 cycles of denaturing at 94ºC for 30 sec.

Optimization of multiplex PCR for parallel detection of 16S rRNA and *gyr*B

Garlic (clove)

Neem (leaf)

Tulsi(leaf)

Miswak (stem)

Soapnut (fruit)

Adathoda (leaf)

Nerium (leaf)

Plate 2. Plant parts (botanicals)

Gradient PCR: Annealing temperature gradients were determined to optimize annealing of primer pairs of 16S rRNA *XAP*F-16S rRNA *XAP*R and *gyr*BF- *gyr*BR in thermocycler gradient PCR. Temperatures from 56 to 59ºC with 1ºC increments; 56, 56.9, 57.9 and 59ºC, were tested for annealing temperature for multiplex PCR.

The multiplex PCR was optimized by using the gradient function of Eppendorf gradient thermocycler. Different annealing temperatures of 56.0ºC, 56.9ºC, 58.1ºC and 59.0ºC was set and PCR was carried out. After the completion of the reaction, the products were kept at 4ºC. (Figueiredo and Torres, 2007). The amplified fragments were electrophorized in 1.0 per cent agarose gel. A medium range marker was included on gel.

3.4.6 Colony PCR

Single colony of *Xap* was picked up with a sterile pipette tip and added directly to the PCR mixture as a template for DNA amplification. Colony PCR mixture contained

1. 10X assay buffer with 15 mM $MgCl_2$: 2.0 µl
2. dNTPs mix (10 mM) : 1.0 µl
3. Primer F(5 pM/µl) : 1.0 µl
4. Primer R(5 pM/µl) : 1.0 µl
5. Sterile distilled water : 11.5 µl
6. *Taq* DNA polymerase (3.0 U/µl) : 0.5µl

A small loopful of culture was added to 25µl aliquots by scratching 2-3 mm across the surface of a colony using a disposable tip on a 20µl pipettor, then mixed twice while gently swirling the tip in the mixture. The PCR tubes were given small spin, and were placed in a PCR thermocycler. The PCR was performed in a JH Bio thermocycler. The procedure is as follows: initial denaturation at 95°C for 5min; 30 cycles of denaturation for 30s at 92°C, annealing for 30s at 60°C, and extensión for 45s at 72°C; the final extensión at 72°C for 10 min, followed by cooling to 4°C until the sample was recovered. Amplicons were visualized with an UV transilluminator after resolving 5µl of the products in 1% agarose gel and staining with ethidium bromide. The primers used in this study were *gyr*B. The sequences of forward and reverse primers used were, respectively as follows

*gyr*BF 5'- GTTGATGCTGTTCACCAGCG 3'

*gyr*BR 5'- CATTCATTTCGCCCAAGCCC 3'

3.5 Prospecting the botanicals and selected nutrients for *in vitro* and *in vivo* inhibition of *Xap*

3.5.1 *In vitro* evaluation of botanicals on the growth of *Xap* 10 isolate

Extracts from certain plants are known to have antibacterial properties. Recently prospecting botanicals in pest and diseases management has been successful. Plant based pesticides, which are relatively safe, economical and non-hazardous can be used successfully for the management of bacterial diseases in crop plants. The present investigation was aimed at screening plant extracts for their antibacterial properties against *X. a.* pv. *punicae*. The following plant extracts were selected:

Common name	Botanical name	Plant parts used
Garlic	*Allium sativum* (L.)	Clove
Soapnut	*Sapindus mukorossi* (L.)	Fruit
Miswak	*Salvadora persica* (L.)	Stem
Adahtoda	*Adahtoda vessica* (L.)	Leaf
Tulsi	*Ocimum sanctum* (L.)	Leaf
Nerium	*Nerium oleander* (L.)	Leaf
Neem	*Azadirachta indica* (L.)	Leaf
Adahtoda + Soapnut	*Adahtoda vessica* (L.) + *Sapindus mukorossi* (L.)	Leaf + Fruit
Adahtoda + Miswak	*Adahtoda vessica* (L.) + *Salvadora persica* (L.)	Leaf + Stem
Miswak + Soapnut	*Salvadora persica* (L.) + *Sapindus mukorossi* (L.)	Stem + Fruit

Plant parts were collected and washed in distilled water, 100 grams of fresh sample was chopped and macerated in a sterilized pestle and mortar by adding 100 ml of distilled steriled water (1:1 w/v). The extract was filtered through Watman No. 1 filter paper; filtrate thus obtained was used as a stock solution. To study the antibacterial mechanism of plant extracts, inhibition zone assay method was followed (Plate 2).

A loopfull (72 h old) of *Xap*10, multiplied on NGA (15ml) in Petri Plates was mixed with 5 ml distilled sterilized water. Solution of 300 µl was spread to NGA. Sterilized filter paper discs (Whatman No. 1) measuring 5 mm diameter were soaked for 10 minutes in 5 per cent and 10 per cent of above mentioned plant extracts and placed on the surface of the NGA medium which was seeded with *X. a.* pv. *punicae*. The sterilized filter paper discs were dipped in sterilized water served as control. The inoculated plates were incubated at 28±1^{0}C for 96 h.

Observations were recorded by measuring the diameter of the inhibition zone around the filter paper disc for each plant extract at 5 and 10 per cent concentrations in millimeter and analysed statistically. The experiment was repeated twice.

3.5.2 *In vitro* evaluation of micro and secondary nutrients on the growth of *Xap*10

Effect of nutrients for their ability to inhibit the growth and development of *X. a.* pv. *punicae* were tested. Nutrients were diluted at four concentrations and inhibition zone assay was done by following the above mentioned method.

Following are the nutrients (secondary and micro) at four concentrations (0.05, 0.1, 0.25 and 0.5 per cent) were tested *in vitro.*

Nutrients (Micro and Secondary)	Source
Zinc	$ZnSO_4$
Copper	$CuSO_4$
Manganese	$MnSO_4$
Magnesium	$MgSO_4$
Calcium	$CaSO_4$
Boron	Sodium tetra borate
Sulfur	Wettable Sulfur
Iron	$FeSO_4$
Multinutrients	Micron special (boron, $ZnSO_4$ and $FeSO_4$)
Untreated control	-

Dilutions of different nutrient sources were made in sterile distilled water. A loopfull (72 h old) of *Xap*10, multiplied on NGA (15ml) was mixed with 5ml sterile distilled water. 300µl (5x10^7 cfu/ml) of this solution was spread to NGA. Sterilized filter paper discs (Whatman No. 1) measuring 5 mm diameter were soaked for 10 minutes in 0.05 per cent, 0.1 per cent, 0.25 per cent and 0.5 per cent of above said nutrients and placed on the surface of the NGA medium which was seeded with *X. a.* pv. *punicae*. The Sterilized filter paper discs dipped in sterile distilled water served as control. Four replications were maintained. The inoculated plates were incubated at 28±1^{0}C for 96 h.

Observations were recorded by measuring the diameter of the inhibition zone around the filter paper disc in millimeter and data obtained was analysed statistically. The experiment was repeated twice.

3.5.3 Antibacterial activity of bio-agents for the management of bacterial blight of pomegranate *in vitro*

Five *Pseudomonas fluorescens* strains and eleven Pink Pigmented Facultative Methylotrophs (PPFM) strains obtained from Department of Biotechnology (IABT) and Department of Microbiology, UAS, Dharwad respectively were tested for their antagonistic activity against *Xap*10 as evidenced by inhibition zone assay method. *P. fluorescens* strains were grown and maintained on King's B broth and PPFM strains on AMS (Ammonium mineral salt) broth. Good growth of *P. fluorescens* and PPFM strains were observed on NGA medium.

Strains of *P. fluorescens* : 134, PF-5, 326(4), PF-1, 139.

Strains of Pink Pigmented Facultative Methylotrophs (PPFM) : 20, 71, 32, 10L, 75, 42, 23, 80, 26, 38, H.

Seventy two h old culture of *Xap*10 was grown on NGA (15ml) in Petri Plates was scraped and mixed with 5 ml sterile distilled water. 300µl ($5x10^7$ cfu/ml) of this solution was seeded to NGA. 5 mm dia sterilized filter paper disc (Whatman No. 1) was soaked for 10 minutes in the suspension of *P. fluorescens* and PPFM (a loopful of culture was mixed in 1ml of sterile water). Sterilized filter paper discs soaked in sterile water served as control. Such discs were placed on the surface of the NGA medium seeded with *X. a.* pv. *punicae*. Five replications were maintained. The inoculated plates were incubated at 28 ± 1^0C for 72 h.

The effect of antagonistic bacteria in inhibiting the growth of *X. a.* pv. *punicae* was detected by observing the zone of inhibition around the filter paper disc in each antagonistic bacteria in millimeter and analysed statistically. The experiment was repeated twice.

3.5.4 *In vitro* evaluation of effective botanical, bioagents, nutrients and commercial formulation (5%) of copper either singly or in combination against *Xap*10.

Treatment	Concentration (%)
$CuSO_4$	0.25
Commercial formulation (5%) of copper	0.3
$ZnSO_4$	0.5
P. fluorescens strain 326 (4)	Loopfull in 500 µl sterilized water
PPFM strain 10	Loopfull in 500 µl sterilized water
Soapnut fruit extract	5
$CuSO_4$ + Soapnut fruit extract	0.3 +5
$ZnSO_4$ + Soapnut fruit extract	0.5 +5
Commercial formulation (5%) of copper + Soapnut fruit extract	0.3 +5
P. fluorescens strain 3 + PPFM strain 10L	Loopfull of both cultures in 500µl sterile water
P. fluorescens strain 3 + Soapnut fruit extract	Loopfull of culture in 500µl sterile water + 5
Control	Sterile water

The effective botanical, bioagent, nutrient and commercial formulation (5%) of copper with maximum inhibition zone were combined and tested under *in vitro* condition.

The *Xap*10 was multiplied for 72 h on NGA (15ml) in Petri Plates and was mixed with 5ml sterile distilled water and 300µl of this solution was seeded to NGA. Sterilized filter paper discs (Whatman No. 1) measuring 5 mm diameter were soaked in the solutions of botanical, bioagents, nutrients and commercial formulation (5%) of copper for 10 minutes Sterilized filter paper discs soaked in sterile water served as control. Such discs were placed on the surface of the NGA medium seeded with *Xap*10 contained in the Petri Plates. Three replications were maintained for each concentration. The inoculated plates were incubated at 28 ± 1^0C for 72 h.

The combined effect of botanical and bioagent, nutrient and botanical, commercial formulation (5%) of copper and botanical in inhibiting the growth of *X. a.* pv. *punicae* was detected by observing the inhibition zone around the filter paper disc in each treatment and statistically analysed.

3.6 Field evaluation of selected botanicals, microbial antagonist and nutrient for the management of bacterial blight of pomegranate (var. Bhagwa)

Two field trials were conducted during Mrig bahar (June – August) of 2012-2013 in naturally infected farmer's field at Talkal in Yelaburga Taluk (Koppal District) and Muddaballi of Koppal Taluk (Koppal District) (Plate 3). To study the effect of different combinations of botanical, bioagents, micro and secondary nutrients effective under *in vitro* conditions were evaluated under the field condition. The trials were laid out in Randomized Complete Block Design (RCBD) with four replications comprising of four plants per replication. Variety used for management studies was Bhagwa. Nine sprays were given at 15 days interval starting from August and observations on per cent incidence and disease severity on leaf, twig and fruits were recorded before and after each spray. The details of treatments are furnished below.

Treatment	Details	Concentration (%)
T_1	$CuSO_4$	0.1
T_2	Soapnut	5
T_3	Commercial formulation (5%) of copper	0.3
T_4	*Pseudomonas fluorescens* (strain 326 (4))	1
T_5	*P. fluorescens* (strain 326 (4)) + soapnut	1+5
T_6	*P. fluorescens* (strain 326 (4)) + PPFM (strain 10)	1+1
T_7	*P. fluorescens* (strain 326(4)) → soapnut→ Commercial formulation (5%) of copper→ *P. fluorescens* (strain 326(4)) → COC+Streptocycline sulphate → $CuSO_4$→ *P. fluorescens* (strain 326 (4)) → Commercial formulation (5%) of copper→ soapnut	1→5→0.3→1→0.3 +0.05→0.1→1→0. 3→ 5
T_8	Soapnut→ Commercial formulation (5%) of copper→ *P. fluorescens* (strain 326(4)) → soapnut→ $CuSO_4$→ COC+Streptocycline sulphate → Commercial formulation (5%) of copper→ soapnut→ *P. fluorescens* (strain 326 (4))	5→0.3→1→0.1→0 .3+0.05→0.3→5→ 1
T_9	COC+Streptocycline sulphate	0.3+0.05
T_{10}	Control	-

..→indicates followed by

Details of fields

Details	Location 1	Location 2
Name of the farmer	Mallikarjun Gadagi	Manjunath Reddy
Village,Taluk, District	Talkal, Yelburga, Koppal	Muddaballi, Koppal, Koppal
Variety	Bhagwa	Bhagwa
Age of the orchard	9 years	7 years
Soil type	Red Sandy	Red Sandy
Spacing	15x10 ft	15x10 ft
Total no. of plants/ ha	726	726
No. of plants per treatment	4	4

All other practices such as irrigation, fertigation, insect pest and fungal disease control strategies were similar for both the orchards for different treatments as per the recommendations.

Plate 3. Field view of experimental plot

Observations on incidence and severity on stem, leaf, fruits were recorded before and after the spray using 0 – 6 scale and per cent disease incidence and per cent disease index was worked out.

Severity of disease was recorded by using 0-5 and 0-6 scale on leaf and fruit, respectively as per guidelines given by NRC Pomegranate, Solapur

Grade	Per cent infection	
	Leaf	Fruit and Stem
0	0.00	0.00
1	Upto 1	Upto 1
2	>1-10	>1-10
3	>10-20	>10-20
4	>20-40	>20-40
5	>40-100	>40-70
6	-	>70-100

Per cent disease incidence and per cent disease index of bacterial blight on leaves, stem and fruit was calculated by applying the formula (Wheeler, 1969) given below.

$$\text{Per cent disease incidence on leaves/fruits/twigs} = \frac{\text{Number of infected leaves/fruits/twigs}}{\text{Total leaves/fruits/twigs observed in a set}} \times 100$$

$$\text{Per cent disease index on leaves/fruits/twigs} = \frac{\text{Number of infected leaves/fruits/twigs x disease grade}}{\text{Total leaves/fruits/twigs observed in a set x Max. grade}} \times 100$$

Per cent reduction to control

$$\text{Disease reduction (\%)} = \frac{\text{PDI in control - PDI in treatment}}{\text{PDI in control}} \times 100$$

Where

PDI - per cent disease index.

The yield contributing parameters like fruit number and average fruit weight was recorded from all the treatments and data was analyzed statistically (Sukhatme and Amble, 1985).

EXPERIMENTAL RESULTS

The results of the study undertaken on bacterial blight of pomegranate caused by *Xap* during 2010-11 and 2011-12 are presented as under.

Collection of bacterial blight affected pomegranate plant parts from different agroclimatic zones of Northern Karnataka and other parts of Maharashtra and Andhra Pradesh

The various parts of pomegranate such as leaves, twigs and fruits showing symptoms of bacterial blight were collected from the affected pomegranate gardens in different agro-climatic zones of Northern Karnataka, Maharashtra, Andhra Pradesh states and the isolates were designated as mentioned under materials and methods.

4.1 Symptomatology

Symptoms on leaves

Initially the symptoms appeared as minute water soaked lesions which later turned brown to dark brown surrounded by diffused water soaked margin or yellow halo. Several such spots were seen on single leaf, which increased in size, coalesced to form irregular lesions. Severely infected leaves became yellow, distorted and later defoliated (Plate 4).

Symptoms on twigs and branches

Long and narrow light brown coloured lesions could be seen on the branches and also on the main stems with the advancement of disease, the lesions become rough, cankerous and appeared blackish with the advancement of the disease the bark cracked, dried and died (Plate 5).

Symptoms on fruits

The symptoms first appeared on fruits in the form of small diffused oily lesions; later on they turned brown surrounded by diffused water soaked zones. The lesions which are round in the beginning increased in size became irregular, brown to dark brown. The pericarp of affected fruits cracked in the middle of the lesion in the form of L, Y or star shape. Later on affected fruits split open longitudinally through the lesion exposing arils. The bacterial cells oozed out and dried as thin flakes on the surface of fruits and twigs must have been broken into small flakes and dried in the form of very thin white flakes. The affected plant appears weak, sickly and unthrifty, with several dried and bare branches. Severely infected plants dried (Plate 6).

4.1.1 Isolation, purification, preservation of the causal organism from the diseased pomegranate plant parts and proving pathogenicity

Isolation

Isolation of causal organism was made from the infected parts of pomegranate plant *viz.,* leaves, twigs and fruits on nutrient agar medium. The cultural characters of the bacterial colonies appeared light yellow, raised, convex and glistening after 72 h of incubation.

4.1.2 Purification and preservation of the bacterium isolated from infected plants

The isolated colonies which appeared as pale yellow to dark yellow after 72 h incubation at 28±1^{0}C were picked and purified by single colony isolation technique on NGA medium and suspended in polypropylene tubes containing sterile distilled water and preserved at 4^{o}C. Further, eighteen purified *Xap* isolates were stored at -80 ^{o}C using 50 per cent glycerol as cryoprotectant. These served as stock culture for further molecular studies.

4.1.3 Pathogenicity

The pathogenicity of all eighteen *Xap* isolates were confirmed by spraying the 96 hrs old cell suspension on to the susceptible pomegranate variety Bhagwa as described in Table 2 a, b. and Plate 7.

The characteristic symptoms on leaves were observed after six days to nineteen days of incubation period. Seven isolates *viz.*, *Xap*4, *Xap*7, *Xap*8, *Xap*10, *Xap*12, *Xap*14, *Xap*17 required incubation period upto seven days to appear symptoms on leaves. *Xap*1, *Xap*2, *Xap*3, *Xap*5, *Xap*6,

Initial water soaked lesions

Extened water soaked lesions

Brown to black lesions on leaves

Leaf spot leading to blight

Plate 4: Symptoms of bacterial blight on leaves of pomegranate (var. Bhagva)

Lesions on stem

Stem canker

Stem cracking

Plate 5: Symptoms of bacterial blight on stem of pomegranate

Table 2a: Incubation period of eighteen isolates of Xanthomonas axonopodis pv. punicae

Isolate	Place	Incubation period (Days)
*Xap*1	Matrangi	12
*Xap*2	Kanamadi	15
*Xap*3	Kannur	15
*Xap*4	Kaladagi	9
*Xap*5	Chikkasamshi	10
*Xap*6	Ankalgi	15
*Xap*7	Hiraj	9
*Xap*8	Jumnal	9
*Xap*9	Malegaon	13
*Xap*10	Solapur	9
*Xap*11	Anantpur	10
*Xap*12	Mandarna	8
*Xap*13	Govinakoppa	19
*Xap*14	Lakmapura	7
*Xap*15	Mehaboobnagar	15
*Xap*16	Kerur	15
*Xap*17	Laxmipur	6
*Xap*18	Koppal	11

Table 2b: Grouping based on incubation period

Incubation period (days)	Isolate	Total
Upto 9	*Xap*4, *Xap*7, *Xap*8, *Xap*10, *Xap*12, *Xap*14, *Xap*17	07
10-15	*Xap*1, *Xap*2, *Xap*3, *Xap*5, *Xap*6, *Xap*9, *Xap*11, *Xap*15, *Xap*16, *Xap*18	10
>15	*Xap*13	01

Lesions at later stage

Oily spot on fruit

Splitting of fruit

Fruit craking

Plate 6. Symptoms of bacterial blight on fruit of pomegranate

10 days

14 days

16 days

19 days

22 days

27 days

Plate 7: Stages of leaf infection by Xap 11 on var. Bhagwa

*Xap*9, *Xap*11, *Xap*15, *Xap*16 and *Xap*18 required ten to fifteen days incubation period and *Xap*13 required more than fifteen days for start of appearance of the symptoms on leaves as small, water soaked at earlier period, brown to black coloured lesions, which later on developed into angular to irregular shaped spots along the veins and veinlets of the leaf lamina leading to marginal necrosis. Reisolations were carried out from these lesions for each isolate and comparisons were made with the original culture to confirm the identity of the pathogen. The isolates from artificially inoculated plants yielded the bacterial colonies similar to the original ones.

4.1.4 Hypersensitive reaction on tobacco

All the 18 isolates of bacterium injected into the intercellular spaces of *Nicotiana tobacum* leaves produced characteristic water soaked lesions after 48 h of inoculation and produced desiccated confluent brown necrotic area in 90 h of inoculation, indicated that pathogenicity was also proved on tobacco leaves (Plate 8).

4.1.5 Morphological character

The colony characters of eighteen *Xap* isolates observed are presented in Table 3 and Plate 9. All the isolates showed entire circular colonies. Sparse mucoidness character was exhibited by *Xap*3, *Xap*6, *Xap*8, *Xap*11, *Xap*12, *Xap*13, *Xap*15, *Xap*17 and *Xap*18, where as rest other showed thick mucoid character. Colony colour was light yellow in *Xap*3, *Xap*6, *Xap*11, *Xap*12, *Xap*15, *Xap*17 and *Xap*18, dark yellow in *Xap*16, rest other isolates had medium yellow colour. All isolates showed convex colony. Highly raised, glittering colony was observed in *Xap*1, *Xap*4, *Xap*5, *Xap*8, *Xap*10, *Xap*16 and *Xap*18, rest other exhibited slightly raised, glittering colony except *Xap*12, which was flat and opaque.

Bacterium is rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative, capsulated, non-spore forming, having single polar flagellum and measured 0.25 – 0.4 $\times$ 1.25 – 3.00 μm in size. The cells readily stained with common stains such as crystal violet.

4.1.6 Growth phase study of *Xap*.

There was a gradual increase in the growth of bacterium (*Xap*10) at different interval (Table 4). The growth started after 40 hrs *i.e.*, 0.108 OD. Maximum growth was observed at 128 and 136 hrs after inoculation with OD value 0.274 *i.e.*, stationary phase, after 136 hrs there was gradual decrease in the growth *i.e.*, decline phase. The dynamics of the bacterial growth was studied by plotting the cell growth (absorbance) versus the incubation time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve of the bacterium.

4.2 Physiological variability

4.2.1 Temperature sensitivity

Temperature influences the rate of growth, metabolism and morphological characters of the bacterium. The bacterium can thrive under wide range of temperature and there is minimum, optimum and maximum for each species and for a particular strain.

All the sixteen *Xap* isolates were tested at temperatures 28^0C and 37^0C on LBA (Table 5). Excellent growth was observed at 28 ± 1^0C with all the *Xap* isolates, however the isolates failed to grow at 37 ± 1^0C. Growth of *Xap* was started 72 hrs after inoculation. Maximum growth was observed after 120 hrs of inoculation at 28 ± 1^0C. At 37 ± 1^0C, no growth was observed upto 120 hrs in all isolates.

4.2.2 Effect of different pH levels

The hydrogen ion concentration or pH of the medium influences the growth of bacterium. Every organism has its own minimum, maximum and optimum pH regimes for growth. In order to know the optimum pH requirement for each of the isolate the experiment was conducted and data are presented in Table 6.

Growth of all isolates at pH 7 was significantly superior to (mean OD value 0.320) other pH levels, followed by pH 6 (mean OD value 0.273) while growth at pH 8 was minimum (mean OD value 0.051).

Among the isolates, *Xap*14 and *Xap*10 produced maximum mean OD value (0.241 and 0.237) which were on par to each other and significantly superior to all other isolates followed by *Xap*12 (0.216).

Table 3: Colony characteristics of different isolates of *Xap* (after 10 days of inoculation)

Isolate Code	Mucoidness	Colour (Yellow shade)	Appearance of the colony (Convex)
*Xap*1	Highly mucoid	Medium	Hhighly raised, glittering
*Xap*2	Highly mucoid	Medium	Slightly raised, glittering
*Xap*3	Slightly mucoid	Light	Slightly raised, glittering
*Xap*4	Highly mucoid	Medium	Highly raised, glittering
*Xap*5	Highly mucoid	Medium	Highly raised, glittering
*Xap*6	Slightly mucoid	Light	Slightly raised, glittering
*Xap*7	Highly mucoid	Medium	Slightly raised, glittering
*Xap*8	Slightly mucoid	Medium	Highly raised, glittering
*Xap*9	Highly mucoid	Medium	Slightly raised, glittering
*Xap*10	Highly mucoid	Medium	Highly raised, glittering
*Xap*11	Slightly mucoid	Light	Slightly raised, glittering
*Xap*12	Slightly mucoid	Light	Flat, opaque
*Xap*13	Slightly mucoid	Medium	Slightly raised, glittering
*Xap*14	Highly mucoid	Medium	Slightly raised, glittering
*Xap*15	Slightly mucoid	Light	Slightly raised, glittering
*Xap*16	Highly mucoid	Dark	Highly raised, glittering
*Xap*17	Slightly mucoid	Light	Slightly raised, glittering
*Xap*18	Slightly mucoid	Light	Highly raised, glittering

Bacterial inffltration in to intercellular spaces

Yellow zone

Necrotic lesion

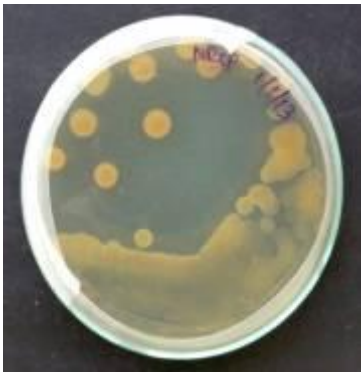
Control

Plate 8: Hypersensitivity test on tobacco leaf

Lower View

Xap9

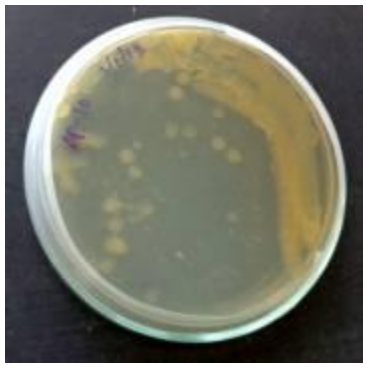
Xap8



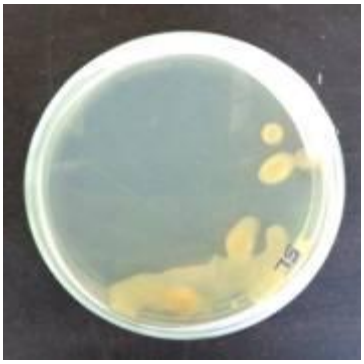
Xap10

Xap11

Xap14



Xap15



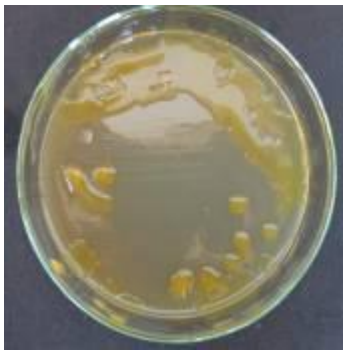
Xap16

Xap17

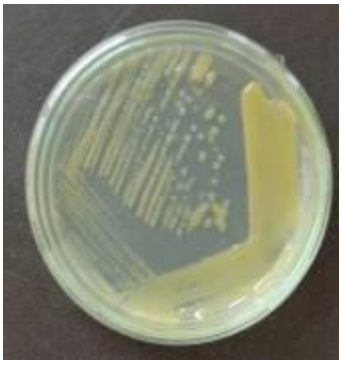
Plate 9 : Cultural variability of isolates of Xanthomonas axonopodis pv.punicae

Plate 9 contd….. **Upper View**

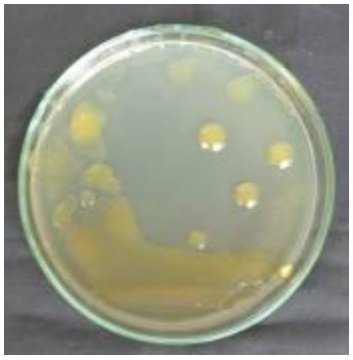
Xap 3



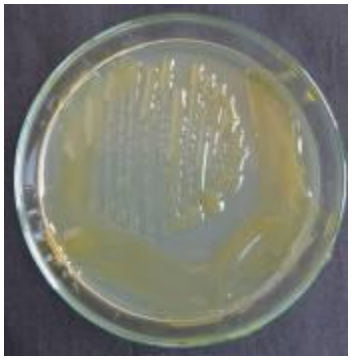
Xap17



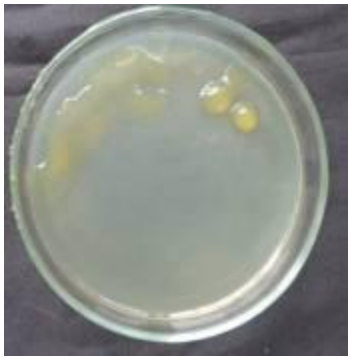
Xap 18



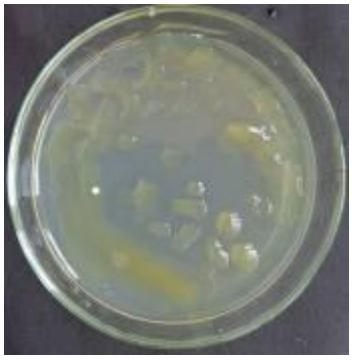
Xap13



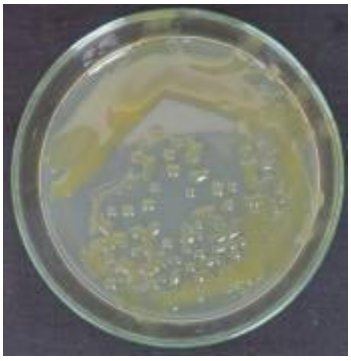
Xap 12



Xap 7



Xap 6



Xap 4

Table 4: *In vitro* growth phase study of *Xap*10

Hours after inoculation	OD (absorbance) at 660 nm
8	0.063
24	0.066
32	0.085
40	0.108
48	0.126
56	0.153
64	0.180
72	0.193
80	0.227
88	0.239
96	0.246
104	0.256
112	0.268
120	0.273
128	0.274
136	0.274
144	0.266
152	0.258
160	0.243
168	0.230
Mean	**0.201**
S.Em **±**	0.006
CD	0.025

Table 5: Growth rate of eighteen isolates of *Xap* at 28ºC and 37ºC

Isolate	Growth rate temperature							
	28ºC				37ºC			
	48 hrs	72 hrs	96 hrs	120 hrs	48 hrs	72 hrs	96hrs	120 hrs
*Xap*1	-	+	+ +	+ + +	-	-	-	-
*Xap*2	-	+	+ +	+ + +	-	-	-	-
*Xap*3	-	+	+ +	+ + +	-	-	-	-
*Xap*4	-	+	+ +	+ + +	-	-	-	-
*Xap*5	-	+	+ +	+ + +	-	-	-	-
*Xap*6	-	+	+ +	+ +	-	-	-	-
*Xap*7	-	+	+ +	+ + +	-	-	-	-
*Xap*8	-	+	+ +	+ + +	-	-	-	-
*Xap*9	-	+	+ +	+ + +	-	-	-	-
*Xap*10	-	+ +	+ + +	+ + +	-	-	-	-
*Xap*11	-	+	+ + +	+ + +	-	-	-	-
*Xap*12	-	+	+ +	+ +	-	-	-	-
*Xap*13	-	+	+ +	+ + +	-	-	-	-
*Xap*14	-	+	+ + +	+ + +	-	-	-	-
*Xap*15	-	+	+ + +	+ + +	-	-	-	-
*Xap*16	-	+	+ +	+ +	-	-	-	-
*Xap*17	-	+ +	+ + +	+ + +	-	-	-	-
*Xap*18	-	+ +	+ + +	+ + +	-	-	-	-

Colony growth : ‘ – ’ no growth, ‘ + ’ less growth (2 mm diameter), ‘ + + ’ moderate growth (2.1-4.0 mm diameter), ‘ + + + ’ maximum growth (>4 mm diameter)

Table 6: Effect of different pH on growth of eighteen isolates of Xap

Isolate	Growth (O. D. value) at 660 nm				
	pH				
	5	6	7	8	Mean
*Xap*1	0.000 (1.000)	0.293 (1.137)	0.261 (1.123)	0.000 (1.000)	0.124 (1.058)
*Xap*2	0.000 (1.000)	0.288 (1.135)	0.319 (1.148)	0.133 (1.064)	0.166 (1.078)
*Xap*3	0.000 (1.000)	0.173 (1.083)	0.195 (1.093)	0.000 (1.000)	0.082 (1.039)
*Xap*4	0.000 (1.000)	0.199 (1.095)	0.381 (1.175)	0.000 (1.000)	0.130 (1.060)
*Xap*5	0.000 (1.000)	0.213 (1.101)	0.243 (1.115)	0.000 (1.000)	0.102 (1.048)
*Xap*6	0.000 (1.000)	0.200 (1.095)	0.249 (1.118)	0.000 (1.000)	0.101 (1.048)
*Xap*7	0.000 (1.000)	0.201 (1.096)	0.252 (1.119)	0.000 (1.000)	0.102 (1.048)
*Xap*8	0.000 (1.000)	0.309 (1.144)	0.391 (1.179)	0.000 (1.000)	0.157 (1.072)
*Xap*9	0.000 (1.000)	0.228 (1.108)	0.258 (1.121)	0.000 (1.000)	0.109 (1.051)
*Xap*10	0.000 (1.000)	0.401 (1.183)	0.469 (1.212)	0.184 (1.088)	0.237 (1.108)
*Xap*11	0.000 (1.000)	0.23 (1.110)	0.315 (1.147)	0.000 (1.000)	0.136 (1.064)
*Xap*12	0.000 (1.000)	0.339 (1.157)	0.391 (1.179)	0.134 (1.065)	0.216 (1.100)
*Xap*13	0.000 (1.000)	0.319 (1.148)	0.286 (1.134)	0.000 (1.000)	0.151 (1.071)

Contd….

Isolate	Growth (O. D. value) at 660 nm				
	pH				
	5	**6**	**7**	**8**	**Mean**
*Xap*14	0.000 (1.000)	0.381 (1.175)	0.416 (1.190)	0.168 (1.081)	0.241 (1.111)
*Xap*15	0.000 (1.000)	0.291 (1.136)	0.356 (1.164)	0.000 (1.000)	0.162 (1.075)
*Xap*16	0.000 (1.000)	0.323 (1.150)	0.278 (1.130)	0.000 (1.000)	0.150 (1.070)
*Xap*17	0.000 (1.000)	0.318 (1.148)	0.323 (1.150)	0.174 (1.083)	0.203 (1.095)
*Xap*18	0.000 (1.000)	0.206 (1.098)	0.385 (1.177)	0.123 (1.059)	0.178 (1.083)
Mean	**0.000 (1.000)**	**0.273 (1.128)**	**0.320 (1.149)**	**0.051 (1.024)**	0.161 (1.075)
Source	Isolates	pH	Isolates x pH		
S.Em **±**	0.001	0.001	0.002		
CD @ 1%	0.004	0.002	0.009		

* $\sqrt{x + 1}$ transformed values

In general pH 6 and pH 7 were preferred by all isolates. It could be clearly observed that there was a wide variation in the growth among different isolates at pH levels.

4.2.3 Nacl sensitivity

Growth of all isolates at zero per cent NaCl concentration was significantly superior to (mean OD value 0.354) other treatments which was followed by one per cent (mean OD value 0.223) while growth at 3 and 4 per cent was zero (Table 7 and Plate 10).

Among the isolates, *Xap*2 and *Xap*10 produced maximum OD value 0.231 and 0.230 which were on par with each other and was significantly superior to all other isolates followed by *Xap*14 (0.206).

In general zero per cent NaCl concentration was found to be preferred by all isolates. It could be clearly observed that there is a wide variation in the growth among different isolates at different NaCl concentrations.

4.2.4 Fuscan pigment production on LBA without NaCl

Fuscan pigment production is the characteristic feature of *Xap* which was observed after five especially beneath the lawn of bacterium in the medium or in the broth. All eighteen isolates produced fuscan pigment on LB agar after nine days of inoculation (Table 8 and Plate 11). Only three isolates *viz.,* *Xap*10, *Xap*11, *Xap*15 produced fuscan pigment 5 days after inoculation, three isolates *viz*., *Xap*8, *Xap*17, *Xap*18 produced fuscan after 6 days of inoculation, three isolates *viz*., *Xap*2, *Xap*3, *Xap*14 after 7 days of inoculation, five isolates *viz*., *Xap*1, *Xap*4, *Xap*9, *Xap*12, *Xap*13 after 8 days of inoculation.

4.3 Assessment of baseline sensitivity of *Xap* isolates against major bactericides

Five bactericides were tested at 100, 250, 500, 750 and 1000 ppm concentrations against eighteen isolates of *Xap*. The inhibition was calculated after six days of incubation period. Among the isolates total mean of maximum inhibition zone was found in *Xap*14 (1.64 cm) which is followed by *Xap*2 (1.52 cm) and *Xap*12 (1.45 cm). Least inhibition zone was found in *Xap*17 (1.02 cm).

Among the bactericides, K-Cycline was effective against all isolates of *Xap*, which is significantly superior to rest of the bactericides and is followed by streptocycline and plantomycine. Least inhibition was found in bronip (2-Bromo-2-nitro-propane-1, 3 diol).

Between the concentrations of each bactericide, efficacy was significant from lower to higher concentration with greater efficacy at higher concentrations.

Among the different concentrations of streptocycline at 1000 ppm maximum inhibition zone was recorded against *Xap*14 (3.33 cm) followed by 3.27 cm at 750 ppm. *Xap*1 and *Xap*2 recorded an inhibition zone of 3.13 cm at 1000 ppm and 3.00 cm at 750 ppm. Least inhibition was found at 100 ppm in *Xap*6 and *Xap*13 isolates with the inhibition zone of 1.30 cm (Table 9a and Plate 12).

Plantomycine at 1000 ppm has given maximum inhibition zone of 1.93 cm against *Xap*4 isolate. It is followed by 1.80 cm at 1000 ppm against *Xap*2 and 1.67 cm at 1000 ppm against *Xap*14. Least inhibition was observed at 100 ppm with the inhibition zone of 0.60 cm against *Xap*9. Zero inhibition was observed in *Xap*1. *Xap*4, *Xap*5, *Xap*6, *Xap*7, *Xap*8, *Xap*10, *Xap*11, *Xap*13, *Xap*15, *Xap*16, *Xap*17, *Xap*18 at 100 ppm, *Xap*1, *Xap*4, *Xap*5, *Xap*6, *Xap*8, *Xap*11, *Xap*13, *Xap*15, *Xap*16, *Xap*17, *Xap*18 at 250 ppm, *Xap* 5, *Xap*8, *Xap*11, *Xap*16, *Xap*17, *Xap*18 at 500 ppm and *Xap*17 at 750 ppm concentration (Table 9b).

K-Cycline at 1000 ppm has given maximum inhibition zone of 3.37 cm against *Xap*14 followed by 3.23 cm against *Xap*4 and 3.07 cm against both *Xap*1 and *Xap*12. Least inhibition was observed at 100 ppm (1.80 cm) against *Xap*7 (Table 9c and Plate 13).

Bronip at 1000 ppm was found effective against *Xap*7 with the inhibition zone of 1.97 cm followed by *Xap*18 and *Xap*3 at 1000 ppm. *Xap*3 at 100, 250, 500, 750, 1000 ppm concentration has given inhibition zone of 0.60, 0.70, 0.80, 0.83 and 1.33 cm respectively. *Xap*6 (0.70 cm), *Xap*16 (0.60 cm) and *Xap*18 (1.07 cm) were inhibited at 750 ppm (Table 9d).

COC at 1000 ppm with the inhibition zone of 3.17 cm followed by 2.20 cm at 750 ppm against *Xap*7, 1.83 cm at 1000 ppm and 1.73 cm at 750 ppm against *Xap*14 found effective. Least inhibition

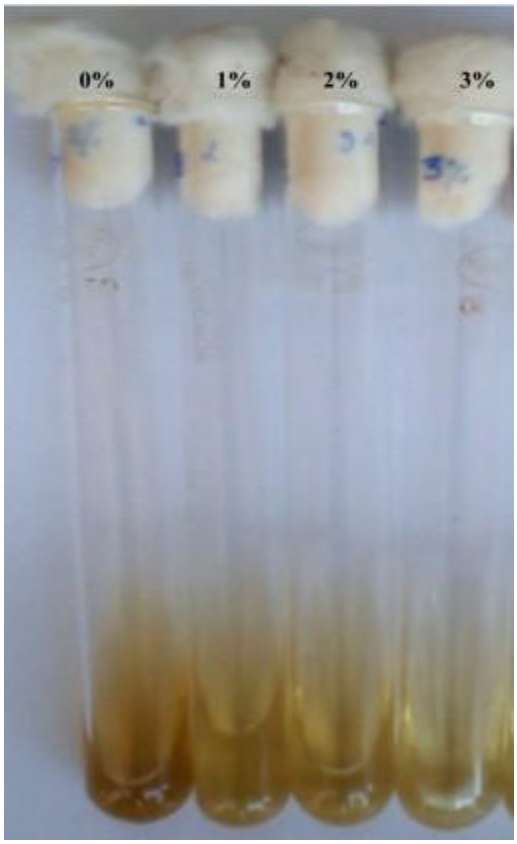
Table 7: Effect of NaCl concentrations on the growth of eighteen isolates of Xap under in vitro

Isolate	Growth (O. D. value) at 660 nm					
	NaCl concentrations (%)					
	0	1	2	3	4	Mean
*Xap*1	0.400 (1.183)*	0.199 (1.095)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.149 (1.031)
*Xap*2	0.389 (1.179)	0.295 (1.138)	0.242 (1.114)	0.000 (1.000)	0.000 (1.000)	0.231 (1.107)
*Xap*3	0.281 (1.132)	0.257 (1.121)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.134 (1.063)
*Xap*4	0.386 (1.177)	0.399 (1.183)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.196 (1.090)
*Xap*5	0.244 (1.115)	0.080 (1.039)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.081 (1.038)
*Xap*6	0.250 (1.118)	0.096 (1.047)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.086 (1.041)
*Xap*7	0.256 (1.121)	0.095 (1.046)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.088 (1.042)
*Xap*8	0.399 (1.183)	0.185 (1.089)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.146 (1.068)
*Xap*9	0.260 (1.122)	0.258 (1.122)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.129 (1.061)
*Xap*10	0.477 (1.215)	0.267 (1.126)	0.176 (1.084)	0.000 (1.000)	0.000 (1.000)	0.230 (1.106)
*Xap*11	0.434 (1.197)	0.246 (1.116)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.170 (1.078)
*Xap*12	0.398 (1.182)	0.248 (1.117)	0.126 (1.061)	0.000 (1.000)	0.000 (1.000)	0.193 (1.090)

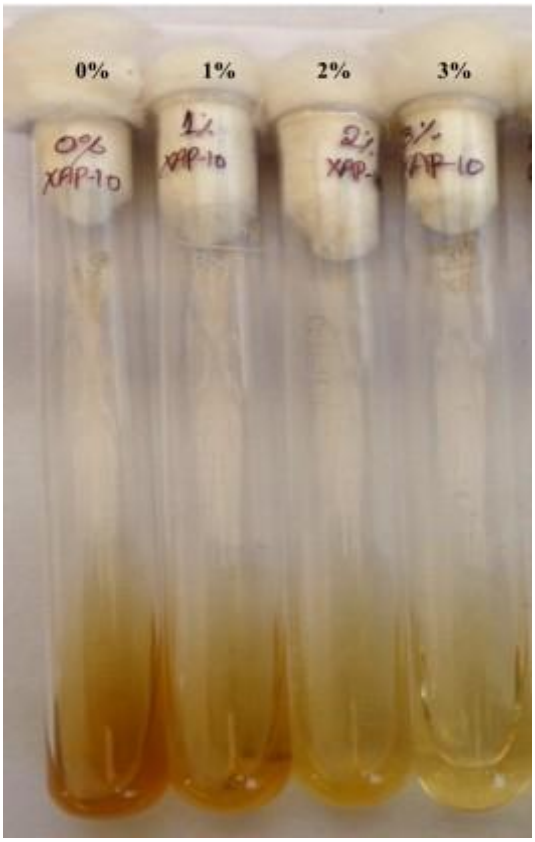
Contd…

Isolate	Growth (O. D. value) at 660 nm					
	NaCl concentrations (%)					
	0	**1**	**2**	**3**	**4**	**Mean**
*Xap*13	0.297 (1.139)	0.082 (1.040)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.094 (1.044)
*Xap*14	0.411 (1.188)	0.287 (1.134)	0.126 (1.061)	0.000 (1.000)	0.000 (1.000)	0.206 (1.095)
*Xap*15	0.387 (1.178)	0.274 (1.129)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.165 (1.076)
*Xap*16	0.287 (1.134)	0.126 (1.061)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.103 (1.048)
*Xap*17	0.426 (1.194)	0.223 (1.106)	0.167 (1.080)	0.000 (1.000)	0.000 (1.000)	0.204 (1.095)
*Xap*18	0.398 (1.182)	0.200 (1.095)	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)	0.149 (1.069)
Source	Isolates	NaCl	Isolates x NaCl			
S.Em ±	0.001	0.001	0.002			
CD @ 1%	0.004	0.004	0.008			

* $\sqrt{x + 1}$ transformed values

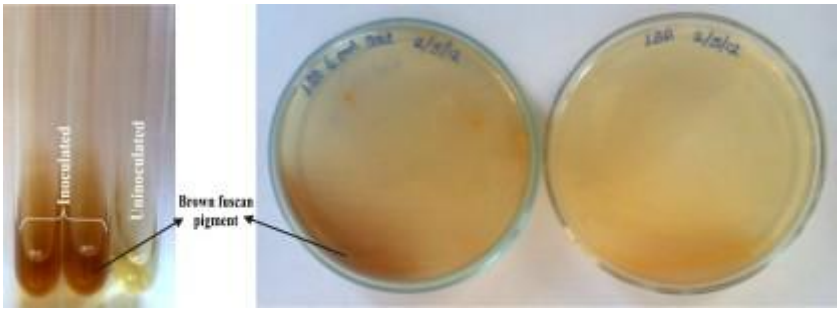


Xap 2



Xap10

Plate10: NaCl sensitivity test at different concentrations



LB agar

Plate11: Fuscan pigment production by Xanthomonas axonopodis pv.punicae

Table 8: Fuscan pigmentation from eighteen isolates of *Xap* on Luria Bertani's Agar without NaCl

	No. of days to appear fuscan pigment					
Isolate	**5**	**6**	**7**	**8**	**9**	**10**
*Xap*1	-	-	-	+ + +	+ + +	+ + +
*Xap*2	-	-	+ + +	+ + + +	+ + + +	+ + + +
*Xap*3	-	-	+ + +	+ + +	+ + + +	+ + + +
*Xap*4	-	-	+ + +	+ + +	+ + +	+ + +
*Xap*5	-	-	-	-	+ + +	+ + +
*Xap*6	-	-	-	-	+ + +	+ + +
*Xap*7	-	-	-	-	+ + +	+ + +
*Xap*8	-	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
*Xap*9	-	-	-	+ + +	+ + +	+ + + +
*Xap*10	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
*Xap*11	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
*Xap*12	-	-	-	+ + +	+ + +	+ + +
*Xap*13	-	-	-	+ + +	+ + +	+ + +
*Xap*14	-	-	+ + +	+ + +	+ + + +	+ + + +
*Xap*15	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
*Xap*16	-	-	-	-	+ + +	+ + +
*Xap*17	-	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
*Xap*18	-	+ + +	+ + +	+ + +	+ + +	+ + + +

-Indicates no fuscan pigment production
+ + + indicates slight fuscan pigmentation started
+ + + + indicates full fuscan pigmentation

Table 9: Sensitivity of *Xap* isolates against bactericides

a) Streptocycline

Isolate	Inhibition zone (cm) at different concentrations (ppm)					
	100	**250**	**500**	**750**	**1000**	**Mean**
*Xap*1	2.20 (1.79)*	2.80 (1.95)	2.90 (1.97)	3.00 (2.00)	3.13 (2.03)	**2.81 (1.99)**
*Xap*2	2.40 (1.84)	2.50 (1.87)	2.73 (1.93)	3.00 (2.00)	3.13 (2.03)	**2.75 (1.93)**
*Xap*3	1.37 (1.54)	1.57 (1.60)	1.80 (1.67)	2.02 (1.74)	2.27 (1.81)	**1.81 (1.67)**
*Xap*4	2.00 (1.73)	2.20 (1.79)	2.35 (1.83)	2.60 (1.90)	2.90 (1.97)	**2.41 (1.84)**
*Xap*5	1.47 (1.57)	1.57 (1.60)	1.83 (1.68)	1.97 (1.72)	2.13 (1.77)	**1.79 (1.67)**
*Xap*6	1.30 (1.52)	1.43 (1.56)	1.60 (1.61)	1.83 (1.68)	2.07 (1.75)	**1.65 (1.62)**
*Xap*7	2.03 (1.74)	2.17 (1.78)	2.67 (1.91)	2.93 (1.98)	2.97 (1.99)	**2.55 (1.88)**
*Xap*8	2.10 (1.76)	2.23 (1.80)	2.37 (1.83)	2.40 (1.84)	2.50 (1.87)	**2.32 (1.82)**
*Xap*9	1.53 (1.59)	1.63 (1.62)	1.77 (1.66)	2.03 (1.74)	2.23 (1.80)	**1.84 (1.68)**
*Xap*10	2.00 (1.73)	2.07 (1.75)	2.17 (1.78)	2.43 (1.85)	2.43 (1.85)	**2.22 (1.79)**
*Xap*11	2.07 (1.75)	2.13 (1.77)	2.43 (1.85)	2.73 (1.93)	2.57 (1.89)	**2.39 (1.84)**
*Xap*12	2.30 (1.82)	2.37 (1.83)	2.60 (1.90)	2.90 (1.97)	3.10 (2.02)	**2.65 (1.91)**
*Xap*13	1.30 (1.52)	1.43 (1.56)	2.00 (1.73)	2.07 (1.75)	2.40 (1.84)	**1.84 (1.68)**
*Xap*14	2.60 (1.90)	2.60 (1.90)	2.93 (1.98)	3.27 (2.06)	3.33 (2.08)	**2.95 (1.98)**
*Xap*15	2.27 (1.81)	2.23 (1.80)	2.57 (1.89)	2.63 (1.91)	2.77 (1.94)	**2.49 (1.87)**
*Xap*16	1.43 (1.56)	1.77 (1.66)	2.13 (1.77)	2.27 (1.81)	2.50 (1.87)	**2.02 (1.74)**
*Xap*17	1.83 (1.68)	2.07 (1.75)	2.17 (1.78)	2.37 (1.83)	2.43 (1.85)	**2.17 (1.78)**
*Xap*18	1.37 (1.54)	1.48 (1.58)	1.73 (1.65)	1.93 (1.71)	2.03 (1.74)	**1.71 (1.64)**
Mean	**1.86 (1.69)**	**2.01 (1.73)**	**2.26 (1.80)**	**2.47 (1.86)**	**2.61 (1.90)**	**2.24 (1.8)**

* $\sqrt{\times + 1}$ transformed values

b) Plantomycine

Isolate	Inhibition zone (cm) at different concentrations (ppm)					
	100	**250**	**500**	**750**	**1000**	**Mean**
*Xap*1	0.00 (1.0.0)	0.00 (1.00)	0.97 (1.40)	1.07 (1.44)	1.23 (1.49)	0.65 **(**1.26**)**
*Xap*2	0.70 (1.30)	1.00 (1.41)	1.00 (1.41)	1.10 (1.45)	1.80 (1.67)	1.22 **(**1.44**)**
*Xap*3	0.77 (1.33)	0.93 (1.39)	1.00 (1.41)	1.10 (1.45)	1.40 (1.55)	1.04 **(**1.42**)**
*Xap*4	0.00 (1.00)	0.00 (1.00)	1.00 (1.41)	1.17 (1.47)	1.93 (1.71)	**0.82 (1.31)**
*Xap*5	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.27 (1.50)	1.43 (1.56)	**0.54 (1.21)**
*Xap*6	0.00 (1.00)	0.00 (1.00)	1.03 (1.42)	1.27 (1.50)	1.53 (1.59)	**0.76 (1.30)**
*Xap*7	0.00 (1.00)	1.00 (1.41)	1.07 (1.44)	1.40 (1.55)	1.53 (1.59)	**1.00 (1.39)**
*Xap*8	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.17 (1.47)	1.43 (1.56)	**0.52 (1.20)**
*Xap*9	0.60 (1.26)	0.70 (1.30)	0.87 (1.37)	1.03 (1.43)	1.27 (1.51)	**0.89 (1.37)**
*Xap*10	0.00 (1.00)	0.70 (1.30)	0.73 (1.31)	1.07 (1.44)	1.17 (1.47)	**0.73 (1.30)**
*Xap*11	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.53 (1.23)	1.07 (1.44)	**0.32 (1.13)**
*Xap*12	0.80 (1.34)	1.07 (1.44)	1.40 (1.55)	1.50 (1.58)	1.40 (1.55)	**1.23 (1.49)**
*Xap*13	0.00 (1.00)	0.00 (1.00)	0.93 (1.39)	1.07 (1.44)	1.43 (1.56)	**0.68 (1.27)**
*Xap*14	0.87 (1.36)	0.90 (1.38)	1.40 (1.55)	1.47 (1.57)	1.67 (1.63)	**1.26 (1.49)**
*Xap*15	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	0.97 (1.40)	1.07 (1.43)	**0.55 (1.22)**
*Xap*16	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.00 (1.41)	1.17 (1.47)	**0.43 (1.17)**
*Xap*17	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.93 (1.39)	**0.18 (1.07)**
*Xap*18	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.20 (1.48)	1.37 (1.54)	**0.51 (1.20)**
Mean	**0.21 (1.09)**	**0.35 (1.15)**	**0.67 (1.28)**	**1.08 (1.43)**	**1.38 (1.54)**	**0.73 (1.29)**

* $\sqrt{x + 1}$ transformed values

c) K- Cycline

Isolate	Inhibition zone (cm) at different concentrations (ppm)					
	100	**250**	**500**	**750**	**1000**	**Mean**
*Xap*1	2.30 (1.82)	2.50 (1.87)	2.60 (1.90)	3.00 (2.00)	3.07 (2.01)	**2.69 (1.92)**
*Xap*2	2.37 (1.83)	2.50 (1.87)	2.57 (1.89)	2.67 (1.91)	2.83 (1.96)	**2.58 (1.89)**
*Xap*3	2.07 (1.75)	2.20 (1.79)	2.40 (1.84)	2.63 (1.91)	2.97 (1.99)	**2.45 (1.85)**
*Xap*4	2.00 (1.73)	2.40 (1.84)	2.70 (1.92)	2.93 (1.98)	3.23 (2.06)	**2.65 (1.90)**
*Xap*5	2.17 (1.78)	2.27 (1.81)	2.53 (1.88)	2.77 (1.94)	2.97 (1.99)	**2.54 (1.88)**
*Xap*6	2.03 (1.74)	2.33 (1.83)	2.43 (1.85)	2.50 (1.87)	2.80 (1.95)	**2.42 (1.85)**
*Xap*7	1.80 (1.67)	2.03 (1.74)	2.07 (1.75)	2.07 (1.75)	2.40 (1.84)	**2.07 (1.75)**
*Xap*8	2.63 (1.91)	2.77 (1.94)	2.63 (1.91)	2.87 (1.96)	2.97 (1.99)	**2.77 (1.94)**
*Xap*9	2.03 (1.74)	2.00 (1.73)	2.03 (1.74)	2.23 (1.80)	2.40 (1.84)	**2.14 (1.07)**
*Xap*10	2.17 (1.78)	2.43 (1.85)	2.27 (1.81)	2.50 (1.87)	2.87 (1.96)	**2.43 (1.86)**
*Xap*11	1.97 (1.72)	2.23 (1.80)	2.52 (1.87)	2.43 (1.85)	2.70 (1.92)	**2.37 (1.83)**
*Xap*12	2.43 (1.85)	2.37 (1.83)	2.60 (1.90)	2.63 (1.91)	3.07 (2.02)	**2.62 (1.90)**
*Xap*13	2.10 (1.76)	2.43 (1.85)	2.57 (1.89)	2.63 (1.91)	2.87 (1.96)	**2.52 (1.87)**
*Xap*14	2.63 (1.91)	2.73 (1.93)	2.87 (1.96)	3.07 (2.01)	3.37 (2.09)	**2.98 (1.98)**
*Xap*15	2.07 (1.75)	2.17 (1.78)	2.13 (1.77)	2.40 (1.84)	2.57 (1.89)	**2.26 (1.81)**
*Xap*16	2.07 (1.75)	2.13 (1.77)	2.37 (1.83)	2.53 (1.88)	2.80 (1.95)	**2.38 (1.83)**
*Xap*17	2.37 (1.83)	2.43 (1.85)	2.53 (1.88)	2.67 (1.91)	2.83 (1.96)	**2.57 (1.89)**
*Xap*18	1.97 (1.72)	2.07 (1.75)	2.37 (1.83)	2.57 (1.89)	2.73 (1.93)	**2.34 (1.82)**
Mean	**2.18 (1.78)**	**2.33 (1.82)**	**2.45 (1.86)**	**2.62 (1.90)**	**2.86 (1.96)**	**2.48 (1.86)**

* $\sqrt{x + 1}$ transformed values

d) Bronip (2-Bromo-2-nitro-propane-1, 3 diol)

Isolate	Inhibition zone (cm) at different concentrations (ppm)					
	100	**250**	**500**	**750**	**1000**	**Mean**
*Xap*1	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	**0.00 (1.00)**
*Xap*2	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.70 (1.30)	**0.14 (1.06)**
*Xap*3	0.60 (1.26)	0.70 (1.30)	0.80 (1.34)	0.83 (1.35)	1.33 (1.53)	**0.85 (1.36)**
*Xap*4	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.13 (1.46)	**0.23 (1.09)**
*Xap*5	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.80 (1.34)	**0.16 (1.07)**
*Xap*6	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.70 (1.30)	0.93 (1.39)	**0.33 (1.14)**
*Xap*7	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.97 (1.72)	**0.39 (1.14)**
*Xap*8	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	**0.15 (1.06)**
*Xap*9	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	7.00 (1.30)	**0.14 (1.06)**
*Xap*10	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	**0.00 (1.00)**
*Xap*11	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.67 (1.29)	**0.13 (1.06)**
*Xap*12	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.70 (1.30)	**0.14 (1.06)**
*Xap*13	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.77 (1.33)	**0.15 (1.07)**
*Xap*14	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.93 (1.39)	**0.19 (1.08)**
*Xap*15	0.00 (1.00)	0.00 (1.00)	0.00 (100)	0.00 (1.00)	0.00 (1.00)	**0.00 (1.00)**
*Xap*16	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.60 (1.26)	1.00 (1.41)	**0.32 (1.13)**
*Xap*17	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	**0.00 (1.00)**
*Xap*18	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.07 (1.44)	1.43 (1.56)	**0.50 (1.20)**
Mean	**0.03 (1.01)**	**0.04 (1.02)**	**0.04 (1.02)**	**0.18 (1.08)**	**0.77 (1.31)**	**0.21 (1.11)**

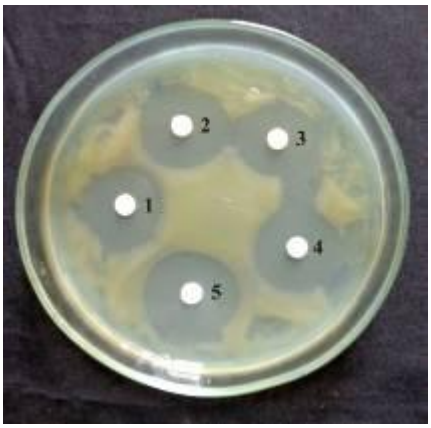
* $\sqrt{x + 1}$ transformed values

e) Copper-oxychloride

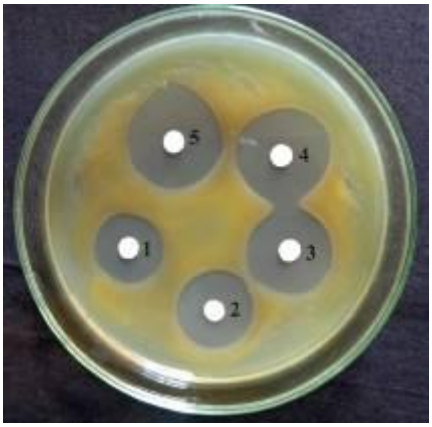
Isolates	Inhibition zone (cm) at different concentrations (ppm)						
	100	**250**	**500**	**750**	**1000**	**Mean**	**Grand mean**
*Xap*1	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	0.90 (1.38)	1.13 (1.46)	0.55 (1.23)	**1.34 (1.47)**
*Xap*2	0.00 (1.00)	1.00 (1.41)	1.10 (1.45)	1.40 (1.55)	1.50 (1.58)	1.00 (1.40)	**1.52 (1.55)**
*Xap*3	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.80 (1.34)	1.13 (1.46)	0.39 (1.16)	**1.31 (1.49)**
*Xap*4	0.00 (1.00)	0.83 (1.35)	0.97 (1.40)	1.23 (1.49)	1.37 (1.54)	0.88 (1.36)	**1.40 (1.50)**
*Xap*5	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	0.83 (1.35)	0.87 (1.37)	0.49 (1.21)	**1.10 (1.41)**
*Xap*6	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.77 (1.33)	1.10 (1.45)	0.37 (1.16)	**1.11 (1.41)**
*Xap*7	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	2.20 (1.79)	3.17 (2.04)	1.07 (1.37)	**1.42 (1.51)**
*Xap*8	0.00 (1.00)	0.00 (1.00)	0.77 (1.33)	0.90 (1.37)	1.13 (1.46)	0.56 (1.23)	**1.26 (1.45)**
*Xap*9	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.03 (1.42)	1.23 (1.49)	0.45 (1.18)	**1.09 (1.41)**
*Xap*10	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.90 (1.38)	0.83 (1.35)	0.35 (1.15)	**1.15 (1.42)**
*Xap*11	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	0.67 (1.29)	1.10 (1.45)	0.50 (1.21)	**1.14 (1.41)**
*Xap*12	0.00 (1.00)	0.00 (1.00)	0.60 (1.26)	1.07 (1.44)	1.37 (1.54)	0.61 (1.25)	**1.45 (1.52)**
*Xap*13	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.97 (1.40)	0.19 (1.08)	**1.08 (1.40)**
*Xap*14	0.00 (1.00)	0.00 (1.00)	0.70 (1.30)	1.73 (1.65)	1.83 (1.68)	0.85 (1.33)	**1.64 (1.57)**
*Xap*15	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.67 (1.29)	0.13 (1.06)	**1.09 (1.39)**
*Xap*16	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.73 (1.31)	1.07 (1.44)	0.36 (1.15)	**1.10 (1.41)**
*Xap*17	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.93 (1.39)	0.19 (1.08)	**1.02 (1.36)**
*Xap*18	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	1.10 (1.45)	0.22 (1.09)	**1.06 (1.39)**
Mean	**0.00 (1.00)**	**0.10 (1.00)**	**0.35 (1.04)**	**0.84 (1.15)**	**1.25 (1.34)**	**0.51 (1.11)**	**1.24 (1.49)**
Source	I	B	IXB	C	IXC	BXC	IXBXC
S.Em **±**	0.003	0.002	0.008	0.002	0.008	0.004	0.017
CD @ 0.01	0.013	0.007	0.031	0.007	0.031	0.016	0.067

* $\sqrt{x + 1}$ transformed values

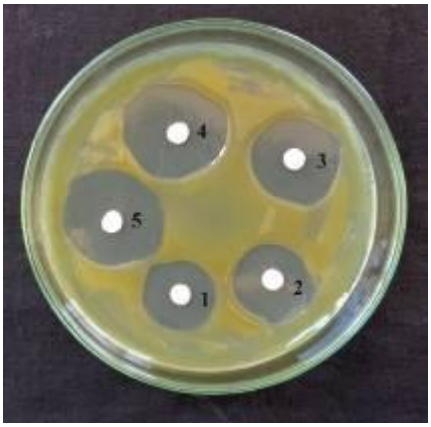
Xap4



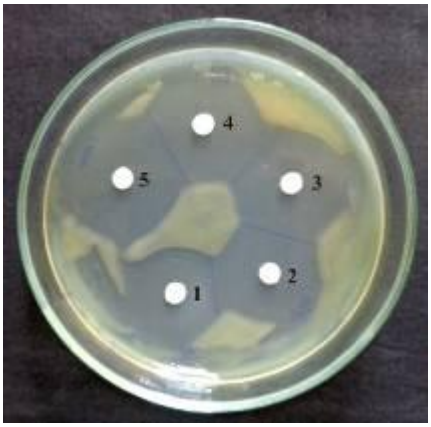
Xap8



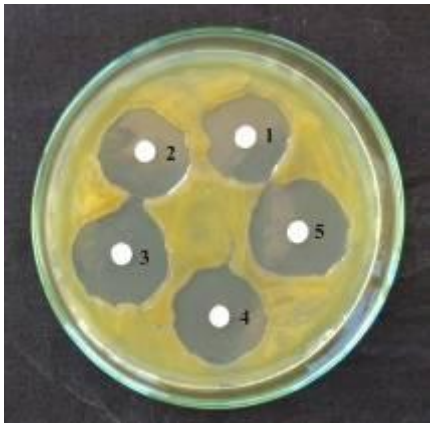
Xap10



Xap11



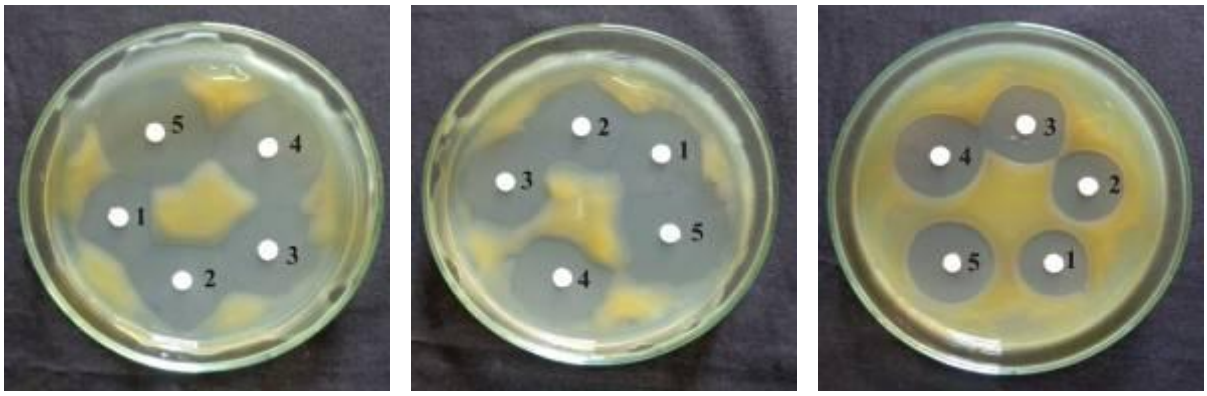
Xap15



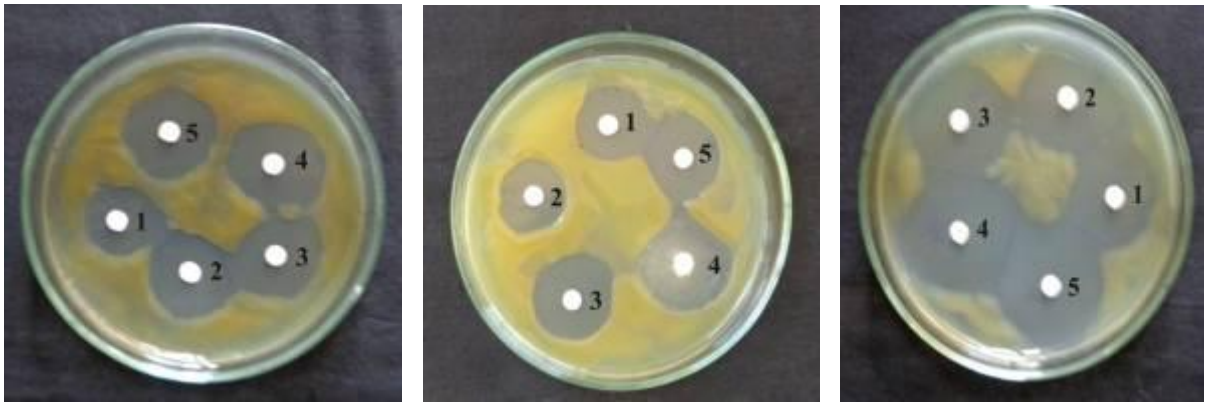
Xap14

1 = 100 ppm 2 = 250 ppm 4 = 750 ppm 5 = 1000 ppm

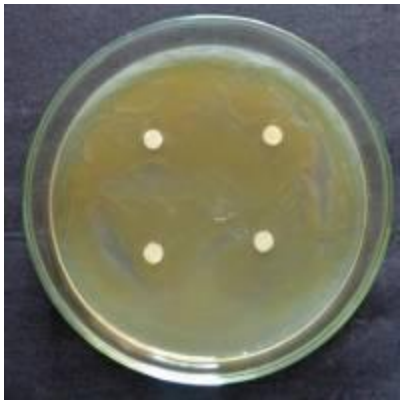
Plate 12 : Sensitivity of Xap isolates against strepocycline after 10 days of inoculation



Xap4 **Xap8** **Xap10**



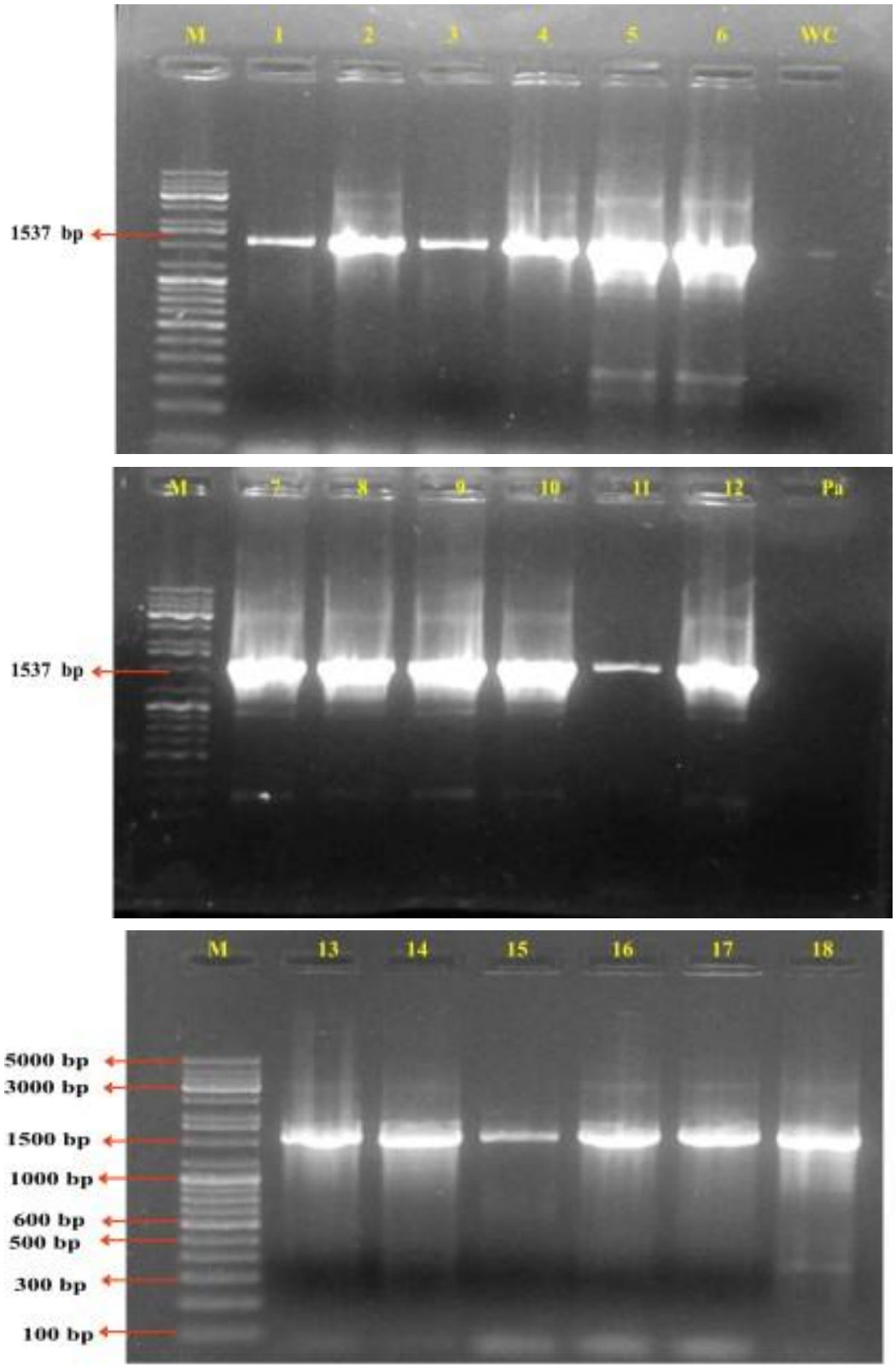
Xap11 **Xap 14** **Xap15**



Control

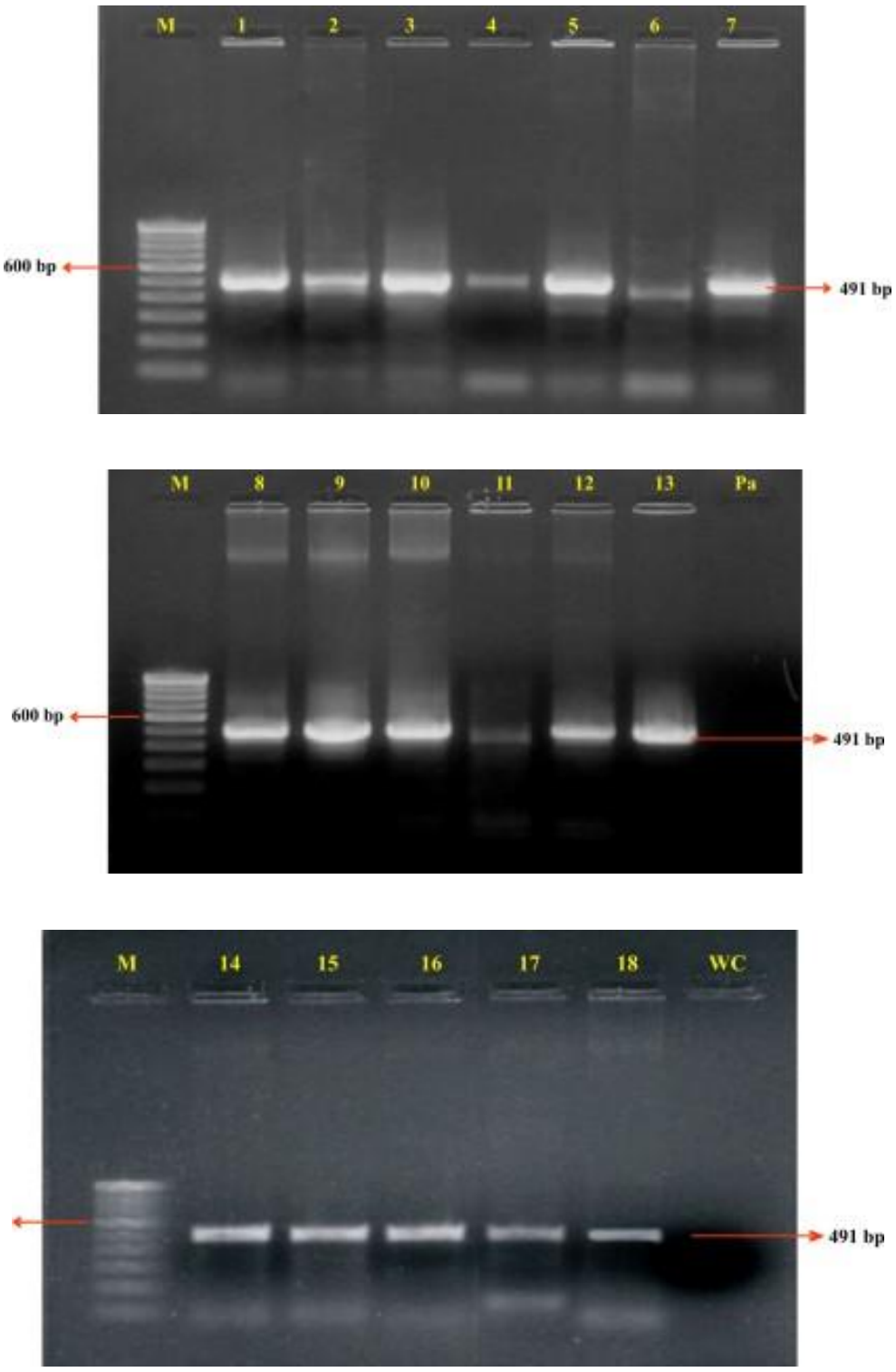
1 = 100 ppm **2 = 250 ppm** **4 = 750 ppm** **5 = 1000 ppm**

Plate 13: Sensitivity of Xap isolates against K-Cycline after 10 days of inocuilation:



M = Medium range marker (5000 bp), 1 to 18 = Isolates of *Xanthomonas axonopodis* pv. *punicae* from Karnataka, Maharashtra, Andhra Pradesh, WC = Water control and Pa = *Pantoea agglomeran,s* respectively

Plate 14: Amplification of Xanthomonas pv. Punicae using 16sr RNA primer from bacterial DNA



M = 100 bp marker, 1 to 18 = isolates of Xanthomonoas axonopodis pv. Punicae from Karnataka, Maharashtra Andhra Pradesh, wc = Water control and pa = Pantoea agglomerans respectively

Plate 15: Amplification of *Xanthomonas axonopodis* pv. *punicae* using gyrB primer set from bacterial DNA

zone was found in *Xap*12 (0.60 cm) and *Xap*15 (0.67 cm). No inhibition was found at 100 ppm for all isolates of *Xap* (Table 9e).

4.4 16S rRNA (Ribosomal RNA) analysis and phylogeography of *Xap* isolates

Isolation of genomic DNA and amplification of 16S rRNA and *gyr*B region

The extraction of total genomic DNA was carried out following a modified protocol as described by Mondal and Kumar (2011). Thus obtained DNA was confirmed by running on 1.0 per cent agarose gel electrophoresis. In all isolates the yield of DNA was sufficient for the analysis.

A 1,537 bp region of 16S rRNA gene was amplified using forward 5′AGTGAAGAGTTTGATCCTGGC3′ and reverse-5′AGGAGGTGATCCAGCCGC3′ primers. A 491 bp region of *gyr*B was targeted using forward-5′GTGCGGCGCTGAT T C 3 ′ and reverse - 5 ′ CGTGTCGGGGTTGAC 3′ primers from *Xap* strains through polymerase chain reaction (PCR). An amplicon of 1537 bp and 491 bp amplicons for 16S rRNA and *gyr*B respectively were obtained in all samples (Plate 14 and Plate 15).

Sequences of 16S rRNA gene from *Xap*

The sequences obtained using forward primers were assembled using Vector NTI software. The nucleotide sequences of *Xap* 16S rRNA gene of all nine isolates of *Xap* is given below.

>AP2

CTCAGAGTTTCGGGGGATACTGGCGGCGGCCTAACACATGCAAGTCGAACGGCAGCACAGTAA
GAGCTTGCTCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCG
TGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGA
CCTTCGGGCTTCGCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCC
CACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAG
TCGGTTAATACCCGAGTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGC
AGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGG
TGGTGATTTAAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGGTC
ACTAGAGTGTGGTAGAGGGTAGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGA
GGAACATCCGTGGCGAAAGCGGCTACCTGGACCAACACTGACACTGAGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGC
AATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCCGCCGCCTGGGGAGTACGGTCGCAGA
CTGAAACTCAAGGAATTTGACGGGGCCCGCACAGCGTGAATATGTGTTTAATTCGATGCACGCG
AAGAACCTTACCTGTCTGACATCACGACTTTCAAGATGGATTGTTGCGTCGGACATGAACTGTTG
CTGCATGGCTGTCATCAACTCAGATCCTGAAGTTTCGGTAAGTTCCCCACAAAGCGCAACATATG
TCATAAGTTGCAGCCGTATTGTAGAGCTTGAGGACTGCGGTTGACAAACAGATGATCACA

>4F

CGACCGGGCCTGGCGGCGGCCTAACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTC
TTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAA
CGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTT
CGCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGA
CGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTG
GGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACC
CGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT
ACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTGATTTAAGT
CTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGGTCACTAGAGTGTGGT
AGAGGGTAGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCCGTGG
CGAAAGGCGGCTACCTGGACCAACACTGACACTGAGCACGAAGCGTGGGGAGCAAACAGGATT
AATACCCTGGTAGTCCACGCCCTAAACGATGCGACTGGATGTTGGGTGCAATTTGGCACGCAGT
ATCGAGCTAACGCGTTAAGTCGTCGCCTTGGGGAGTACGGTCGCAGACTGAAACTCAAAGAATT
TGACGGGGGCCGCATAGCGGTGGAGTAAGTGTTTATTCGATGCATCGCAAAGCTTACTGGTCTT
TGACTTCACAGACTTCCCGAGATGAATGGTGCTTCCGAACCTGGACACAAGTCTCTGCCTGCTGT
CCTCCACTTCGA

>7F

TTGGTTAGTTGGTTTAAAGGGGGGGAGTTGGAGGCGGCCTAACACATGCAAGTCGAACGGCAG
CACAGTAAGAGCTTGCTCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTA
CTCTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAG
CGGAGGACCTTCGGGCTTCGCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGT
AAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
GATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGA
AAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTG
CGTAGTGGTGATTTAAGTCTGTTGTGAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTG
GGTCACTAGAGTGTGGTAGAGGGTAGCGGATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCG
GGAGGACATCCGTGGCGAAGCGGCTACCTTGACCACACTGACACTGAGCACGAAAGCGTGGGA
GCAAACATGATTAATTCCTTGTAGTCCACGCCCTAACGATGCGACTGGATGTCGGTTG

>KA3

GTTGCGGTTGTAGGACATACTGGCGGCGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAG
AGCTTGCTCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGT
GGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGAC
CTTCGGGCTTCGCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCC
ACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGATATTGGACAATGGGCGCAAGCCTGATCCAGCA
TGCCGCGTGGTGAGACGCCTTCGGGTTGTAAGCCCCTTGTTGGGAAGAAGCAGTCGTTAAACCG
ATGTTCTGACGGACCACGATAACACCGGCTACTTTCGTGGCCACAACCCCCGGTAATACAAGGG
GTGCAAGCGTTTACTCCGGAATTTACTGGGGCGTAAAAGCGTTGCCGTAAGGTGGGTGAATTTA
AATTCTGGTTGTGGAAAGCCCCTGGGGCTTCAACCTGGGGAAATTGCATTGGATAACTGGGGTC
CACTTAAATTGGGGGTAAAAAGGGTAACCGGAAATTCCCCGGTGGTACCAATGGAAATTGGCTA
AAAAACCCGGGAAGGAACCTTTCGTGGGAGAATGGGGGGGTAGCTTTGAACCATAGGGTGAAAA
TAGAGGTGTGAAAGCCCGGGGAAA

>LAX

GTCATAGCTGGCGGCAGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTA
TGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAACG
TAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTCG
CGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACG
ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGG
GTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCC
GATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTATGTGGTGATTTAAGTC
TGTTGTGAAAGCCCTGGGCTCAACCTGGGATTTGCATTGGATACTGGGTCACTAAATGTGGTAGA
GGATATCGAATTCCCGTGTAAAAAGGGAATGCTTT

>KA5

GTTAAGAGCTGGCGGCAGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTT
ATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAAC
GTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTC
GCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGGCCCCCCAGGGGGA
CGATCCCGTAGCTGGGTCTGAAAAGGAGGATCACCCACACTGGAAACTGAGACACGGTCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCC
GCGTGGGTGAAGAAGGCCTTCGGGTTGTAGAGCCCTTTTTTTTTGAAAAAAAAACAATCTCTTAA
TACACGGGGGGGGGGAAGGTCCCCCCAGAATGTGTCCCCGGGTACTTAAAGGCACAACCTCTC
TAGGAAAATTGGTGGGAGTCGCAATCCAAATAAGGGTCCCCGCGGTGCCCGAATAACACCATAT
ACCCCGCGTTTGTGCGGGGCCCCCACATTCCTTTGAGTTTGATCTTGGGACGTACTCTCGGGGC
GGAGAAATTACGCGTCAGCTCAATATGGGTGC

>MAL

GGGGAGTTACCTGGCGGCGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTC
TTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAA
CGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTT

CGCGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGA
CGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTG
GGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGCACACAAACGCACTCTGTTAATAC

>KA-8

GGTCATACTGGCGGCAGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTA
TGGGTGGGAGAGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAACGT
AGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCCTCG
CGCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACG
ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGG
GTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCC
GAGTGTTCTGACGGTACCCACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGTGGTGATTTAAGTCT
GTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGGTCACTAGAGTGTGGTAG
AGGGTAGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCCGTGGCG
AAAGCGGCTACCTGGACCAACACTGACACTGAGCACGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCCTAACGATGCGAACTGGGATTGTTGGGTGCAATTTGGCACGCAG
TATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGGAGTACGTCGCAAGACTGAAACTCAAAGG
AATTTGACGGGGCCCGCACTAGCCGGGGAAGTAAGTGTAATTCGATGCCACGCTGACGAACTAT
ACCTGAGCTCTGACA

>HIR

CCAGAGCTGGCGGCAGGCCTACACATGCAAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTAT
GGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTTTCGTGGGGGATAACGT
AGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTCGC
GCGGTTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGA
TCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGG
GGTGAAAAAGGCCTTCGGGGTTGTAACGCCCTTTTTTTTTTTTAAAAAAAACCAGTCCGTTAATT

Nucleotide sequences of nine *Xap* isolates 16S rRNA region were analyzed using bioinformatics tools at National Center for Biotechnology Information (NCBI) BLAST programme. Based on sequence comparison, all the fragment of 16S rRNA gene sequences of nine isolates were confirmed as *Xap Xap*-85 strain 16S rRNA gene, partial sequence; ribosomal RNA. The list of nine isolates, identified as, genBank accession number, per cent similarity with reference are presented in Table 10. Fragment of 16S rRNA gene sequences of *Xap* of Lakmapur, Laxmipur and Malegaon isolates showed 98 per cent similarity with NCBI published sequence of *Xap* -85 strain 16S rRNA gene (JN109175.1), Kaladagi and Hiraj isolates showed 97 per cent similarity with *Xap* -85 strain 16S rRNA gene (JN109175.1), Govinkoppa isolate showed 96 per cent similarity with *Xap* -5 IARI strain 16S rRNA (JN036622), while Anantpur and Kanamadi isolates showed 95 per cent similarity with *Xap* AP-5 strain 16S rRNA (JQ067629) and *Xap* -85 strain 16S rRNA (JN109175.1) respectively, and Matarangi isolate showed 91 per cent similarity with *Xap* -5 IARI strain 16S rRNA (JN036622).

4.4.1 Phylogenetic analysis

A dendrogram was constructed using neighbor-joining tree method for the nucleotide sequences of 16S rRNA region of all nine *Xap* isolates. Published sequence of *Xap* 85 strain, 16S rRNA gene (JN109175.1), *Xap*-5 IARI strain 16S rRNA (JN036622), *Xap* AP-5 strain 16S rRNA (JQ067629), *X. oryzae*, *X. campestris* pv. *mangifaraeindica, X. a.* pv. *malvacearum*, *X. citri, Pantoea agglumerans* were downloaded from NCBI and was used in the construction of dendrogram (Fig 1a and 1b).

In the present study, *Pantoea agglumerans* (FJ611839) was used as out group to interpret the clustering of nine *Xap* isolates. Phylogram results of nine *Xap* isolates revealed that there are two major clusters, cluster A comprising all *Xanthomonas* species, whereas cluster B comprises *Pantoea agglumerans*. A glance towards dendrogram reveals that there is not much diversity among the nine isolates of *Xap*. As expected branching of *Pantoea agglumerans* was altogether different having wide divergence with separate branch in dendrogram.

Table 10: Comparison and identity of fragment of the 16S rRNA gene of *Xap* of pomegranate growing districts of Karnataka, Maharashtra and Andhra Pradesh with that of referred gen bank available in NCBI

Sample ID	Isolate code	District	Identified as	Gene Bank Accession Number	Reference	Per cent Similarity
AP-2	*Xap*11	Anantpur	*Xap* AP-5 strain 16S rRNA, partial sequence; ribosomal RNA	JQ067629	Mondal *et al*., 2011	95
4F	*Xap*13	Govinakoppa	*Xap* -5 IARI strain 16S rRNA, partial sequence; ribosomal RNA	JN036622	Mondal and Mani , 2011	96
7F	*Xap*14	Lakmapur	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN109175.1	Mondal and Mani , 2011	98
KA-3	*Xap*1	Matrangi	*Xap* -5 IARI strain 16S rRNA, partial sequence; ribosomal RNA	JN036622	Mondal and Mani , 2011	91
LAX	*Xap*17	Lakshmipur	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN109175.1	Mondal and Mani , 2011	98
KA-5	*Xap*2	Kanamadi	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN109175.1	Mondal and Mani , 2011	95
MAL	*Xap*9	Malegaon	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN109175.1	Mondal and Mani , 2011	98
KA-8	*Xap*4	Kaladagi	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN109175.1	Mondal and Mani , 2011	97
HIR	*Xap*7	Hiraj	*Xap* -85 strain 16S rRNA, partial sequence; ribosomal RNA	JN036623	Mondal and Mani , 2011	97

4.4.2 Molecular detection of *Xap* using 16S rRNA primers

a) By direct DNA isolation from infected samples

The extraction of total genomic DNA from naturally infected plant parts was carried out. Thus obtained DNA was used as a template. A 1537 bp region of 16S rRNA gene was amplified and separated on 1 per cent agarose gel with medium range marker as standard DNA marker (Plate 16).

b) By colony PCR

The detection of *Xap* from infected samples recovered on semi-selective media without DNA isolation. Single colony of *Xap* was picked up with a sterile pipette tip and added directly to the PCR mixture as a template for DNA amplification. Amplification of 16S rRNA at 1537 bp using a set of primers and annealing temperature for 16S rRNA gene was 60°C for 1 minute (Plate 17).

4.4.3 Multiplex polymerase chain reaction

Primers developed for the 16S rRNA and *gyr*B genes were combined for the development of a multiplex PCR protocol. The multiplex polymerase chain reaction was performed using template containing DNA from *Xap* targeting 16S rRNA and *gyr*B gene. Each of the primer pairs specific for the target loci were used for PCR amplifications. Multiplex polymerase chain reaction was performed at different gradient annealing temperatures and periods, with a temperature ranging from 56 to 59ºC with 1ºC increments to find out the common annealing temperature that support the amplification of 16S rRNA and *gyr*B gene of *Xap*. High intensity of amplification of the gene specific products of 1537 bp and 491 bp corresponding to 16S rRNA and *gyr*B were obtained at annealing temperature of 57.5º C for 1 minute as compared to other temperatures (Plate 18).

4.5 Prospecting the botanicals and selected nutrients for *in vitro* and *in vivo* inhibition of *Xap*

4.5.1 Effect of plant extracts on growth inhibition of *Xap* under *in vitro*

Plant extracts are known for safe, ecofriendly and cost effective means of managing the crop diseases. The present study was conducted with nine plant extracts screened at 5 per cent and 10 per cent concentrations against the growth of *Xap*10 under *in vitro* conditions. The results obtained are presented in Table 11 and Plate 19.

Among the plant extracts tested, soapnut extract was very effective in inhibiting the growth of the pathogen by 1.46 cm diameter which is significantly superior to all other plant extracts tested, followed by adahtoda + soapnut (1.24 cm) and miswak + soapnut (1.00 cm). Garlic bulb extract (0.81 cm), adahtoda + miswak extract (0.83 cm), adahtoda leaf extract (0.73 cm) and miswak stem extract (0.65 cm) was found moderately effective. The other plant extracts *viz.*, tulsi, nerium and neem lef extract were observed completely ineffective with zero efficacy. Among the different concentrations used, higher concentration level at 10 per cent was found to be superior to lower concentration level at 5 per cent.

Interaction effect between the plant extracts and concentrations revealed that soapnut extract at 10 per cent concentration was significantly superior to other treatments with highest inhibition zone of 1.98 cm diameter followed by adahtoda + soapnut (1.48 cm) at 10 per cent concentration. The next best effective extracts were adahtoda + miswak extract (0.98 cm) at 10 per cent and soapnut extract (0.95 cm) at 5 per cent concentration. Adahtoda leaf extract (5 per cent) was found least effective with an inhibition zone of 0.63 cm diameter.

4.5.2 *In vitro* evaluation of micro and secondary nutrients on the growth of *Xap*

Among the nine nutrients tested, $CuSO_4$ has recorded highest mean inhibitory zone of 0.89 cm which is significantly superior to other micro and secondary nutrients tested followed by micron special (boron, $MgSO_4$ and $ZnSO_4$) 0.41 cm at all concentrations tested. $MnSO_4$, $CaSO_4$, boron, sulfur, $FeSO_4$ were failed to inhibit the pathogen. $CuSO_4$ has recorded maximum inhibition zone of 0.60 cm at 0.05 per cent, 0.75 cm at 0.1 per cent and 0.95 cm at 0.2 per cent and 1.25 cm at 0.5 per cent concentrations respectively (Table 12 and Plate 19).

4.5.3 Effect of antagonistic organisms on the growth of *Xap* under *in vitro*

The study conducted revealed that among the five different strains of *P. fluorescens* tested strain No. 326(4) and PF1 has recorded maximum inhibitory zone of 3.10 cm and 3.00 cm

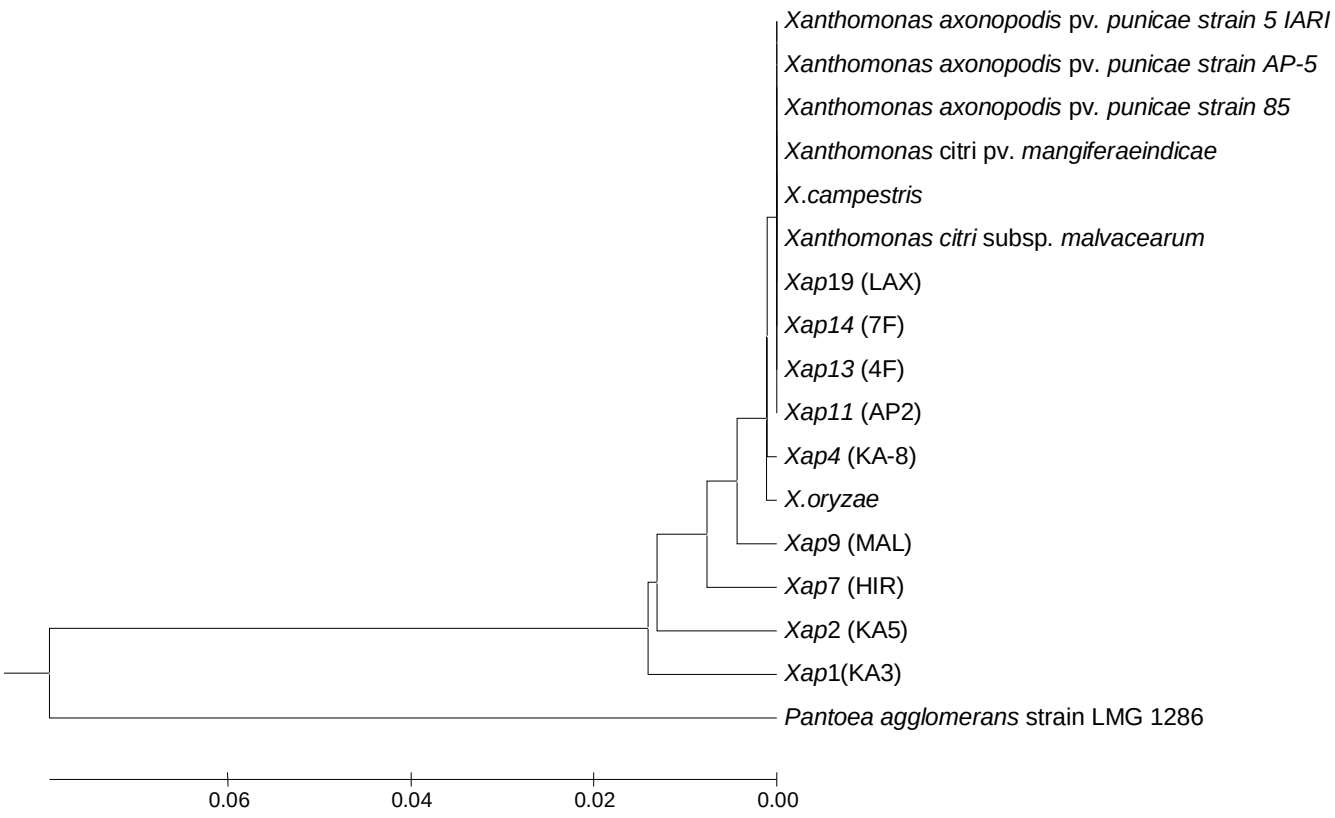


Fig. 1a: Phylogenetic relationship based on 16S rRNA gene sequences analysis of *Xap* strains from Karnataka, Andhra Pradesh and Maharashtra with the 16S rRNA sequences of selected *Xanthomonas*. Sequences of *Pantoea agglomerans* LMG 1286 was included as out-group

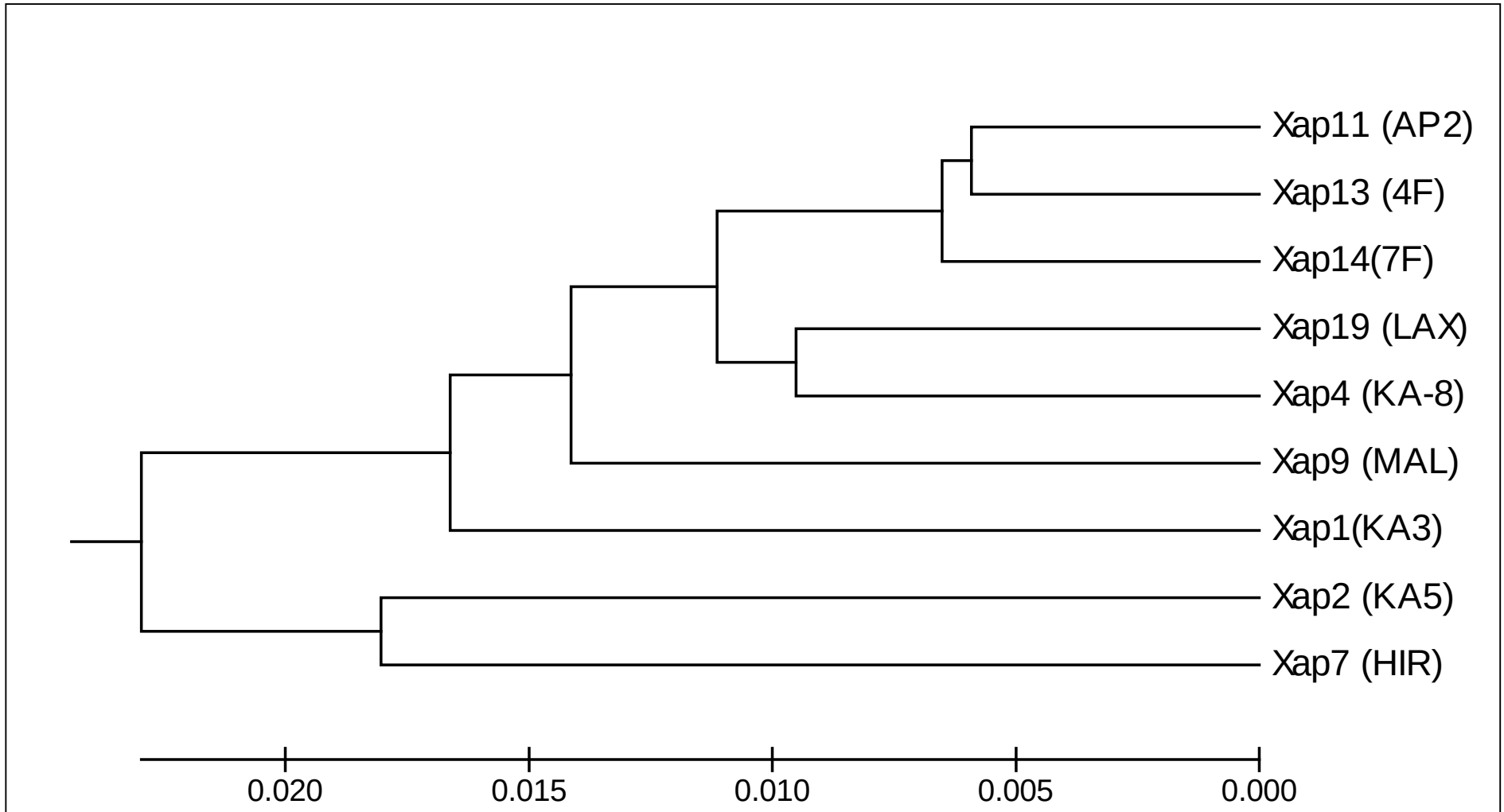


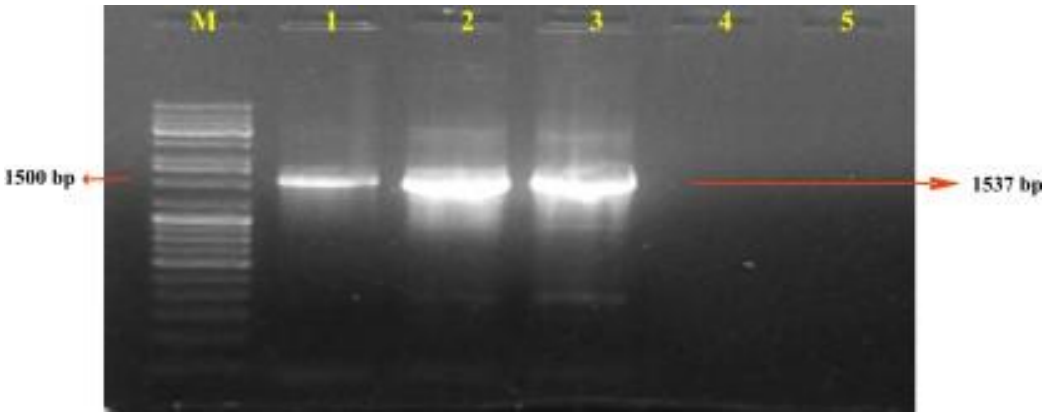
Fig. 1b: Phylogenetic relationships based on 16S rRNA gene sequence analysis of *Xap* strains from Karnataka, Andhra Pradesh and Maharashtra

respectively against *X. axonopodis* pv. *punicae* and these are significantly superior to other strains tested and there is no significant difference between these two strains followed by strain No. 139 (2.78 cm), 134 (2.72 cm) and PF5 (2.65 cm) (Table 13 and Plate 19).

Table 11: Bio-efficacy of different plant extracts against *Xap*10 under *in vitro* conditions

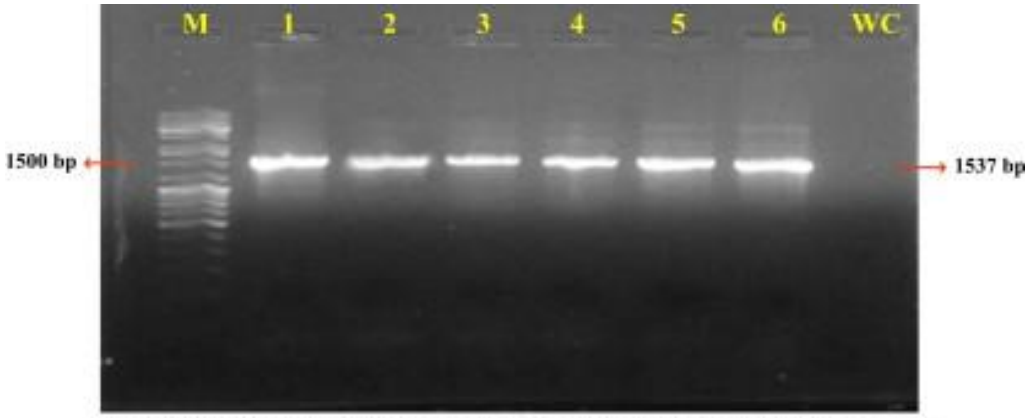
Name of plants	Parts used	Inhibition zone (mean diameter in cm)		
		5%	**10%**	**Mean**
Garlic (*Allium sativum L.*)	Clove	0.73 (1.31)*	0.90 (1.38)	0.81 (1.35)
Soapnut (*Sapindus mukorossi L.)*	Fruit	0.95 (1.40)	1.98 (1.72)	1.46 (1.57)
Meswak (*Salvadora persica L.)*	Stem	0.70 (1.26)	0.80 (1.30)	0.65 (1.28)
Adathoda (*Adathoda vessica L.)*	Leaf	0.63 (1.27)	0.83 (1.35)	0.73 (1.31)
Tulsi (*Ocimum sanctum L.)*	Leaf	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Nerium (*Nerium oleander L.)*	Leaf	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Neem (*Azadirachta indica L.)*	Leaf	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Adathoda + Soapnut *(A. vessica L.+ S. Mukorossi L.)*	Leaf + Fruit	1.00 (1.41)	1.48 (1.57)	1.24 (1.50)
Adathoda + Meswak *(A. vessica L.+ Salvadora persica L.)*	Leaf + Stem	0.68 (1.29)	0.98 (1.41)	0.83 (1.35)
Meswak + Soapnut (*Salvadora persica L. + S. mukorossi L.)*	Stem + Fruit	0.83 (1.35)	1.18 (1.47)	1.00 (1.41)
Mean		**0.49 (1.22)**	**0.73 (1.32)**	**0.61 (1.27)**
Source	S.Em **±**	**CD at 1%**		
Plant extracts (P)	0.01	0.03		
Concentration (C)	0.004	0.01		
Interaction (P × C)	0.01	0.04		

* $\sqrt{\times + 1}$ transformed values



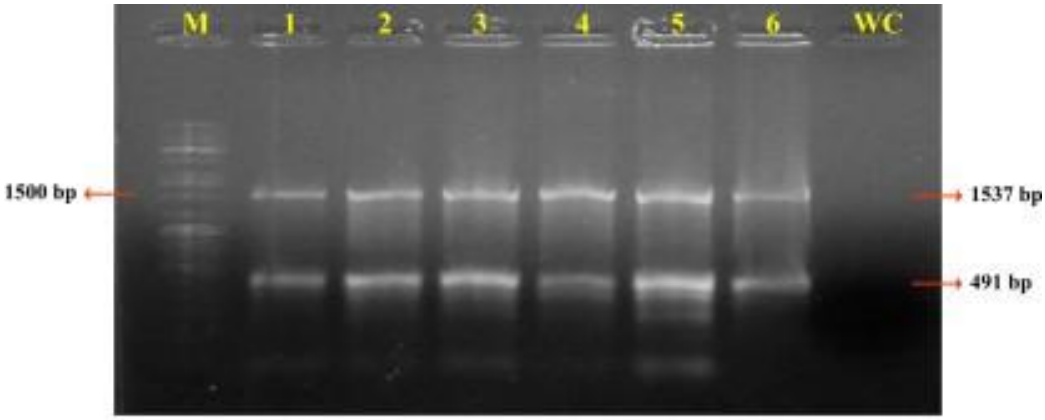
M = Medium range marker (5000 bp), 1 = Bacterial DNA (+ve control), 2 and 3= DNAs naturally infected leaf and fruit sample, 4 and 5 = healthy leaf and fruit respectively.

Plate 16: Amplification of *Xanthomonas axonopodis* pv. *punicae* using 16S rRNA primer set from naturally infected samples



M = Medium range marker (5000 bp), 1 to 6 = isolates of *Xanthomonas axonopodis* pv. *punicae* from Karnataka, Maharashtra, Andhra Pradesh, WC= Water control, respectively

Plate 17: Colony polymerase chain reaction based detection of *Xanthomonas axonopodis* pv. *punicae* by using 16S rRNA primer set



M = Medium range marker (5000 bp), 1 to 6 = isolates of *Xanthomonas axonopodis* pv. *punicae* from Karnataka, Maharashtra, Andhra Pradesh, WC= Water control, respectively

Plate 18:Multiplex polymerase chain reaction based detection of *Xanthomonas axonopodis* pv. *punicae* by using gyrB and *Xap* 16S rRNA primer set

Table 12: *In vitro* evaluation of micro and secondary nutrients against *Xap*10

Nutrient	Inhibition zone (cm) at				
	0.05%	**0.1%**	**0.25%**	**0.5%**	**Mean**
$ZnSO_4$	0.00 (1.00)*	0.00 (1.00)	0.00 (1.00)	0.63 (1.27)	0.16 (1.07)
$CuSO_4$	0.60 (1.26)	0.75 (1.32)	0.95 (1.40)	1.25 (1.50)	0.89 (1.37)
$MnSO_4$	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
$MgSO_4$	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.60 (1.26)	0.15 (1.07)
$CaSO_4$	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Boron	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Sulfur	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
$FeSO_4$	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Micron	0.00 (1.00)	0.00 (1.00)	0.65 (1.28)	1.00 (1.41)	0.41 (1.17)
Control	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Mean	**0.06 (1.03)**	**0.13 (1.05)**	**0.21 (1.09)**	**0.39 (1.17)**	**0.16 (1.07)**
Sources	**Nutrient**	**Concentration**	**Nutrients x Concentration**		
S.Em ±	0.002	0.001	0.004		
CD at 1%	0.01	0.01	0.02		

* $\sqrt{x+1}$ transformed values

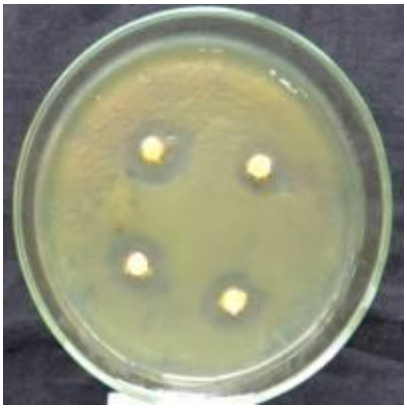
Table 13: *In vitro* evaluation of *Pseudomonas fluorescens* strains against *Xap*10

***Pseudomonas fluorescens* strains**	**Inhibition zone (mean diameter in cm)**
	Mean
134	2.73 (1.93)*
PF-5	2.65 (1.91)
326 (4)	3.10 (2.02)
PF-1	3.00 (2.00)
139	2.78 (1.94)
Mean	**2.37 (1.80)**
SEm±	0.03
CD at 1%	0.13

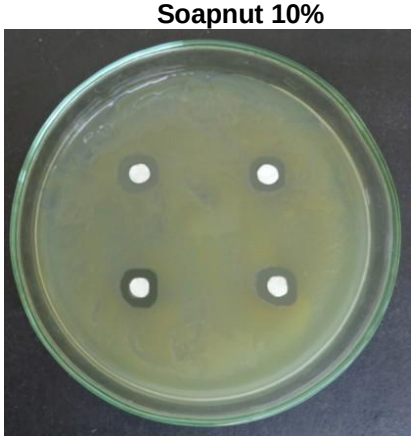
* $\sqrt{x + 1}$ transformed values

Streptocycline 0.05% + COC 0.3%

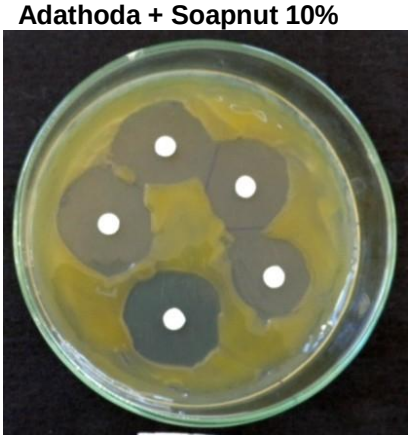
Soapnut 10%



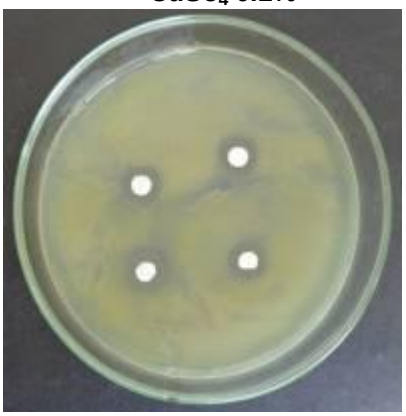
Adathoda + Soapnut 10%



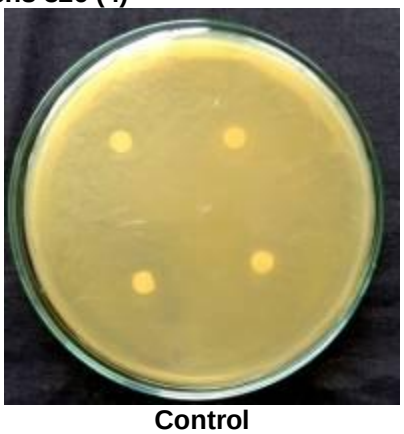
$CuSo_4$ 0.1%



Pseudomonas fluorescens 326 (4)



PPFM 10L



Control

Plate 19: *In vitro* evaluation of bactericide, botanical, bioagent and secondary micronutrient on the growth of *Xap*10

Among the eleven different strains of Pink Pigmented Facultative Methylotrophs (PPFM) tested, strain No. 10 has recorded highest inhibitory zone (0.90 cm) which is significantly superior to rest of the strains followed by 75 (0.80 cm). Other strains of PPFM were failed to inhibit the growth of *Xap* (Table 14 Plate 19).

4.6 *In vitro* evaluation of effective botanical, bioagents, nutrient and commercial formulation (5%) of copper either singly or in combination against *Xap*10

Botanicals, bioagents, nutrients and commercial formulation (5%) of copper were tested in alone under *in vitro* condition by inhibition zone assay method. Among them effective botanicals, bioagents, nutrients and commercial formulation (5%) of copper were selected in different combination to assess the combined efficacy (Table 15 and Plate 20).

Among the botanicals soapnut, among bioagents *Pseudomonas fluorescens* strain (326(4)) and PPFM strain 10L, among nutrients $CuSO_4$ and $ZnSO_4$ and commercial formulation (5%) of copper were tested in alone and in combination against *Xap* by inhibition zone assay method under *in vitro*.

COC (0.3 per cent) + Streptocycline (0.05 per cent) was selected as standard check which was given highest inhibition zone of 3.13 cm diameter and is significantly superior to rest of the treatments followed by *P. f* strain 326(4) loopfull culture in 500 µl sterilized water +soapnut at 5 per cent (2.58 cm), *P. f.* starin 326(4) loopfull culture in 500 µl sterilized water + PPFM strain 10 loopfull culture in 500 µl sterilized water (2.36 cm) and *P. f.* 326(4) loopfull culture in 500 µl sterilized water (2.29 cm). Least inhibition zone was observed in $ZnSO_4$ (0.65 cm) at 0.5 per cent.

4.7 Field evaluation of selected botanicals, microbial antagonist, commercial formulation (5%) of copper and nutrient against bacterial blight of pomegranate

4.7.1 Orchard 1

This experiment was carried out during Mrigbahar (June-December, 2012) to test the field efficacy of effective botanical, bioagent, nutrient and commercial formulation (5%) of copper, which showed better inhibition under *in vitro* condition. Individual plant was considered as each replication. Before spray infection on stem, leaf and fruits were non-significant.

a) Stem infection

Initially the orchard was affected with bacterial blight on stem with 62.50 to 70.00 per cent incidence. At the end of ninth spray minimum stem infection was observed in T_9 (COC+Streptocycline sulphate, 0.3 + 0.05%), T_5 (*Pseudomonas fluoroscens* strain 326 (4) + Soapnut, 1 + 5%) and T_4 (*Pseudomonas fluoroscens* strain 326 (4), 1%) with values of 12.5, 15.00 and 15.00 per cent respectively and significantly superior to rest of the treatments which were on par with each other followed by T_3 (Commercial formulation (5%) of copper, 0.3%) (20.00 %). The maximum disease incidence was observed (68.75%) in untreated control (T_{10}) (Table 16).

The per cent disease reduction to control was maximum (81.81%) in T_9 followed by T_5 and T_4 (78.18 %). T_3 have reduced the disease to the tune of 70.90 per cent.

Disease severity on stem was significantly reduced in T_9, T_5 and T_4 with 8.75, 10.83 and 11.25 per cent respectively and found to be on par with each other, followed by T_1 treatment (17.92%), compaired to the initial per cent disease incidence before the spray (64.16 to 52.08 per cent) (Table 17).

Per cent disease reduction to control after 9th spray was found maximum (85.53%) in T_9, followed by T_5 (82.09%). The next best treatment is T_4 where 81.40% disease reduction was noticed.

b) Leaf infection

Table 18, data indicates that initial per cent disease incidence on leaves was with a range of 45.00 to 58.75 per cent. At the end of 9th spray, T_9 has minimum per cent disease incidence of 18.75, T_5 with 20.00 per cent, these two were significantly superior to rest of the treatments and on par with each other, followed by T_4 (22.25%) and T_3 (25.00%) and they were statistically differ with each other. However, maximum per cent stem infection was 47.50 per cent in untreated control (T_{10}).

Table 14: *In vitro* evaluation of PPFM (Pink Pigmented Facultative Methylotrophs) strains against *Xap*10

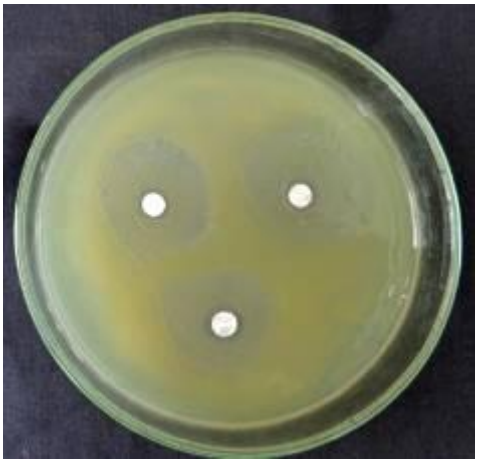
PPFM Strains	Mean inhibition zone (cm)
20	0.00 (1.00)*
71	0.00 (1.00)
32	0.00 (1.00)
10	0.90 (1.40)
75	0.80 (1.34)
42	0.00 (1.00)
23	0.00 (1.00)
80	0.00 (1.00)
26	0.00 (1.00)
38	0.00 (1.00)
H	0.00 (1.00)
Control	0.00 (1.00)
Mean	**0.15 (1.06)**
SEm±	0.006
CD at 1%	0.020

* $\sqrt{x + 1}$ transformed values

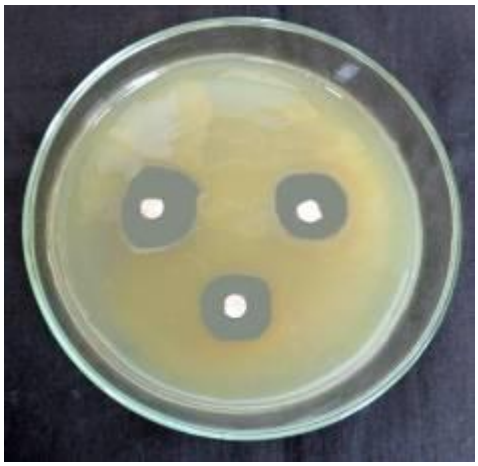
Table 15: *In vitro* evaluation of effective botanical, bioagents, nutrients and Commercial formulation (5%) of copper alone and in combination on *Xap*10

Treatment	Concentration (%)	Mean inhibition zone (cm)
$CuSO_4$ (Himedia)	0.1	1.05 (1.43)*
Commercial formulation (5%) of copper	0.3	1.21 (1.49)
$ZnSO_4$	0.25	0.65 (1.28)
Pseudomonas fluoroscens strain 326 (4)	Loopfull in 500 µl sterilized water	2.29 (1.81)
PPFM strain 10 L	Loopfull in 500 µl sterilized water	0.85 (1.36)
Soapnut	5	1.33 (1.53)
$CuSO_4$ + Soapnut	0.1 + 5	1.18 (1.48)
$ZnSO_4$ + Soapnut	0.25 + 5	0.91 (1.37)
Commercial formulation (5%) of copper+ Soapnut	0.3 + 5	1.78 (1.67)
P. fluoroscens strain 326 (4) + PPFM strain 10 L	Loopfull in 500 µl sterilized water	2.36 (1.83)
P. fluoroscens strain 326 (4) + Soapnut	Loopfull in 500 µl sterilized water + 5	2.58 (1.89)
COC + Streptocycline	0.3 + 0.05	3.13 (2.03)
Control	-	0.00 (1.00)
Mean		**1.49**
SEm±		0.041
CD @ 1%		0.161

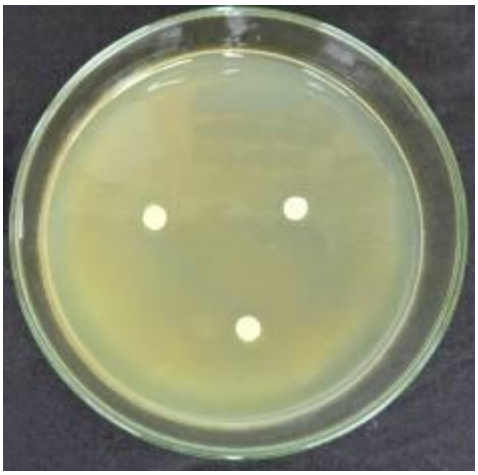
* $\sqrt{x + 1}$ transformed values



Pseudomonas fluorescens 326 (4) + Soapnut 5%



Pseudomonas fluorescens 326 (4) + PPFM (10 L)



Control

Plate 20: *In vitro* evaluation of botanical and bioagent in combination against growth of *Xap*10

Table 16: Orchard 1; Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on stem (Orchard 1)

Tr. No.	Treatment details	Dosage (%)	Per cent disease incidence on stem at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	62.50 (52.52)*	55.00 (47.91)	45.00 (42.14)	22.50 (28.24)	67.27
T_2	SN	5	65.00 (53.81)	60.00 (50.79)	45.00 (42.14)	27.50 (31.57)	60.00
T_3	5% Cu	0.3	62.50 (52.30)	52.50 (46.47)	42.50 (40.69)	20.00 (26.58)	70.90
T_4	*P. f.*	1	62.50 (52.30)	47.50 (43.58)	40.00 (39.25)	15.00 (22.51)	78.18
T_5	*P. f.* + SN	1 + 5	62.50 (52.52)	45.00 (42.14)	35.00 (36.24)	15.00 (22.51)	78.18
T_6	*P. f.* + PPFM	1 + 1	67.50 (55.69)	62.50 (52.30)	47.50 (43.58)	27.50 (31.57)	60.00
T_7	*P.f.* □ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	67.50 (55.47)	65.00 (53.81)	52.50 (46.47)	30.00 (33.23)	56.36
T_8	SN □ Cu (5%)□ *P. f.*□SN□CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	70.00 (56.97)	67.50 (55.31)	52.50 (46.47)	30.00 (33.23)	56.36
T_9	COC + SC	0.3+0.05	62.50 (52.52)	40.00 (39.25)	32.50 (34.73)	12.50 (20.48)	81.81
T_{10}	Control	-	67.50 (55.31)	68.75 (56.05)	68.25 (55.79)	68.75 (56.04)	
	SEm ± CD at 5%		2.11 NS	1.42 4.12	1.41 4.29	1.62 4.68	

* Figures in the parenthesis are arc sin transformed values

CS=Copper Sulfate; SN=Soapnut; 5% Cu= 5% Copper Commercial Formulation; *P. f. = Pseudomonas fluorescens* Strain 326(4)*;* PPFM= Pink Pigmented Facultative Methylotroph Strain 10; COC+SC=Copperoxychloride+Streptocycline

The per cent disease reduction to control, it was maximum of 60.53 per cent in T_9 followed by T_5 (57.89%) and T_4 (52.63%).

Table 17: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on stem (Orchard 1)

Tr. No.	Treatment details	Dosage (%)	Per cent disease index on stem at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	54.58 (47.70)*	47.92 (43.82)	35.42 (36.52)	17.92 (24.91)	70.38
T_2	SN	5	56.66 (48.87)	52.50 (46.47)	39.58 (38.95)	20.42 (26.82)	66.24
T_3	5% Cu	0.3	57.50 (49.41)	48.33 (44.07)	35.42 (36.52)	18.33 (25.34)	69.70
T_4	*P. f.*	1	52.08 (46.22)	39.58 (38.99)	30.00 (33.19)	11.25 (19.32)	81.40
T_5	*P. f.* + SN	1 + 5	52.08 (46.26)	37.50 (37.75)	24.58 (29.70)	10.83 (19.11)	82.09
T_6	*P. f.* + PPFM	1 + 1	64.16 (53.51)	60.00 (50.86)	41.67 (40.19)	22.92 (28.54)	62.11
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	58.75 (50.10)	56.67 (48.87)	41.67 (40.19)	22.50 (28.30)	62.80
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	60.83 (51.30)	58.75 (50.07)	43.75 (41.42)	23.75 (29.16)	60.74
T_9	COC + SC	0.3+0.05	52.08 (46.26)	33.33 (35.28)	22.92 (28.57)	8.75 (17.04)	85.53
T_{10}	Control	-	59.17 (50.37)	59.08 (50.32)	60.00 (50.79)	60.50 (51.09)	
	SEm ± CD at 5%		2.31 NS	1.69 4.89	1.48 4.27	1.38 3.99	

* Figures in the parenthesis are arc sin transformed values

Table 18: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on leaves (Orchard 1)

Tr. No.	Treatment details	Dosage	Per cent disease incidence on leaves at different spray intervals				% disease reduction
		(%)	Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	51.25 (45.75)*	38.75 (38.51)	36.25 (37.03)	25.00 (30.02)	47.37
T_2	SN	5	45.00 (42.11)	42.50 (40.70)	41.25 (39.98)	33.75 (35.52)	28.95
T_3	5% Cu	0.3	46.25 (42.84)	33.75 (35.52)	31.25 (33.99)	25.00 (30.02)	47.37
T_4	*P. f.*	1	46.25 (42.78)	32.50 (34.76)	28.75 (32.42)	22.25 (28.30)	52.63
T_5	*P. f.* + SN	1 + 5	46.25 (42.78)	30.00 (33.19)	27.50 (31.62)	20.00 (26.58)	57.90
T_6	*P. f.* + PPFM	1 + 1	52.50 (46.53)	41.25 (39.98)	35.00 (36.29)	27.50 (31.62)	42.11
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	52.50 (46.47)	43.75 (41.43)	37.50 (37.77)	31.25 (33.99)	34.21
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	55.00 (47.91)	48.75 (44.31)	42.50 (40.70)	35.00 (36.26)	26.32
T_9	COC + SC	0.3+0.05	47.50 (43.52)	27.50 (31.62)	25.00 (30.02)	18.75 (25.63)	60.53
T_{10}	Control	-	58.75 (50.07)	57.50 (49.35)	51.25 (45.74)	47.50 (43.59)	
	SEm ±		1.99	0.94	0.72	0.78	
	CD at 5%		NS	2.7	2.07	2.27	

* Figures in the parenthesis are arc sin transformed values

Table 19: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on leaves (Orchard 1)

Tr. No.	Treatment details	Dosage (%)	Per cent disease index on leaves at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	49.25 (44.55)*	36.75 (37.31)	32.75 (34.87)	20.00 (26.58)	54.74
T_2	SN	5	43.25 (41.03)	40.25 (39.39)	35.00 (36.27)	27.00 (31.31)	40.22
T_3	5% Cu	0.3	44.50 (41.76)	32.00 (34.44)	28.25 (32.05)	18.75 (25.63)	58.65
T_4	*P. f.*	1	38.50 (37.94)	26.00 (30.66)	25.75 (30.49)	15.50 (23.19)	65.36
T_5	*P. f.* + SN	1 + 5	44.50 (41.70)	25.50 (30.28)	20.50 (26.91)	13.00 (21.11)	74.30
T_6	*P. f.* + PPFM	1 + 1	50.50 (45.33)	39.00 (38.66)	31.50 (34.13)	22.00 (27.96)	50.83
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	50.25 (45.16)	41.75 (40.23)	35.50 (36.55)	26.50 (30.97)	40.78
T_8	SN □ Cu (5%)□ *P.f.*□SN□CS□COC + SS □ Cu (5%)□ SN□ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	52.50 (46.47)	46.50 (42.99)	40.50 (39.51)	29.75 (33.02)	34.07
T_9	COC + SC	0.3+0.05	41.00 (39.49)	23.25 (28.82)	18.75 (25.63)	12.00 (20.28)	73.18
T_{10}	Control	-	59.00 (50.31)	58.00 (49.73)	51.25 (45.74)	45.25 (42.27)	
	SEm ± CD at 5%		1.86 5.40	1.74 5.04	1.33 3.84	0.9 2.83	

* Figures in the parenthesis are arc sin transformed values

Table 20: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on fruits (Orchard 1)

Tr. No.	Treatment details	Dosage (%)	Per cent disease incidence on fruits at different spray intervals			% disease reduction
			After 4 sprays	After 6sprays	After 9sprays	
T_1	CS	0.1	42.50 (40.69)	35.00 (36.24)	24.50 (28.24)	54.63
T_2	SN	5	47.50 (43.58)	37.50 (37.75)	30.00 (33.23)	44.44
T_3	5% Cu	0.3	37.50 (37.75)	32.50 (34.73)	24.50 (28.24)	54.63
T_4	*P. f.*	1	37.50 (37.75)	30.00 (33.23)	20.00 (26.58)	62.96
T_5	*P. f.* + SN	1 + 5	37.50 (37.75)	30.00 (33.23)	16.50 (24.54)	69.44
T_6	*P. f.* + PPFM	1 + 1	42.50 (40.69)	37.50 (37.75)	27.50 (31.57)	49.07
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	52.50 (46.47)	47.50 (43.58)	37.50 (37.75)	30.56
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	55.00 (47.91)	45.00 (42.14)	35.00 (36.24)	35.19
T_9	COC + SC	0.3+0.05	37.50 (37.75)	20.00 (26.58)	12.50 (20.48v	76.85
T_{10}	Control	-	52.50 (46.47)	53.00 (46.75)	54.00 (47.32)	
	SEm ± CD at 5%		1.49 4.31	1.32 3.82	1.48 4.27	

* Figures in the parenthesis are arc sin transformed values

T_9 : Streptocycline 0.05% + COC 0.3%

T_5 : Pseudomonas fluorescens 326 (4) 1% + Soapnut 5%

T_4 : Pseudomonas fluorescens 326 (4) 1%

Untreated

Plate 21: Field efficacy of effective botanical, bioagent and bactericide

Table 21: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on fruits (Orchard 1)

Tr. No.	Treatment details	Dosage (%)	Per cent disease incidence on fruits at different spray intervals			% disease reduction
			After 4 sprays	After 6sprays	After 9sprays	
T_1	CS	0.1	30.00 (33.10)	26.00 (30.63)	15.50 (23.14)	70.36
T_2	SN	5	35.50 (36.55)	26.50 (30.85)	22.50 (28.29)	56.10
T_3	5% Cu	0.3	28.00 (31.91)	31.00 (33.76)	21.50 (27.49)	58.05
T_4	*P. f.*	1	36.00 (36.78)	22.50 (28.29)	10.50 (22.76)	79.51
T_5	*P. f.* + SN	1 + 5	22.50 (28.29)	16.50 (23.91)	9.50 (17.80)	81.46
T_6	*P. f.* + PPFM	1 + 1	40.50 (39.50)	35.50 (36.55)	26.00 (30.60)	49.27
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	39.50 (38.90)	35.50 (36.55)	28.00 (31.91)	45.37
T_8	SN □ Cu (5%)□ *P.f.*□SN□CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	41.50 (40.06)	33.50 (35.35)	26.50 (30.85)	48.29
T_9	COC + SC	0.3+0.05	20.50 (26.85)	11.00 (19.32)	7.00 (15.05)	86.34
T_{10}	Control	-	50.00 (45.02)	50.75 (45.45)	51.25 (45.74)	
	SEm ± CD at 5%		2.03 5.86	1.53 4.41	1.55 4.48	

* Figures in the parenthesis are arc sin transformed values

Table 22: Influence of management treatments for bacterial blight of pomegranate on fruit yield (Orchard 1)

Tr. No.	Treatment details	Per cent disease index on fruit after 9th spray	Total number of fruits/plant	Total number of healthy fruits/plant	Average weight of the fruit (g)	Fruit yield/ tree (kg)	Estimated fruit yield (tones/ha)
T_1	CS	15.50	107.00	54.00	262.25	14.16	10.28
T_2	SN	22.50	97.00	52.50	247.50	12.99	9.43
T_3	5% Cu	21.50	101.50	51.00	262.75	13.40	9.73
T_4	*P. f.*	10.50	112.00	58.00	285.00	16.52	12.00
T_5	*P. f.* + SN	9.50	113.50	60.00	277.50	16.65	12.09
T_6	*P. f.* + PPFM	26.00	96.50	50.50	241.00	12.17	8.83
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	28.00	90.00	48.50	235.75	11.44	8.30
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	26.50	91.00	56.00	236.25	13.23	9.61
T_9	COC + SC	7.00	116.00	67.00	292.75	19.60	14.23
T_{10}	Control	51.25	111.00	32.50	201.50	6.61	4.80
	SEm ±	1.55	0.91	1.48	3.99	0.42	0.31
	CD at 5%	4.48	2.65	4.30	11.57	1.23	0.89

T_9 :Streptocycline 0.05% + CoC 0.3%

T_5: Pseudomonas 326 (4) 1% + Soapnut 5%

T_4: Pseudomonas 326 (4) 1%

Untreated

Plate 22: Field efficacy of effective botanical, bioagent and bactericide

Table 19 indicated that before spray the initial per cent disease severity on leaves in the orchard was 38.50 to 59.00 per cent. After 9th spray lowest per cent disease severity was observed in T_9 (12.00%), T_5 (13.00%) and T_4 (15.50%). They were on par with each other and significantly superior to other treatments. The per cent disease severity on leaves was maximum (45.25%) in untreated control. The per cent disease reduction to control was maximum in T_5 treatment (74.30%) followed by T_9 (73.18%).

c) Fruit infection

The per cent disease incidence before spray on fruit was in the range of 37.5 to 52.5 per cent in the orchard (Table 20). At the end of 9th spray per cent disease incidence on the fruit was lowest in T_9 (12.50%) which is significantly superior to rest of the treatments. Followed by T_5 (16.50%) and T_4 (20.00%) significantly they are on par with each other followed by T_3 (24.50%) and T_1 (24.50%). The maximum per cent fruit infection was observed in untreated control plot (54.00%). Per cent disease reduction to initial disease was maximum in T_9 (76.85%) and T_5 (69.44%), followed by T_4 treatment (62.96%) (Plate 21).

Table 21, indicated that in untreated control, the per cent disease severity on fruits was attained maximum of (51.25%), it was observed minimum in T_9 (7.00%), T_5 (9.50%) and T_4 (10.50%), which were on par with each other and significantly superior to rest of the treatments followed by T_1 (15.50%). Per cent disease reduction to control was maximum in T_9 (86.34%), T_5 (81.46%) and T_4 (79.51%) followed by T_1 (70.36%).

d) Yield estimation

The maximum disease severity was observed in untreated control (38.00%) after 9^{th} spray. While minimum disease severity was found in T_9 (7.00%) and T_5 (9.50%) and T_4 (10.50%) which were on par with each other and significantly superior to other treatments followed by T_1 (15.50) (Table 22 and Plate 22).

The total number of fruits/plant was maximum in T_9 (116.00), T_5 (113.00) and T_4 (112.00) which were on par each other and significantly superior to other treatments, followed by T_1 (107.00). The data indicated that total number of fruits per plant was not affected by the different treatments. However, number of healthy fruits per plant was maximum in T_9 (67.00) which is significantly superior to rest of the treatments followed by T_4 (60.00), T_5 (58.00) and T_8 (56.00) they were on par with each other.

Average weight of fruit was maximum in T_9 (292.75 g) followed by T_5 (285.00 g), T_4 (277.5 g) and they significantly differed with each other but superior to rest of the treatments. Fruit yield per tree was maximum in T_9 (19.60 kg) followed by T_5 (16.65 kg) and T_4 (16.52 kg). Estimated fruit yield per tree was maximum in T_9 (14.23 kg), T_5 (12.09 kg) and T_4 (12.00 kg).

4.7.2 Orchard 2

a) Stem infection

Initially the orchard was affected with bacterial blight on stem with 55.00 to 62.50 per cent incidence. At the end of ninth spray maximum per cent disease incidence was observed in untreated control (62.00%). Minimum stem infection was observed in T_9 (10.00), T_5 (12.50) and T_4 (15.00) they are on par with each other and significantly differed from other treatments (Table 23).

Maximum disease reduction was observed in T9 (83.87%) to control followed by T_5 (79.84%) and T_4 (75.81%).

The per cent disease severity on stem was in the range of 48.33 to 54.58 per cent before spray (Table 24). After 9^{th} spray per cent disease severity on stem significantly reduced in T_9, T_5 and T_4 with 7.08, 9.17 and 10.83 per cent, respectively and they were on par with each other.

Maximum disease reduction was observed in T_9 (86.83%) to control after 9^{th} spray followed by T_5 (82.94%), T_4 (79.85%).

b) Leaf infection

The per cent disease incidence on leaves was with a range of 47.50 to 53.75 per cent (Table 25) before spray. At the end of 9th spray, T_9 has minimum per cent disease incidence of 13.75, T_5 with 15.00 per cent and T_4 with 17.00 per cent, these three were significantly superior to rest of the treatments and on par with each other. However, maximum per cent disease incidence on leaves was

Table 23: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on stem (Orchard 2)

Tr. No.	Treatment details	Dosage (%)	Per cent disease incidence on stem at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	55.00 (47.91)*	47.50 (43.58)	35.00 (36.24)	20.00 (26.57)	67.74
T_2	SN	5	60.00 (50.79)	55.00 (47.91)	40.00 (39.25)	22.50 (28.24)	63.71
T_3	5% Cu	0.3	55.00 (47.91)	47.50 (43.58)	37.50 (37.75)	17.50 (24.54)	71.77
T_4	*P. f.*	1	57.50 (49.35)	42.50 (40.69)	27.50 (31.57)	15.00 (22.51)	75.81
T_5	*P. f.* + SN	1 + 5	57.50 (49.35)	37.50 (37.75)	25.00 (29.90)	12.50 (20.47)	79.84
T_6	*P. f.* + PPFM	1 + 1	60.00 (50.86)	52.50 (46.47)	37.50 (37.75)	22.50 (28.24)	63.71
T_7	*P.f.*□SN□5%Cu□*P. f.*□COC + SC □ CS□ *P. f.*□ 5% Cu□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	60.00 (50.79)	55.00 (47.91)	40.00 (39.25)	25.00 (29.90)	59.68
T_8	SN□5%Cu□*P.f.*□SN□CS□COC + SC □ Cu (5%)□ SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	62.50 (52.30)	52.50 (46.47)	42.50 (40.69)	25.00 (29.90)	59.68
T_9	COC + SC	0.3+0.05	57.50 (49.35)	35.00 (36.24)	22.50 (28.24)	10.00 (18.44)	83.87
T_{10}	Control	-	60.00 (50.79)	60.25 (50.94)	61.25 (51.53)	62.00 (51.97)	
	SEm ±		1.30 NS	1.53	1.45	1.63	
	CD at 5%				4.41	4.18	4.72

* Figures in the parenthesis are arc sin transformed values

Table 24: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on stem (Orchard 2)

Tr. No.	Treatment details	Dosage (%)	Per cent disease index on stem at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	48.33 (44.07)*	41.67 (40.19)	27.92 (31.79)	15.83 (23.44)	70.55
T_2	SN	5	52.50 (46.47)	48.33 (44.07)	35.00 (36.27)	16.67 (24.06)	68.99
T_3	5% Cu	0.3	50.00 (45.02)	43.75 (41.39)	31.25 (33.97)	16.25 (23.51)	69.77
T_4	*P. f.*	1	49.00 (44.45)	35.42 (36.52)	20.42 (26.82)	10.83 (19.11)	79.85
T_5	*P. f.* + SN	1 + 5	48.75 (44.31)	31.25 (33.97)	17.92 (24.86)	9.17 (17.49)	82.94
T_6	*P. f.* + PPFM	1 + 1	54.50 (47.61)	50.42 (45.27)	32.92 (34.96)	18.75 (25.58)	65.12
T_7	*P.f.*□SN□5%Cu□*P.f.* □COC + SC □ CS□*P.f.*□5% Cu□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	52.50 (46.47)	47.92 (43.82)	31.67 (34.24)	19.17 (25.72)	64.33
T_8	SN□5%Cu□*P.f.*□SN□CS □COC + SC □ Cu (5%)□ SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	54.58 (47.67)	46.25 (42.87)	35.42 (36.52)	20.00 (26.39)	62.79
T_9	COC + SC	0.3+0.05	49.25 (44.59)	29.17 (32.65)	15.83 (23.39)	7.08 (15.42)	86.83
T_{10}	Control	-	52.50 (46.47)	52.75 (46.60)	53.25 (46.89)	53.75 (47.17)	
	SEm ±		1.01	1.98	1.6	1.58	
	CD at 5%		NS	5.71	4.64	4.55	

* Figures in the parenthesis are arc sin transformed values

Table 25: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on leaves (Orchard 2)

Tr. No.	Treatment details	Dosage	Per cent disease incidence on leaves at different spray intervals				% disease reduction
		(%)	Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	47.50 (43.59)*	30.00 (33.23)	22.50 (28.30)	19.50 (24.69)	52.7
T_2	SN	5	50.00 (45.02)	36.25 (37.03)	28.75 (32.42)	25.00 (30.02)	39.39
T_3	5% Cu	0.3	48.75 (44.31)	30.00 (33.23)	25.00 (30.02)	21.25 (27.44)	48.48
T_4	*P. f.*	1	47.50 (43.59)	28.75 (32.42)	20.00 (26.58)	17.00 (24.69)	57.58
T_5	*P. f.* + SN	1 + 5	48.75 (44.31)	27.50 (31.62)	18.75 (25.63)	15.00 (22.80)	63.64
T_6	*P. f.* + PPFM	1 + 1	47.50 (43.58)	38.75 (38.51)	30.00 (33.23)	25.00 (30.02)	39.39
T_7	*P.f.*□SN□5%Cu□*P. f.* □COC + SC □ CS□ *P.f.*□5% Cu□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	50.00 (45.02)	40.00 (39.25)	31.25 (33.96)	28.75 (32.42)	30.30
T_8	SN□5%Cu□*P.f.*□SN□ CS□COC + SC□5%Cu□SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	47.50 (43.59)	45.00 (42.15)	36.25 (37.03)	32.50 (34.76)	21.21
T_9	COC + SC	0.3+0.05	48.75 (44.31)	25.00 (30.02)	16.25 (23.74)	13.75 (21.71)	66.67
T_{10}	Control	-	53.75 (47.18)	50.00 (45.02)	48.75 (44.31)	41.25 (39.98)	
	SEm ±		NS	0.51	0.82	0.79	
	CD at 5%		NS	1.47	2.37	2.28	

* Figures in the parenthesis are arc sin transformed values

41.25 per cent in untreated control (T_{10}).Maximum disease reduction was observed in T_9 (66.67%) to control followed by T_5 (63.64%) and T_4 (57.58%).

The per cent disease severity on leaves in the orchard was 45.25 to 53.50 per cent (Table 26). After 9th spray lowest per cent disease severity was observed in T_9 (8.75%), T_5 (9.75%), T_4 (11.00%), which were on par with each other and significantly superior to rest of the treatments. The per cent disease severity on leaves was maximum (39.25%) in untreated control. The per cent disease reduction to control was maximum in T_9 treatment (77.71%), T_5 (75.16%) and T_3 (71.97%).

c) Fruit infection

At the end of 9th spray per cent disease incidence on the fruit was lowest in T_9 (10.00%) and T_5 (12.50%) which were significantly superior to rest of the treatments and on par with each other, followed by T_4 (17.50%), T_3 (22.00%) and T_1 (22.00%). The maximum per cent fruit infection was observed in untreated control plot (52.75%). Per cent disease reduction to initial disease was maximum in T_9 (80.58%) and T_5 (75.73%), followed by T_4 treatment (66.02%) (Table 27).

Table 28, indicated that at the end of 9^{th} spray, disease has reduced in T_9 (5.50%) and T_5 (7.00%) which was significantly superior to rest of the treatments and they were on par with each other. Next best treatments were T_4 (13.00%), T_1 (14.00%) and T_3 (19.00%). Per cent disease reduction to control was maximum in T_9 (88.78%), T_5 (85.71%), T_4 (73.47%) followed by T_1 (71.43%).

d) Yield estimation

Per cent disease severity after 9^{th} spray was maximum in untreated control (40.50%). The minimum per cent disease severity was found in T_9 (5.50%) followed by T_5 (7.00%), they are significantly superior to rest of the treatments and on par with each other. T_4 (13.00%) and T_1 (14.00%) were next best treatments (Table 29).

The total number of fruits/plant was maximum in T_9 (70.25) followed by T_8 (69.00), T_5 (68.75) and T_4 (65.28). The data indicated that total number of fruits per plants was not affected by the different treatments. However number of healthy fruits per plant was maximum in T_9 (60.00) followed by T_5 (56.5), T_8 (55.75), T_4 (51.25) and T_7 (50.25), they were significantly superior to rest of the treatments and on par with each other.

Average weight of fruit was maximum in T_9 (412.5 g) followed by T_5 (402.0 g), T_4 (400.0 g), T_8 (393.8 g) and significantly superior to rest of the treatments. Fruit yield per tree was maximum in T_9 (19 kg) followed by T_5 (18.5), T_4 (17.75 kg), they are significantly superior to rest of the treatments and on par with each other.

4.7.3 Pooled data

Pooled data of influence of botanical, bioagent, nutrient, bactericide and commercial formulation (5%) of copper on bacterial blight of pomegranate tested at two location presented below.

a) Stem infection

Pooled data obtained after 9^{th} spray revealed that stem infection was significantly very less in the T_9 (11.25%), T_5 (13.75%) and T_4 (15.00%). They were significantly superior to rest of the treatments and on par with each other. Per cent disease incidence in untreated control (T_{10}) was 65.38% (Table 30a).

Per cent disease severity on stem at the end of 9^{th} spray was minimum in T_9 (7.92%), T_5 (10.00%) and T_4 (11.04%). They were significantly superior to rest of the treatments and on par with each other. Per cent disease incidence in untreated control (T_{10}) was 57.13% (Table 30b).

b) Leaf infection

Per cent leaf infection after 9^{th} spray was minimum in T_9 (16.25%) and T_5 (17.50%) which were significantly superior to rest of the treatments followed by T_4 (20.00%). Maximum per cent disease incidence was found in untreated control (44.38%) (Table 30c).

Per cent disease severity on leaf at the end of 9^{th} spray was minimum in T_9 (10.38%), T_5 (11.38), T_4 (13.75%) which were significantly superior to rest of the treatments and on par with each other followed by T_1 (17.00%). Maximum per cent disease severity was observed in untreated control (42.25%) (Table 30d).

c) Fruit infection

Table 26: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on leaves (Orchard 2)

Tr. No.	Treatment details	Dosage (%)	Per cent disease index on leaf at different spray intervals				% disease reduction
			Before spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	47.50 (43.59)*	28.50 (32.26)	20.25 (26.69)	14.00 (21.93)	64.33
T_2	SN	5	47.50 (43.59)	34.25 (35.83)	24.50 (29.63)	20.00 (26.57)	49.04
T_3	5% Cu	0.3	46.25 (42.86)	28.50 (32.26)	22.50 (28.30)	16.00 (23.50)	59.24
T_4	*P. f.*	1	47.50 (43.59)	23.00 (28.66)	18.00 (25.08)	11.00 (20.27)	71.97
T_5	*P. f.* + SN	1 + 5	46.25 (42.86)	23.50 (28.93)	14.00 (21.93)	9.75 (18.16)	75.16
T_6	*P. f.* + PPFM	1 + 1	45.50 (42.38)	36.75 (37.31)	27.00 (31.29)	20.00 (26.57)	49.04
T_7	*P.f.*□SN□5%Cu□*P. f.* □COC + SC □ CS□ *P. f.*□ 5% Cu□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	47.50 (43.58)	38.00 (38.06)	26.25 (30.76)	24.50 (29.62)	37.58
T_8	SN□5%Cu□*P.f.*□SN□CS □COC + SC □ Cu (5%)□ SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	45.25 (42.27)	42.75 (40.84)	34.50 (35.95)	27.75 (31.73)	29.3
T_9	COC + SC	0.3+0.05	45.00 (42.14)	21.25 (27.44)	12.25 (20.40)	8.75 (17.20)	77.71
T_{10}	Control	-	53.50 (47.04)	50.00 (45.02)	48.75 (44.31)	39.25 (38.78)	
	SEm ± CD at 5%		1.09 NS	1.26 3.64	1.2 3.46	1.01 2.92	

* Figures in the parenthesis are arc sin transformed values

Table 27: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on fruits (Orchard 2)

Tr. No.	Treatment details	Dosage (%)	Per cent disease incidence on fruit at different spray intervals			% disease reduction
			After 4 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	35.00 (36.24)	27.50 (31.57)	22.00 (26.58)	57.28
T_2	SN	5	40.00 (39.25)	32.50 (34.73)	27.50 (31.57)	46.60
T_3	5% Cu	0.3	32.50 (34.73)	25.00 (29.90)	22.00 (26.58)	57.28
T_4	*P. f.*	1	30.00 (33.23)	22.50 (28.24)	17.50 (24.54)	66.02
T_5	*P. f.* + SN	1 + 5	27.50 (31.57)	17.50 (24.54)	12.50 (20.48)	75.73
T_6	*P. f.* + PPFM	1 + 1	35.00 (36.24)	32.50 (34.73)	25.00 (29.90)	51.46
T_7	*P.f.*□SN□5%Cu□*P. f.*□COC + SC □ CS□ *P. f.*□ 5% Cu□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	45.00 (42.14)	40.00 (39.25)	35.00 (36.24)	32.04
T_8	SN□5%Cu□*P.f.*□SN□CS □COC + SC □ Cu (5%)□ SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	47.50 (43.58)	37.50 (37.75)	32.50 (34.73)	36.89
T_9	COC + SC	0.3+0.05	25.00 (29.90)	15.00 (22.51)	10.00 (18.44)	80.58
T_{10}	Control	-	50.00 (45.02)	52.50 (46.47)	52.75 (46.60)	
	SEm ± CD at 5%		1.4 4.05	1.69 4.87	1.49 4.29	

* Figures in the parenthesis are arc sin transformed values

Table 28: Field evaluation of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease severity on fruits (Orchard 2)

Tr. No.	Treatment details	Dosage (%)	Per cent disease index on fruit at different spray intervals			% disease reduction
			After 4 sprays	After 6 sprays	After 9 sprays	
T_1	CS	0.1	24.50 (29.57)	21.00 (27.08)	14.00 (21.93)	71.43
T_2	SN	5	30.00 (33.19)	23.00 (28.51)	20.50 (26.85)	58.16
T_3	5% Cu	0.3	24.50 (29.57)	23.50 (28.93)	19.00 (25.83)	61.22
T_4	*P. f.*	1	28.50 (32.26)	16.50 (23.97)	13.00 (20.97)	73.47
T_5	*P. f.* + SN	1 + 5	16.50 (23.91)	9.50 (17.80)	7.00 (15.05)	85.71
T_6	*P. f.* + PPFM	1 + 1	33.00 (35.04)	30.50 (33.54)	24.00 (29.16)	51.02
T_7	*P.f.*□SN□5%Cu□*P. f.*□ COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	1□5□0.3□1□ 0.3+0.05 □0.1□1□0.3□ 5	33.50 (35.35)	30.00 (33.19)	26.50 (30.85)	45.92
T_8	SN□5%Cu□*P.f.*□SN□CS □COC + SC □ Cu (5%)□ SN□*P.f.*	5□ 0.3□1□0.1□0.3+0.05 □0.3□5□1	35.50 (36.55)	28.00 (31.91)	24.50 (29.57)	50.00
T_9	COC + SC	0.3+0.05	14.00 (21.74)	8.00 (16.27)	5.50 (13.53)	88.78
T_{10}	Control	-	47.50 (43.58)	48.25 (44.02)	49.00 (44.45)	
	SEm ± CD at 5%		1.56 4.49	1.52 4.38	1.72 4.98	

* Figures in the parenthesis are arc sin transformed values

Table 29: Influence of management treatments for bacterial blight of pomegranate on fruit yield (Orchard 2)

Tr. No.	Treatment details	Per cent disease index on fruit after 9th spray	Total number of fruits/plant	Total number of healthy fruits/plant	Average weight of the fruit (g)	Fruit yield/ tree (kg)	Estimated fruit yield (tons/ha)
T_1	CS	14.00	60.50	38.00	367.5	14.50	9.67
T_2	SN	20.50	60.00	40.00	380.0	15.50	10.33
T_3	5% Cu	19.00	59.75	44.75	373.8	16.00	10.67
T_4	*P. f.*	13.00	65.25	51.25	400.0	17.75	11.83
T_5	*P. f.* + SN	7.00	68.75	56.50	402.5	18.50	12.33
T_6	*P. f.* + PPFM	24.00	60.50	44.00	369.0	14.50	9.67
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	26.50	63.75	50.25	385.0	14.00	9.33
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	24.50	69.00	55.75	393.8	15.00	10.00
T_9	COC + SC	5.50	70.25	60.00	412.5	19.00	12.67
T_{10}	Control	49.00	61.5	26.5	383.8	8.50	5.67
	SEm ±	1.72	1.24	1.74	3.99	0.53	
	CD at 5%	4.98	4.43	5.04	11.51	1.53	

Table 30a: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by*Xap*: Per cent disease incidence on stem: (pooled data)

Treatment	Per cent Disease Incidence on stem at different spray intervals				Mean
	Before Spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	58.75 (50.21)*	51.25 (45.74)	40.00 (39.19)	21.25 (27.41)	42.88 (40.75)
T_2	62.50 (52.30)	57.50 (49.35)	42.50 (40.69)	25.00 (29.90)	46.75 (43.06)
T_3	58.75 (50.10)	50.00 (45.02)	40.00 (39.22)	18.75 (25.56)	41.75 (40.05)
T_4	60.00 (50.83)	45.00 (42.14)	33.75 (35.41)	15.00 (22.51)	37.63 (37.49)
T_5	60.00 (50.94)	41.25 (39.94)	30.00 (33.07)	13.75 (21.49)	34.75 (35.70)
T_6	63.75 (53.27)	57.50 (49.38)	42.50 (40.66)	25.00 (29.90)	47.13 (43.31)
T_7	63.75 (53.13)	60.00 (50.86)	46.25 (42.86)	27.50 (31.57)	50.38 (45.22)
T_8	66.25 (54.64)	60.00 (50.89)	47.50 (43.58)	27.50 (31.57)	50.75 (45.45)
T_9	60.00 (50.94)	37.50 (37.75)	27.50 (31.49)	11.25 (19.46)	32.13 (33.99)
T_{10}	63.75 (53.05)	64.50 (53.46)	64.75 (53.61)	65.38 (53.98)	64.55 (53.49)
S.Em±	1.47	1.07	0.89	1.26	
CD @ 5%	4.28	3.10	2.58	3.66	

* Figures in the parenthesis are arc sin transformed values

Table 30b: Per cent disease severity on stem (pooled data)

Treatment	Per cent Disease Index on stem at different spray intervals				Mean
	Before Spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	51.46 (45.88)*	44.79 (42.01)	31.67 (34.15)	16.88 (24.18)	36.17 (36.67)
T_2	54.58 (47.67)	50.42 (45.27)	37.29 (37.61)	18.54 (25.44)	40.29 (39.16)
T_3	53.75 (47.22)	46.04 ()42.73	33.33 (35.24)	17.29 (24.43)	36.92 (37.14)
T_4	50.00 (45.02)	37.50 (37.75)	25.21 (30.01)	11.04 (19.22)	30.02 (32.73)
T_5	50.00 (45.04)	34.38 (35.86)	21.25 (27.28)	10.00 (18.30)	27.33 (30.94)
T_6	60.63 (51.31)	55.21 (48.06)	37.29 (37.57)	20.83 (27.06)	43.02 (40.82)
T_7	55.63 (48.28)	52.29 (46.34)	36.67 (37.21)	20.83 (27.01)	41.63 (39.99)
T_8	57.71 (49.48)	52.50 (46.47)	39.58 (38.97)	21.88 (27.77)	43.06 (40.86)
T_9	50.00 (45.04)	31.25 (33.97)	19.38 (25.98)	7.92 (16.23)	25.94 (29.90)
T_{10}	55.83 (48.32)	55.92 (48.42)	56.63 (48.83)	57.13 (49.12)	56.30 (48.64)
S.Em±	1.45	1.54	1.24	1.19	
CD @ 5%	4.21	4.47	3.6	3.46	

* Figures in the parenthesis are arc sin transformed values

Table 30c: Per cent disease incidence on leaf (pooled data)

Treatment	Per cent Disease Incidence on leaves at different spray intervals				Mean
	Before Spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	49.38 (44.67)*	34.38 (35.87)	29.38 (32.66)	21.25 (27.35)	32.81 (34.73)
T_2	47.50 (43.57)	39.38 (38.87)	35.00 (36.20)	29.38 (32.77)	37.06 (37.43)
T_3	47.50 (43.57)	31.88 (34.38)	28.13 (32.00)	23.13 (28.73)	31.31 (33.91)
T_4	46.88 (43.18)	30.63 (33.59)	24.38 (29.50)	20.00 (26.49)	28.69 (32.18)
T_5	47.50 (43.54)	28.75 (32.41)	23.13 (28.63)	17.50 (24.69)	26.88 (30.95)
T_6	50.00 (45.05)	40.00 (39.24)	32.50 (34.76)	26.25 (30.82)	36.06 (36.83)
T_7	51.25 (45.75)	41.88 (40.34)	34.38 (35.86)	30.00 (33.21)	38.38 (38.22)
T_8	51.25 (45.75)	46.88 (43.23)	39.38 (38.87)	33.75 (35.51)	42.63 (40.74)
T_9	48.13 (43.91)	26.25 (30.82)	20.63 (26.88)	16.25 (23.67)	25.06 (29.71)
T_{10}	56.25 (48.62)	53.75 (47.19)	50.00 (45.02)	44.38 (41.78)	51.44 (45.85)
S.Em±	1.02	0.59	0.43	0.5	
CD @ 5%	2.97	1.71	1.24	1.45	

* Figures in the parenthesis are arc sin transformed values

Table 30d: Per cent disease severity on leaf (pooled data)

Treatment	Per cent Disease Index on leaves at different spray intervals				Mean
	Before Spray	After 3 sprays	After 6 sprays	After 9 sprays	
T_1	47.13 (43.35)*	32.63 (34.79)	26.50 (30.78)	17.00 (24.26)	29.79 (32.76)
T_2	45.38 (42.31)	37.25 (37.61)	29.75 (32.95)	23.50 (28.94)	32.38 (34.51)
T_3	45.38 (42.31)	30.25 (33.35)	25.38 (30.18)	17.38 (24.57)	28.44 (31.98)
T_4	38.25 (37.97)	24.50 (29.66)	21.88 (27.78)	13.75 (21.74)	24.25 (29.20)
T_5	45.38 (42.28)	24.50 (29.61)	17.25 (24.42)	11.38 (19.64)	21.51 (27.09)
T_6	48.00 (43.86)	37.88 (37.99)	29.25 (32.71)	21.00 (27.27)	32.91 (34.84)
T_7	48.88 (44.37)	39.88 (39.14)	30.88 (33.66)	25.50 (30.30)	35.51 (36.45)
T_8	48.88 (44.37)	44.63 (41.91)	37.50 (37.73)	28.75 (32.38)	39.70 (38.98)
T_9	41.25 (39.80)	22.25 (28.13)	15.50 (23.02)	10.38 (18.74)	19.81 (25.88)
T_{10}	56.25 (48.68)	54.00 (47.38)	50.00 (45.02)	42.25 (40.53)	50.78 (45.47)
S.Em±	1.18	1.06	0.65	0.93	
CD @ 5%	3.43	3.07	1.89	2.69	

* Figures in the parenthesis are arc sin transformed values

Per cent fruit infection after 9th spray was minimum in T_9 (11.25) which was significantly superior to rest of the treatments followed by T_5 (15.00%) and T_4 (18.75%). Maximum per cent disease incidence was observed in untreated control (52.75%) (Table 30e).

Per cent disease severity on fruit after 9th spray was minimum in T_9 (6.25%) and T_5 (8.25%) were significantly superior to rest of the treatments followed by T_4 (14.00%) and T_1 (14.75%). Maximum per cent disease severity was observed in untreated control (50.13%) (Table 30f).

d) Yield estimation

Results on pooled data to location revealed that (Table 31), significantly maximum number of fruit per tree was observed in T_9 (93.13) and T_5 (91.13) followed by T_4 (88.63). Total number of healthy fruit per tree was significantly maximum in T_9 (63.50) and T_5 (58.25) followed by T_4 (54.63). Average weight of the fruit was significantly maximum in T_9 (352.63 g), followed by T_5 (343.75 g) and T_4 (338.75 g). Significantly highest fruit yield per tree was maximum in T_9 (22.15 kg) followed by T_5 (19.69 kg) and T_4 (18.51 kg). Estimated yield per ha was maximum in T_9 (16.08 tons), T_5 (14.30 tons) and T_4 (13.44 tons).

Looking into the economics worked out by taking yield parameter into consideration, although highest net profit of Rs. 10,16135 was realized with cost:benefit ratio of 1 :5.29 in COC+Streptocycline (0.3+0.05%) it is not recommended at the time of fruit maturity. So, next best option was *Pseudomona fluorescens* (1%) with net profit of Rs. 8,24466 and cost:benefit ratio of 1:4.76 for bacterial blight management in pomegranate.

Table 30e: Per cent disease incidence on fruit (pooled data)

Treatment	Per cent Disease Incidence on fruits at different spray intervals			Mean
	After 4 sprays	After 6 sprays	After 9 sprays	
T_1	38.75 (38.47)	31.25 (33.9)	21.25 (27.41)	31.07 (33.69)
T_2	43.75 (41.42)	35.00 (36.24)	28.75 (32.40)	36.79 (37.26)
T_3	35.00 (36.24)	28.75 (32.32)	21.25 (27.41)	29.29 (32.58)
T_4	33.75 (35.49)	26.25 (30.73)	18.75 (25.56)	27.68 (31.50)
T_5	32.50 (34.66)	23.75 (28.89)	15.00 (22.51)	25.18 ()29.65
T_6	38.75 (38.47)	35.00 (36.24)	26.25 (30.73)	34.11 (35.61)
T_7	48.75 (44.30)	43.75 (41.42)	36.25 (36.99)	43.39 (41.19)
T_8	51.25 (45.74)	41.25 (39.94)	33.75 (35.49)	41.79 (40.23)
T_9	31.25 (33.82)	17.50 (24.54)	11.25 (19.46)	21.43 (26.93)
T_{10}	51.25 (45.74)	51.88 (46.10)	52.75 (46.60)	51.25 (45.74)
S.Em±	0.79	0.92	1.2	
CD @ 5%	2.3	2.68	3.48	

* Figures in the parenthesis are arc sin transformed values

Table 30f: Per cent disease severity on fruit (pooled data)

Treatment	Per cent Disease index on fruits at different spray intervals			Mean
	After 4 sprays	After 6 sprays	After 9 sprays	
T_1	27.25 (31.34)	23.50 (28.85)	14.75 (22.54)	22.32 (27.92)
T_2	32.75 (34.87)	24.75 (29.68)	21.50 (27.57)	27.57 (31.51)
T_3	26.25 (30.74)	27.25 (31.35)	20.25 (26.66)	25.86 (30.40)
T_4	32.25 (34.52)	19.50 (26.13)	14.00 (21.87)	22.39 (27.70)
T_5	19.50 (26.10)	13.00 (20.85)	8.25 (16.42)	16.11 (22.93)
T_6	36.75 (37.27)	33.00 (35.04)	25.00 (29.88)	32.43 (34.56)
T_7	36.50 (37.13)	32.75 (34.87)	27.25 (31.38)	33.04 (34.99)
T_8	38.50 (38.30)	30.75 (33.63)	25.50 (30.21)	31.86 (34.23)
T_9	17.25 (24.29)	9.50 (17.80)	6.25 (14.29)	13.04 (20.22)
T_{10}	48.75 (44.31)	49.50 (44.74)	50.13 (45.09)	49.39 (44.67)
S.Em±	0.91	1.01	1.06	
CD @ 5%	2.64	2.94	3.08	

* Figures in the parenthesis are arc sin transformed values

Table 31: Influence of management treatments for bacterial blight of pomegranate on fruit yield (Pooled data)

Tr. No.	Treatment	Per cent disease index on fruit after 9th spray	Total number of fruits/plant	Total number of healthy fruits/plant	Average weight of the fruit (g)	Fruit yield/ tree (kg)	Estimated fruit yield (tons/ha)	C:B ratio
T_1	CS	14.75	83.75	46.00	314.88	14.06	10.21	1:3.36
T_2	SN	21.50	78.50	46.25	313.75	14.10	10.24	1:3.18
T_3	5% Cu	20.25	80.63	47.88	318.25	15.06	10.94	1:3.28
T_4	*P. f.*	11.50	88.63	54.63	338.75	18.51	13.44	1:4.76
T_5	*P. f.* + SN	8.25	91.13	58.25	343.75	19.69	14.30	1:4.59
T_6	*P. f.* + MB	25.00	78.50	47.25	305.00	14.20	10.31	1:2.83
T_7	*P.f.*□ SN □ Cu (5%)□*P. f.*□COC + SC □ CS□ *P. f.*□ Cu (5%)□ SN	27.25	76.88	49.38	310.38	15.39	11.17	1:3.71
T_8	SN □ Cu (5%)□ *P. f.*□ SN □CS□COC + SC □ Cu (5%)□ SN □ *P. f.*	25.50	80.00	55.88	315.00	17.59	12.77	1:4.21
T_+	COC + SC	6.25	93.13	63.50	352.63	22.15	16.08	1:5.29
T_{10}	Control	50.13	86.25	29.50	292.63	8.38	6.08	
	SEm ±	1.06	0.769011	1.07	3.02	0.35	0.26	
	CD at 5%	3.08	2.231458	3.11	8.77	1.03	0.75	

DISCUSSION

Pomegranate (*Punica granatum* L.), regarded as a fruit of paradise and is one among the major fruit crops of arid zone all through the world. The fruits, have high medicinal properties, rich in many nutrients and vitamins and are traded both in national and international markets.

Bacterial blight of pomegranate is occurring in devastating form in several parts of Karnataka, Maharahastra, Andhra Pradesh and Tamil Nadu states. It was a dollar earning and booming commercial fruit crop to the farmers upto 2002. The disease posed its severe outbreak in all the pomegranate growing regions of Maharashtra, Karnataka, and Andhra Pradesh which resulted in yield loss both in quality and quantity. During 2008-2009, the disease reached its alarming stage bringing substantial damage to the crop and heavy losses to the farmers. Although, the farmers have adopted all the available and possible protection measures, yet the disease could not be managed effectively due to faster inoculum build up and spread in adjoining fields. Bacterial leaf spot disease of pomegranate was first recorded by Hingorani and Mehta (1952). Later on, Hingorani and Singh (1959) made thorough investigations and reported its occurrence and spread in different parts of the country.

Rangaswamy (1962) observed the bacterial leaf spot of pomegranate in Annamalainagar of the then Madras state. The disease was reported by Sohi *et al.* (1964) in Solan region of Himachal Pradesh. A high yield loss (60-80%) due to the disease was noticed by Chand and Kishun (1991) at IIHR experimental plots in Bangalore. Outbreak of the disease was noticed during 2002 (Anon., 2002) in major pomegranate areas of Bellary and Bijapur districts causing severe losses both in terms of yield and quality. Pomegranate, the boon commercial fruit crop to the farmer turned as a big bane after the outbreak of bacterial blight. The disease was originally reported from Delhi in 1952 (Chand and Kishun, 1991). During the last five years, the disease appeared in epiphytotic form in the major pomegranate growing states including Maharashtra and Andhra Pradesh (Mondal and Sharma, 2009).

However, ample information is available on the symptomatological description and chemical control of the disease, there are many obscure points with regard to variability of the pathogen, which needs thorough elucidation. It is in the light of this background the present study on the variability of *Xap* was undertaken. The results obtained from the present investigation are discussed here under.

5.1 Symptomatology

The disease manifested in the form of minute water soaked lesions on lower surface of the leaves, surrounded by diffused water soaked margin. Correspondingly on the upper leaf surface, small brown to black coloured spots were seen. The spots took the shape of either round or angular to irregular. As the disease progressed, the spots enlarged, (2.0 – 5.0 mm in diameter), coalesced and extended upto midrib in a week time and occupied the major portion of leaf lamina. At later stages, the infected leaves turned yellow, became chlorotic and drop off prematurely. Narrow cankerous brown lesions observed on the main stem, primary and secondary branches, with the advancement of the disease lesion turned purplish black, cracked and the branch dry giving a die-back appearance. On flower buds, small water soaked lesions, appeared which later on turned to brown to black coloured spots leading to dropping of buds under severe incidence.

Symptoms on the fruits appeared in the form of very conspicuous small pin head sized lesions surrounded by diffused water soaked margin. As the fruit increased in size, pericarp with lesions crack in the form of L, Y or star shape. The infected fruit split open partially exposing the arils leading to softening of the rind at the ruptured site. Affected plants appear unthrifty, weak and died.

The similar symptoms have been reported by the earlier workers from New Delhi (Hingorani and Singh, 1959), Haryana (Kanwar, 1976), Tamil Nadu (Rangaswami, 1962), Himachal Pradesh (Sohi *et al.*, 1964), Karnataka (Chand and Kishun, 1991, Manjula and Khan, 2003 and Jalaraddi, 2006) who described similar symptoms on leaves, twigs and fruits. The fruits crack through the lesion and burst exposing the arils. Similar observations were made by Yenjeerappa (2009) and Raju (2010),

5.1.1 Collection of the isolates

The isolates of *Xap*, the causal agent of pomegranate bacterial blight were collected from different districts of Karnataka *viz*., Bagalkot, Bellary, Bijapur and Koppal; Maharashtra *viz*., Solapur, Nasik, Sangli; Andhra Pradesh *viz*., Anantpur. Infected samples showing typical blight symptoms on

leaf, twig and fruits were collected. The culture from each location was considered as an individual isolate. Koch's postulates for all the isolates were proved and hence identification of *X. axonopodis* pv. *punicae* was confirmed based on symptoms. In the pathogenicity test, the symptoms observed were in accordance with the description of Hingorani and Mehta (1952), Kanwar (1976), Chand and Kishun (1991) and Manjula (2002). Reisolated cultures resembled the mother culture.

5.1.2 Isolation and pathogenicity

The causal organism was isolated from the infected leaf, fruit and stem parts by tissue maceration method using NGA medium (NGA). Isolation of the pathogen showed typical, yellow, mucoid bacterial colonies on NGA medium after 72 h of incubation at 28±1^{0}C. Culture of bacterium was purified by streaking single colony on to the NGA medium. Pure culture was maintained in 50 per cent glycerol stocks. Pathogenicity was proved by inoculating bacterial cell suspension (200:800; culture: sterile water) to the 2 month old susceptible pomegranate plants of Bhagwa variety. The characteristic symptoms of the disease appeared at different intervals ie., 6-19 hrs after inoculation in the form of small, water soaked, brown to black coloured lesions, which later on developed into angular to irregular shaped spots along the veins and veinlets of the leaf lamina on middle aged leaves. Reisolation carried out from artificially inoculated plants yielded the bacterial colonies similar to the original culture.

Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity. Infection was readily seen by them on tender leaves artificially inoculated plants in seven to ten days of incubation. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976). He observed the symptoms within four to seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts. The observations made pertaining to the isolation and pathogenicity in the present investigation were in conformity with the work of Manjula (2002), who obtained pure culture of the seven isolates of the pathogen from infected plant parts on nutrient agar medium by dilution plating technique and pathogenicity of all the isolates were proved by spray method of inoculation using 45 days old pomegranate plants of variety Bhagwa.

Chand and Kishun (1993) studied the systemic migration of *X. c.* pv. *punicae* in pomegranate by artificial leaf inoculation in the greenhouse and reported that the bacterium colonized the inter-cellular spaces in mesophyll cells before moving to the petiole and finally down to the node. Invaded cells were killed resulting in nodal blight.

These findings receive support from the reports of Hingorani and Mehta (1952), Hingorani and Singh (1959), Kanwar (1976), Patel *et al.* (1965), Vauterin *et al.* (1995) and Manjula (2002). Chand and Kishun (1991) also reported that the bacterium take more than three days to produce symptoms with an incubation period ranging from 4 to 9 days. Similarly Yenjeerappa (2009) and Raju (2010) reported that the culture of each isolate was purified by streaking suspected single colony on the yeast dextrose calcium carbonate agar and pathogenicity of all the isolates was proved.

5.1.3 Hypersensitive reaction

All the 18 isolates of *Xap* injected into the intercellular spaces of *Nicotiana tabacum* leaves produced characteristic hypersensitive reaction in 30 to 48 h after inoculation. Khan (1989) also reported *X. c*. pv. *vignicola* induced hypersensitive reaction in 16-18 h. However, *X. c.* pv. *vesicatoria X. c.* pv. *mori* and *E. carotovora* sub sp. *carotovora* induced hyper sensitive reaction in tobacco leaves in 18-30 hrs. Our findings were also supported by Ravikumar (1994) and Srinivasachary (1995).

The hypersensitive reaction (HR) is a rapid method to detect pathogenicity of bacteria. It is also used to determine viability of strains. Jabeen *et al.* (2012) reported that the test isolates showed typical hypersensitive reaction on tobacco leaves inoculated by injection infiltration technique. The leaf tissue infiltrated with high concentration of virulent strains developed yellow discoloration necrosis after 48 h, while avirulent strains did not exhibit necrosis and delayed reaction behavior. This is confirmed through various studies conducted by Klement & Goodman (1967) in *Xanthomonas oryzae* pv. *oryzae*.

5.1.4 Identification of the pathogen

Identification of the pathogen was determined with the studies on its morphological and physiological features of the pathogen.

5.1.4.1 Morphological characters

Studies on morphological characteristics of the pathogen indicated that the bacterium was rod shaped with rounded ends occurred singly or rarely in pairs, gram negative, capsulated, non-spore forming with single polar flagellum. The cells measured 0.4 to 0.25 × 1.25 – 3.0 μm in size and were readily stained with crystal violet. The results obtained in the present study on morphological characters are in agreement with the reports of earlier workers namely Hingorani and Singh (1959), Kanwar (1976), Manjula (2002) and Yenjeerappa (2009).

5.1.4.2 Growth phase study of *Xap*

There was gradual increase in the growth of bacterium at different intervals. Maximum growth was observed at 128 and 136 hrs after inoculation with OD value 0.274 *i.e.*, stationary phase, after 136 hrs there was gradual decrease in the growth of the bacterium *i.e.*, decline phase. The bacterium starts utilizing the components of the media and it will increase in its size and cellular mass. The dynamics of the bacterial growth was studied by plotting the cell growth (absorbance) versus the incubation time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve of the bacterium (Fig. 2).

Monod (1949) reviewed the growth of bacterial culture and mentioned quantitative characteristics of the growth cycle, essentially the three growth constants *i.e.*, total growth (G), exponential growth rate (R), and growth lag (L).

5.2 Physiological variability

Temperature is an important factor gtoning growth, reproduction and survival of bacterium. In the present study the temperature 28±1^{0}C was found to be optimum for growth of isolates of *X. axonopodis* pv. *punicae*. These results are in line with work of Bhat and Patel (1954). It is evident that incubation at 28±1^{0}C favoured maximum growth of all eighteen isolates, where as 37^{0}C did not favour any isolates. In such isolates growth appeared at 37^{0}C, they were considered as *Pantoea agglumerans*, a contaminant. Similar results were reported by earlier workers *viz*., Hingorani and Mehta (1952), Hingorani and Singh (1959) and Manjula (2002). Mondal and Kumar (2011) reported the growth of *Xap* at 28±1^{0}C and absence of growth at 37±1^{0}C. Manjula and Khan (2002) also reported that the optimum temperature for the growth of *X. a.* pv. *punicae* was 25-28^{0}C, while minimum and maximum temperature was 10 and 40^{0}C, respectively. The optimum pH required for the growth was 7.0- 7.2 and minimum and maximum was 5 and 8, respectively. Yenjeerappa (2009) reported that among the varied temperature and pH levels tested for the growth of *X. axonopodis* pv. *punicae* temperature of 30^{0}C and pH of 7.0 to 7.5 were found optimum for the good growth of the pathogen.

Growth of all isolates at pH 7 was significantly superior to other treatments and followed by pH 6 The *Xap*14 and *Xap*12 produced maximum mean OD value, which were significantly superior to all other isolates followed by *Xap*10 (Fig. 3).

Similar work on temperature requirement was carried out by Hingorani and Mehta (1952). They found that the pomegranate bacterium grows well at a cordial temperature of 30^{0}C and can tolerate a minimum and maximum temperature of 5 and 40^{0}C, respectively.

Gour *et al.* (2000) also got the similar results while working with *X. axonopodis* pv. *vignicola*, the causal agent of leaf blight of cowpea. They have recorded the maximum growth of the pathogen at a temperature level of 30^{0}C and pH of 7.0. Growth (number of colonies) declined considerably at pH values higher and lower than 7.0 being minimum at a pH of 5.0. Manjula (2002) recorded the highest number of colonies of *Xap* at a temperature of 27^{0}C and pH of 7.2. Madhu (2009) reported that the maximum growth of all isolates of *X. axonopodis* pv. *punicae* at pH 5.0 and the least growth at pH 8.0. Good growth of bacterium was observed between the pH range of 5.0 to 6.0. Mondal and Kumar (2011) reported the growth of *Xap* at pH 6 and above and absence of growth at pH below 5.

Growth of all isolates at zere per cent NaCl concentration was significantly superior to other treatment which was followed by one per cent. The results are in confirmation with Mondal and Kumar (2011), they found that *Xap* tolerates upto 1% NaCl concentration, beyond that no growth of the pathogen was noticed.

All eighteen isolates produced fuscan pigment on LB agar after nine days of inoculation. However, only three isolates *viz., Xap*10, *Xap*11, *Xap*15 produced fuscan pigment 5 days after inoculation, three isolates *viz*., *Xap*8, *Xap*17, *Xap*18 produced fuscan after 6 days of inoculation, three

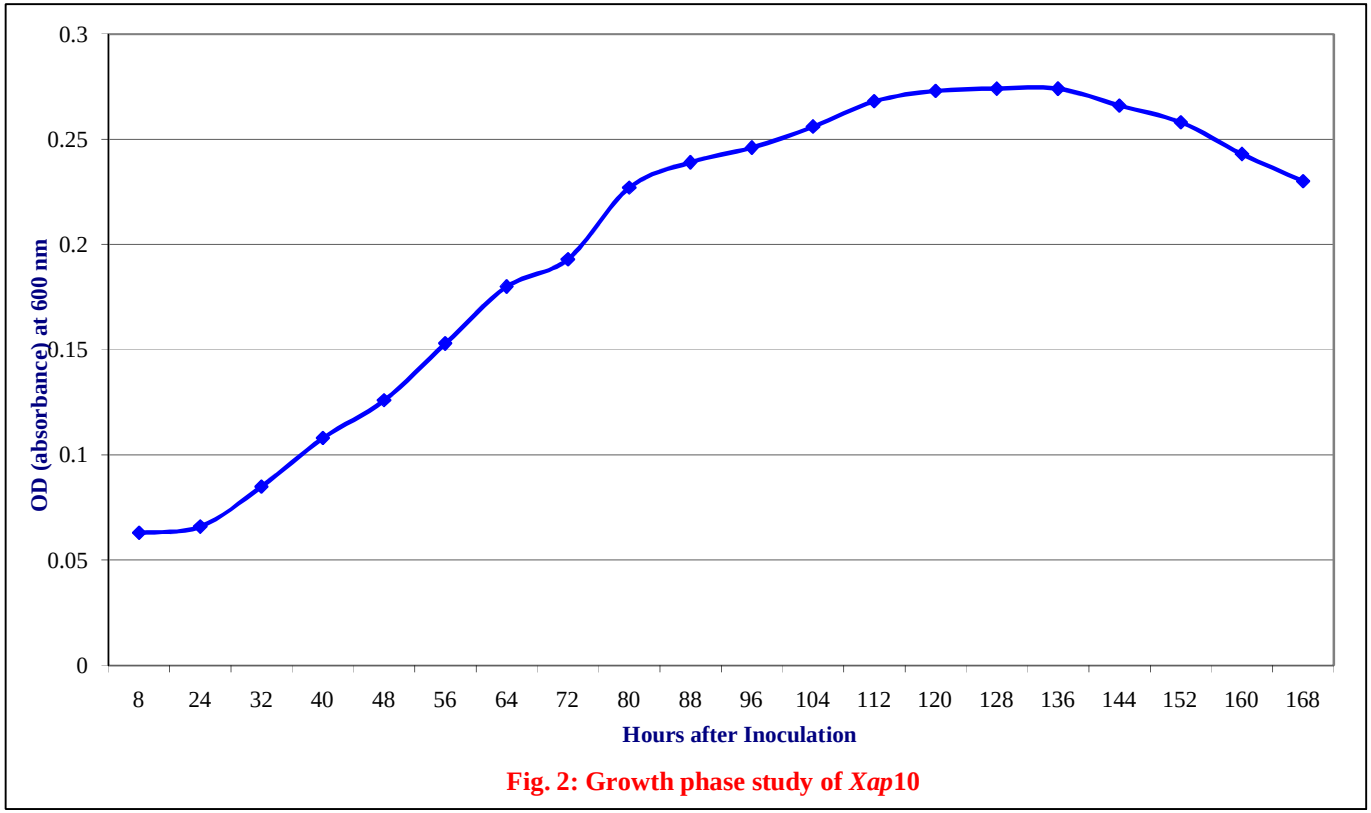


Fig. 2: Growth phase study of *Xap*10

Fig. 2: Growth phase study of *Xap*10

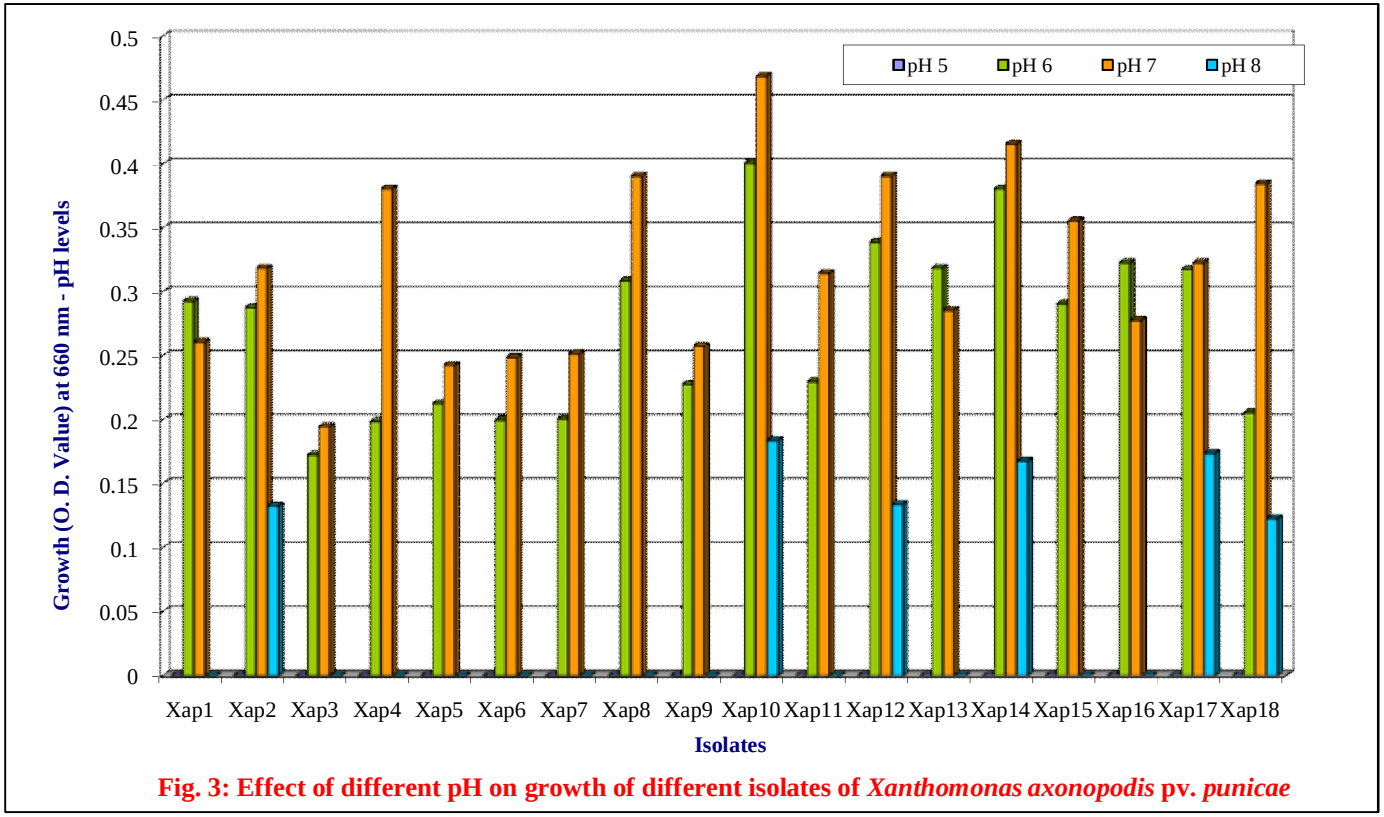


Fig. 3: Effect of different pH on growth of different isolates of *Xanthomonas axonopodis* pv. *punicae*

Fig. 3: Effect of different pH on growth of different isolates of Xanthomonas axonopodis pv. punicae

isolates *viz*., *Xap*2, *Xap*3, *Xap*14 after 7 days of inoculation, five isolates *viz.*, *Xap*1, *Xap*4, *Xap*9, *Xap*12, *Xap*13 after 8 days of inoculation. Fuscan pigment production is the characteristic feature of *Xap* which is observed after 72-96 h of growth especially beneath the lawn of bacterium in the medium or in the liquid medium (Mondal and Kumar, 2011).

Darsonval *et al.* (2009) reported that the pathovar *phaseoli* is divided into four genetic lineages, one of which gathers fuscous strains that produce a brown diffusible pigment (*fuscans*). Fuscous strains are generally more aggressive than the non fuscous ones and were first isolated in Europe.

King *et al.* (1954) routinely used King's medium B for detection of fluorescent Pseudomonas species. They also noted that a brown pigment was produced in King's medium B by *X. c. phaseoli 'fuscans*' variant strain A10. Thus the absence of brown pigment on this medium and modified NBY suggests that no 'fuscans' strains were isolated from symptomless weeds.

Ramos *et al.* (1991) identified the strains of *X. c. phaseoli* on the basis of characteristic yellow colony pigmentation, starch hydrolysis on the semi selective medium MXP, pathogenicity on dry bean leaves and pods, absence of extracellular melanin 'fuscans' pigment on modified nutrient broth yeast extract and King's medium B, and isolation of xanthomonadin pigment with a characteristic R_f value of 0.45 determined by thin layer chromatography.

5.3 Assessment of baseline sensitivity to bactericides among *Xap* isolates

Among the bactericides, K-cyclin was found effective against all isolates of *Xap*, which is significantly superior to rest of the bactericides and is followed by streptocycline and plantomycine. Least inhibition was found in bronip (Fig. 4 and 5).

Between the concentrations of each bactericide, efficacy was significant from lower to higher concentration with greater efficacy at highest concentration.

The effectiveness of the Streptocycline and K-cycline on the growth of some plant pathogenic *Xanthomonas* was also reported by Thirumalachar and Patel (1956), Falico De and Alcaraj (1973), Krishnan and Madu Meeta (1988).

Yenjerappa (2009) reported that bactinash-200 (15.07 mm), bronip (14.67 mm) and plantomycin (13.77 mm) exhibited significantly superior efficacy in inhibiting the growth of *Xap*. The other bactericides *viz.,* K-cycline, copper oxychloride, Bordeaux mixture, bactinashak, bleaching powder and streptocycline were found moderately effective with an inhibition zone ranging from 8.40 to 10.23 mm. In respect of concentration, efficacy of each chemical was significant at relatively higher concentrations.

Raju (2010) reported that Streptocycline + COC with an inhibition zone of 3.3 cm exhibited superior efficacy against *Xap* followed by Streptocycline (2.80 cm) and COC (2.65 cm). However, both the treatments were on par with each other. All other chemicals *viz.,* Streptocycline + Copper hydroxide, Copper hydroxide were found to be moderately effective but were significantly different from each other, Bromopal and Kasugamycin were less effective and were on par with each other. In respect of concentration, efficacy of each chemical was significant at relatively higher concentrations.

Krishnan and Madhumeeta (1988) observed that plantomycin, streptocycline and paushamycin each at 500 ppm concentration were equally effective in inhibiting the growth of *Xanthomonas campestris* pv. *dieffenbachiae* causing bacterial blight of anthurium. Screening of antibiotics/ bactericides under *in vitro* conditions revealed that Streptocycline and Paushamycin was highly effective against the *X. a*. pv. *punicae* at 500 ppm with 25.60 mm and 19.0 mm inhibition zone respectively (Manjula, 2002).

5.4 Genetic diversity of *Xap* from different pomegranate growing areas

Phylogeny based on 16S rRNA gene revealed that all nine strains of *Xap* from Karnataka (Laxmipur, Kaladagi, Govinkoppa, Kanamadi and Matarangi), Maharashtra (Lakmapur, Malegaon and Hiraj) and Andhra Pradesh (Anantpur) formed one cluster sharing 100 per cent identity among them, suggesting that geographical distance does not necessarily contribute to the variation in *Xap* strains with respect to the important housekeeping gene loci, 16S rRNA. The homogeneity within the *Xap* population is also indicative of the similar disease severity across the states in India. *Xap* strains were

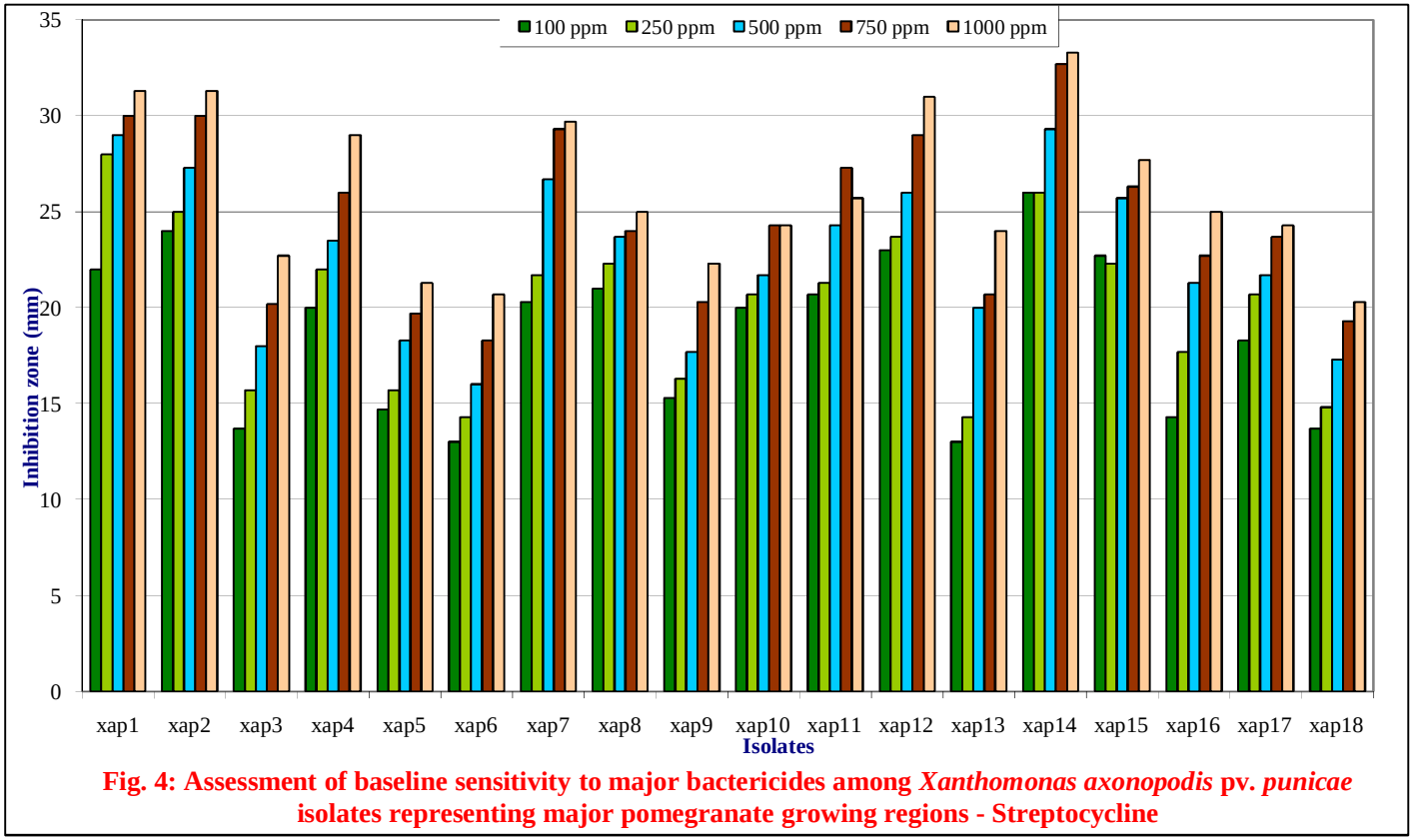


Fig. 4: Assessment of baseline sensitivity to major bactericides among *Xanthomonas axonopodis* pv. *punicae* isolates representing major pomegranate growing regions - Streptocycline

Fig. 4: Assessment of baseline sensitivity to major bactericides among *Xanthomonas axonopodis* pv. *punicae* isolates representing major pomegranate growing regions - Streptocycline

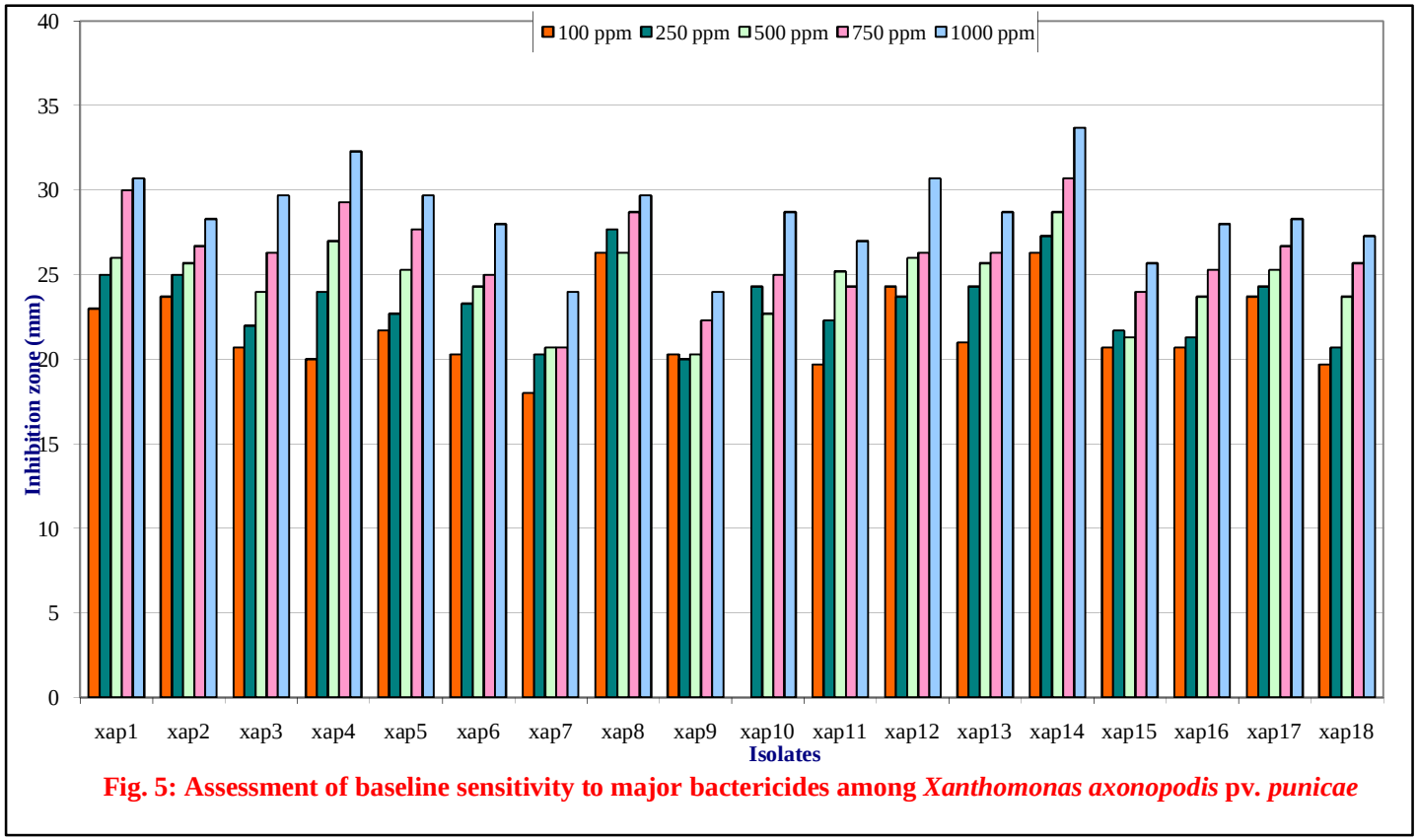


Fig. 5: Assessment of baseline sensitivity to major bactericides among *Xanthomonas axonopodis* pv. *punicae*

Fig. 5: Assessment of baseline sensitivity to major bactericides among *Xanthomonas axonopodis* pv. *punicae* isolates representing major pomegranate growing regions - K-Cycline

phylogenetically related to members of the *X. oryzae*, *X. citri, X. axonopodis* pv*. malvacearum*, *X. a*. pv. *mangiferaeindicae*.

Mondal *et al.* (2012) concluded that geographical variation does not necessarily reflect variation to genetic make- up of the *Xap* strains. This study established that *gyrB* gene would be a good candidate locus to differentiate the *Xap* strains from other closely related strains that could not be differentiated through 16S rRNA gene. Nevertheless, our study demonstrated for the first time that pomegranate bacterial blight pathogen is phylogenetically very close to *Xanthomonas citri* subsp*. malvacearum* infecting cotton. Cotton is extensively grown in the pomegranate growing belts of India predominantly in Maharashtra, thus it is suggested that pomegranate blight pathogen, *Xap* is evolutionarily closely linked to the predominant subspecies *malvacearum* of *Xanthomonas* infecting cotton, an important crop in the pomegranate growing regions of India.

The *gyr*B gene has been demonstrated to offer greater resolution in discriminating pathovars of bacterial species including the genus *Xanthomonas* (Motoshima *et al.,* 2007; Parkinson *et al.,* 2009). Recently, *gyr*B gene based phylogeny resolved the distinctiveness of the species, *Xanthomonas citri* and *X. fuscans*, which were previously classified as pathovars of *X. axonopodis* (Schaad *et al.*, 2007). The species designation of pomegranate blight bacterium has recently been changed from *X. campestris* pv. *punicae* to *X. axonopodis* pv*. punicae* based on *gyr*B sequence (Parkinson *et al.*, 2009). Therefore, they targeted *gyr*B gene, particularly C-terminus variable region, while developing a PCR based detection technique for bacterial blight pathogen, *Xap*. They synthesized a primer set, namely KKM5 and 6 for PCR amplification of 491 bp from *gyr*B gene. Subsequently, a template DNA extraction protocol from plant tissue was optimized and validated. The developed technique could able to detect pathogenic bacteria in all types of the infected pomegranate samples including leaf, fruit and stem. Most notable thing is that, it takes less than 3h to detect the presence of pathogen in the plant samples (50 min for cell extraction from plants, 90 min for PCR and 40 min for electrophoresis). The validation data suggests that the developed PCR detection system could successfully able to detect the presence of blight bacterium in the affected samples with 94.5 per cent success rate. They did not find any amplification in the leaf tissue from tissue culturally raised plants. However, among 4 apparently healthy leaf tissues tested 2 were found positive indicating that the detection system could even work in asymptomatic (but suspected to be diseased) plant samples. However, the sampling of plant tissues to be tested for PCR detection need to be carried out very carefully as the blight bacterium, *Xap* is a mesophyll localized but not systemic one.

5.4.1 Molecular detection of *Xap* using *gyr*B primers by direct colony PCR

Colony PCR can be an efficient and economical method to monitor *Xap* using specific primer pairs. Colony PCR can be a quantitative tool for pathogen detection that may provide more information than standard detection protocols owing to the sensititvity vested with the PCR technology. Direct colony PCR have been applied to detect the population of *Xap* in infected plant material/seed and soil samples using species specific primers (Opina and Tavner, 1997).

Umesha *et al.* (2012) developed a colony PCR - SSCP (single strand confirmation polymorphism) technique to identify *Ralstonia solanacearum* where the PCR product was denature and separated on a non-denaturing polyacrylamide gel. The SSCP banding patterns were detected by silver staining of nucleic acid and compaired with the other phytopathogenic bacteria. Essentially this method did not involve isolation of genomic DNA from the isolates.

5.4.2 Multiplex polymerase chain reaction

The multiplex polymerase chain reaction was performed using template containing DNA from *Xap* targeting 16S rRNA and *gyr*B gene by 16S rRNA and *gyr*B specific primers. High intensity of amplification of the gene specific products of 1537 bp and 491 bp corresponding to 16S rRNA and *gyr*B were obtained at annealing temperature of 57.5^0C for 1 minute as compared to other temperature levels.

Soustrade *et al.* (2010) mentioned that, new multiplex nested PCR assay is less labor intensive and time consuming than isolation on semiselective media. The latter technique requires further evaluation of suspect colonies using other identification methods including serological or molecular techniques and/or pathogenicity tests. A multiplex nested PCR was developed for specific and sensitive detection of *X. axonopodis* pv. *allii* from onion seeds. Due to the high genetic diversity of this pathogen (Gent *et al.*, 2005; Gent *et al.,* 2004; Picard *et al.*, 2008; Roumagnac *et al.,* 2004), two PCR-based sequence-characterized amplified regions (SCARs) identified in RAPD and AFLP analyses were required to detect all isolates of this pathovar. The RAPD technique has been widely

used for SCAR development to obtain PCR diagnostic tools for bacterial plant pathogens (Meng *et al.*, 2004; Soustrade *et al.*, 2006; Trebaol *et al.*, 2001).

5.5 Effect of plant extracts on the growth of *Xap* under *in vitro*

Plant extracts are known for safe, ecofriendly and cost effective means of managing the crop diseases. Among the plant extracts tested, soapnut extract at 10 per cent was found very effective in inhibiting the maximum growth of the pathogen which is significantly superior to all other plant extracts tested. It is followed by adahtoda + soapnut and miswak + soapnut. Between the concentrations, significant difference was observed and higher concentration (10 per cent) was superior to lower concentration (5 per cent) (Fig. 6).

Soapnut plants are rich in secondary metabolites such as saponins which are known to have anti-fungal and antimicrobial activity. Although time tested for safety for thousands of years, soapnuts and saponin are just beginning to be studied in depth to determine all their potential natural benefits (www.soapnuts.pro, 2011). Many biological activities like antimicrobial, antidiabetic, cytotoxic, molluscicidal, fungicidal, anti-inflammatory, *etc.* were shown by fractions of crude extracts and isolated compounds.

Srinivasachary (1995) reported Ocimum plant extract as most effective botanical against the growth of *Xanthomonas campestris* pv. *mori* isolated from mulberry. On the contrary, poor efficacy of the similar plant extract was recorded in the present study against *Xap*. According to Sushma (1997), garlic extract was highly inhibitory to the growth of *Xanthomonas axonopodis* pv. *dieffenbachiae* followed by ocimum and citronella. The antibacterial properties of onion and garlic were attributed to the presence of sulphur as an active principle (Mangamma and Sreeramulu, 1991). Karuna and Khan (1994) reported the effectiveness of Ocimum and Adothoda plant extracts on *Pseudomonas solanacearum* on tomato. *Adatoda zeylanica* was found to be effective against *X. a.* pv. *glycines* (Shobha, 1998) and *X. c.* pv. *vignicola* (Thammaiah *et al.*, 1998). The inhibitory activity of different botanicals such as tulsi, kolangi, pathouli and miswak against *Xap* was reported by Manjula *et al.* (2003).

The roots of *Salvadora persica* contains a steam- distillable oil composed of 10% benzyl nitrate and 90% *Benzylisothiocynate* (BIT) (Mohammad and Turner, 1983). BIT is classified as one of the chemo-preventive agents that are thought to prevent carcinogenic and other genotoxic compounds from reaching or reacting with the target sites on the treated tissues (Ezmirly and El-Nasr, 1981). BIT is reported to have a virucidal activity against herpes simplex virus 1 (HSV-1) at a concentration of 133.3 mg/ml. in addition, it is reported to have a broad-spectru cm bacteriocidal activity (Al Sadhan and Almas, 1999).

Sofrata *et al.* (2011) reported that antibacterial volatiles from miswak (*Salvadora. persica)* root sticks consist of more than 98% of benzyl isothiocyanate (BITC). Secondly, the essential oil obtained by steam distillation revealed two components, BITC (73.8 %) and benzyl nitrile (26.2%). BITC is thus, the main bactericidal factor of *S. persica* root sticks, active both at a distance as a volatile and in extracted form against Gram-negative bacteria. The rapid bacterial killing implied that *S. persi*ca root stick extract might target the bacterial membrane. Electron micro-graphs of the Gram-negative bacterium *A. actinomycetemcomitans* revealed that both essential oil as well as pure commercial BITC gives rise to similar protrusions in the bacterial cell membrane. The membrane protrusions resemble those formed under the influence of antimicrobial peptides (Lehrer *et al.*, 1989). However, as BITC has both lipophilic as well as electrophilic properties we speculate that BITC might penetrate through the outer bacterial membrane and possibly interfere with the bacterial redox systems and thus hamper the ability of the bacterium to maintain its membrane potential. Such an effect of BITC has been demonstrated for mitochondrial membranes (Nakamura *et al.,* 2002).

Sher *et al.* (2011) reported, the chemical constituents of *S. persica* such as essential oils, flavonoids, salvadorine, cyanogenic glycosides, lignans, saponins, alkaloids, tannins, linoleic acid, steraric acid, salvadourea, vitamin C, silica and different salts are also known to possess significant antimicrobial activity (Kamel *et al.*, 1992; Darout *et al.,* 2000; Abd-Rahman *et al.*, 2003).

Jalaraddi (2006) found that both aqueous and alcoholic extracts of garlic, miswak and citronella were inhibitory to growth of the *Xanthomonas axonopodis* pv.*punicae*, while botanical Sufoof-E-Shoneez recorded highest inhibition zone.

Certain botanicals showed broad spectrum antibacterial activity against both type of bacteria, Gram positive and Gram negative, (Rajendra and Alankara, 1987, Garg and Kasera, 1984,

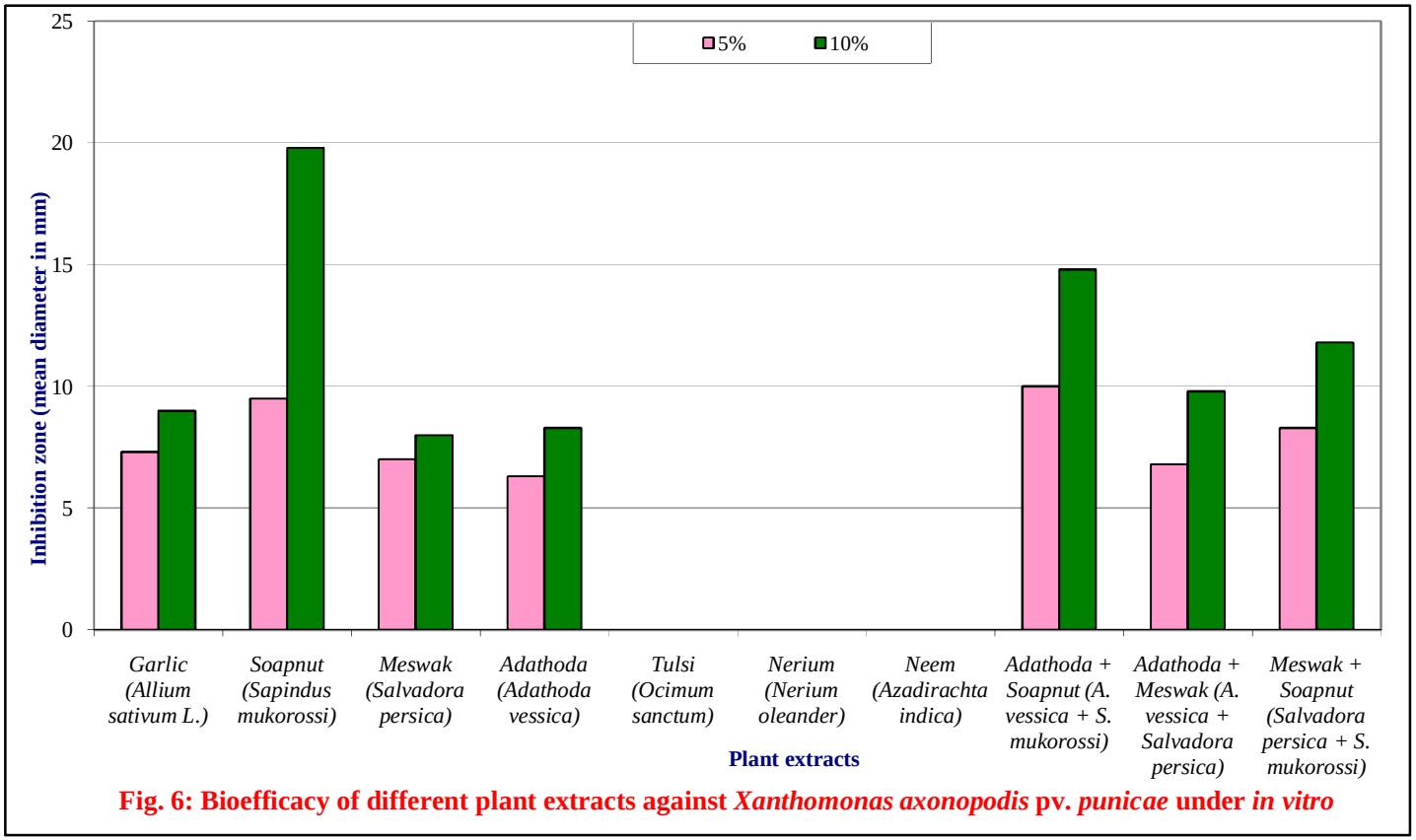


Fig. 6: Bioefficacy of different plant extracts against *Xanthomonas axonopodis* pv. *punicae* under *in vitro*

Fig. 6: Bioefficacy of different plant extracts against *Xanthomonas axonopodis* pv. *punicae* under *in vitro*

Kanthaswamy *et al.*, 1989 and Garg ang Kasera, 1998). The effective control of *Xanthomonas campestris* pathovars using different botanicals was also reported (Garg and Oswal, 1981) and Sushma (1997).

Karuna and Khan (1994) reported the effectiveness of Ocimum and Adotoda plant extracts on *Pseudomonas solanacearum* on tomato. *Adatoda zeylanica* was found to be effective against *X. a.* pv. *glycines* (Shobha, 1998) and *X. c.* pv. *vignicola* (Thammaiah *et al.*, 1998).

5.5.1 *In vitro* evaluation of nutrients on the growth of *Xap*

Among the nine nutrients tested, $CuSO_4$ has recorded highest mean inhibitory zone at all concentrations tested, which is significantly superior to other strains tested followed by micron special (boron, $MgSO_4$ and $ZnSO_4$) and $MnSO_4$, $CaSO_4$, boron, sulfur, $FeSO_4$ were failed to inhibit the pathogen (Fig. 7).

The copper ions are passively absorbed by fungal spores and bacteria during growth and accumulate until their concentration becomes lethal for cells. Copper should be applied before or as the disease starts to spread. The fungicidal and bacteriostatic effects are due to copper ions (Cu^{2+}) that are released in water when combined with different chemical groups of cell proteins and enzyme systems. It is a multi-site operation which makes the selection of resistant mutant strain very improbable. Copper is a contact fungicide, there is a high persistence because the copper ion cannot be altered or degraded by heat/ light (Anon., 2012).

Mondal and Mani (2012) reported the efficacy of nanocopper in suppression of growth as well as in the watersoaked lesions induced by *Xap*. The nanocopper suppressed *Xap* growth at 0.2 ppm, *i.e.*, >10,000 times lower than that usually recommended for Cu-oxychloride. Scanning electron microscopy (SEM) revealed cell wall degradation in nanocopper-treated bacterial cells that failed to colonize plant tissues as well as to produce the characteristic intense water soaking. This study suggests that nanocopper could be employed as an environmentally friendly strategy for the management of pomegranate bacterial blight.

Strong correlation between boron uptake in leaf tissues, residual boron in soil with black rot incidence in cauliflower was reported by Kumar and Kotur (1989). Exogenous supply of boron (0 – 6.4 mg/kg) in low boron containing altisols (hot water soluble soil containing boron of 0.1 mg/kg) indicated that this nutrient has a definite role in susceptibility of cauliflower to black rot caused by *Xanthomonas campestris* pv. *campestris*. Susceptibility was greatly observed in boron deficient (below 0.4 mg/kg) and boron excess (above 1.6 mg/kg) plants than the plants grown with optimum level of boron (0.4 – 1.6 mg/kg).

Ansari and Shridhar (2001) studied the effect of iron on the virulence of *X. o.* pv. *oryzae* by pot culture experiment. Twenty five days old rice seedlings were clip inoculated with 48 h old culture grown in the medium amended with different levels of iron *i.e.*, $FeSO_4$ at 0.20, 2.00, 4.00, 8.00 mg per lit and noticed that increase in lesion length was observed upto 8.0 mg/ lit in susceptible cultivar (IR-8) but restricted upto 4 mg per lit in resistant cultivar (DV-85). Pathogen grown in iron starved medium produced minimum lesion length.

Dordas (2008) reviewed the effect of nutrients, such as N, K, P, Mn, Zn, B, Cl and Si, on disease resistance and tolerance. Among the nutrients, Mn can control a number of diseases as Mn has an important role in lignin biosynthesis, phenol biosynthesis, photosynthesis and several other functions. Zn showed decreased or increased susceptibility to disease in some cases. B was found to reduce the severity of many diseases because it has function on cell wall structure, plant membranes and plant metabolism. Nutrients can reduce disease to an acceptable level or at least to a level at which further control by other cultural practices or conventional organic biocides is more successful and less expensive.

5.6 Effect of antagonistic organisms on the growth of *Xap* under *in vitro*

In the light of present day constraints with the use of chemical pesticides in plant disease management, the biological control as an alternate option is gaining importance and awareness as the approach is ecofriendly and cost effective. Under biological control of plant diseases, various antagonistic organisms have been identified, which fight against the pathogens by different mechanisms *viz.,* competition, lysis, antibiosis, siderophore production and hyper parasistism (Vidyasekaran, 1999).

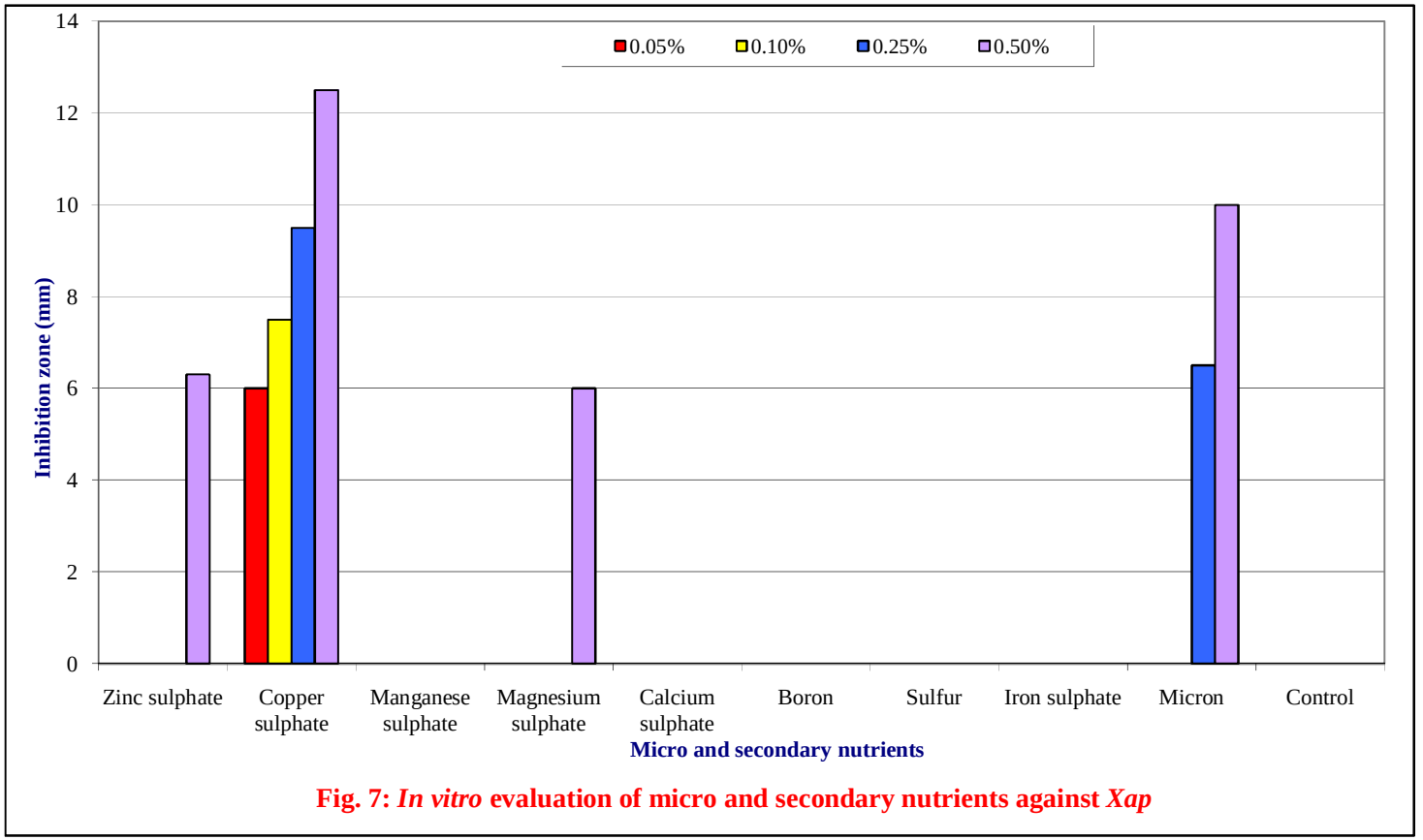


Fig. 7: *In vitro* evaluation of micro and secondary nutrients against *Xap*

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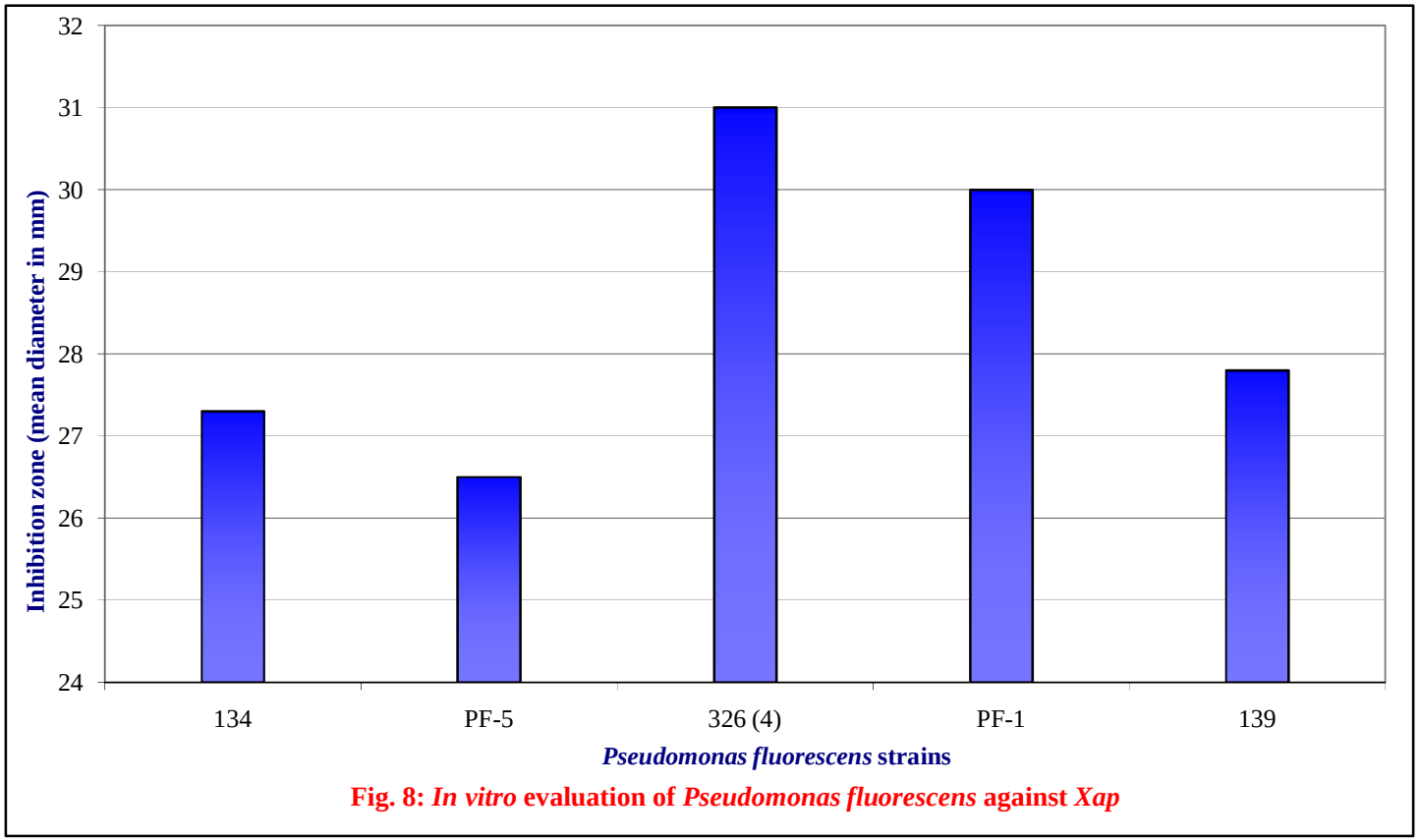

Fig. 8: *In vitro* evaluation of *Pseudomonas fluorescens* against *Xap*

Fig. 8: *In vitro* evaluation of *Pseudomonas fluorescens* against *Xap*

Among the five different strains of *P. fluorescens* tested strain No. 326(4) and PF1 recorded highest inhibitory zone against *X. axonopodis* pv. *punicae* and these were significantly superior to other strains tested and there was no significant difference between these two strains followed by strain No. 139, 134 and PF5 (Fig. 8). The antagonism of *Pseudomonas fluorescens* against some *Xanthomonas* spp. was reported by Unnamalai and Gnanamanickam (1984), Sakthivel *et al.* (1986), Sivamani *et al.* (1987), Safiyazov *et al.* (1995). Antagonistic activity of *Erwinia herbicola* and *Bacillus subtilis* against *Xanthomonas campestris* pv. *viticola* was observed by Chand *et al.* (1991). Similarly Yenjeerappa (2009) and Raju (2010) reported that *Bacillus subtilis* and *Pseudomonas fluorescens* were significantly superior to other antagonists in inhibiting the growth of the pathogen.

Laha *et al.* (1992) stated that fluorescent pigments produced by *Pseudomonas* are sequester Fe^{3-} and are termed as siderophores, which act as inhibitors for the growth of some phytopathogenic bacteria and fungi. Biochemical studies conducted by Valasubramanian *et al.* (1994) showed that efficient strains of *Pseudomonas fluorescens* produces an antibiotic phenazine-1-carboxylic acid (PCA) responsible for hindering the growth of plant pathogenic bacteria. On contrary to the report by earlier workers and findings of present investigation, Manjula (2002) examined the ineffective mechanism of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Xap*.

Among the eleven different strains of Pink Pigmented Facultative Methylotrophs (PPFM) tested, strain No. 10 has recorded highest inhibitory zone of which is significantly superior to rest of the strains followed by 75. Similar results were obtained by Jones *et al.* (2011b) when, Pink Pigmented Facultative Methylobacterium (PPFM) H5 strain, isolated from Bagalkot, has shown maximum inhibition zone (15.00mm) followed by PPFM 42U (14.60mm), PPFM 71 (14.40mm) and PPFM 75L (14.00mm) against *Xap* causing bacterial blight of pomegranate. Similarly, Jones (2010) studied the inhibitory activity of PPFM strains against *Sclerotium rolfsii* and reported that native PPFM 155 produced the highest zone of inhibition (11 mm dia) followed by PPFM 169 which produced 10 mm dia. It can be correlated to the production of siderophore production by several PPFM strains (Neilands and Leong, 1986).

5.7 *In vitro* evaluation of effective botanical, bioagents, nutrient and commercial formulation (5%) of copper singly and in combination against *Xap*

Among the botanicals soapnut, among bioagents *Pseudomonas fluorescens* strain (326(4)) and PPFM strain 10, among nutrients $CuSO_4$ and $ZnSO_4$ and commercial formulation (5%) of copper were tested in alone and in combination against *Xap* by inhibition zone assay method *in vitro*.

COC + Streptocycline at 0.3% + 0.05% was selected as standard check which was given significantly highest inhibition zone to rest of the treatments followed by *P. f* strain 326(4) at 1% +soapnut at 5%, *P. f.* starin 326(4) at 1%+ PPFM strain 10 at 1% and *P. f*. 326(4) at 1%. Least inhibition zone was observed in $ZnSO_4$ at 0.5% (Fig. 9).

5.8 Field evaluation of selected botanicals, microbial antagonist, nutrient, bactericide and commercial formulation (5%) of copper for the control of bacterial blight of pomegranate

Two field trials were conducted during Mrigbahar (June-December, 2012) using effective botanical, bioagent, nutrient, bactericide and commercial formulation (5%) of copper, which has given good result in *in vitro* condition against the incidence and severity of bacterial blight in pomegranate.

During hastabahar cropping season, the incidence and severity of the disease is generally less at the end of the season due to the weather factors, which may not be much favourable for the pathogen. Per cent disease incidence and severity at the end of 9^{th} spray was reduced in all the treatments including untreated control.

The first field trial was conducted at Talkal during Hastabahar (June-December, 2012).

Results revealed that at the end of ninth spray minimum disease on stem was observed in T_9, T_5 and T_4 with values of 12.5, 15.00 and 15.00 per cent respectively and significantly superior to rest of the treatments and they were on par with each other followed by T_3 (20.00%). The maximum disease incidence was observed (52.50%) in untreated control (T_{10}). Before spray per cent disease severity on stem was in the range of 64.16 to 52.08 per cent. After 9^{th} spray per cent disease severity

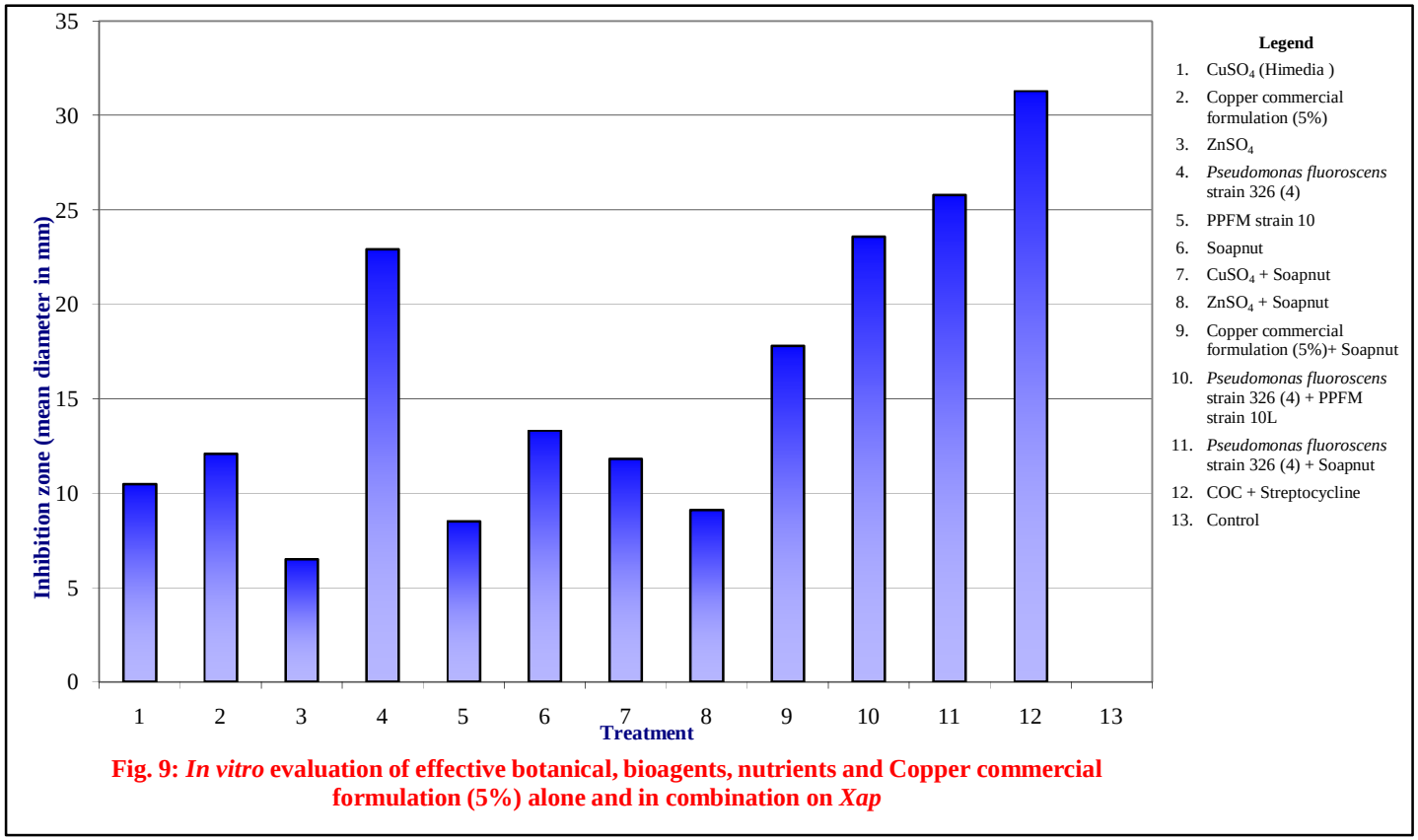

Fig. 9: *In vitro* evaluation of effective botanical, bioagents, nutrients and Copper commercial formulation (5%) alone and in combination on *Xap*

Fig. 9: *In vitro* evaluation of effective botanical, bioagents, nutrients and Copper commercial formulation (5%) alone and in combination on *Xap*

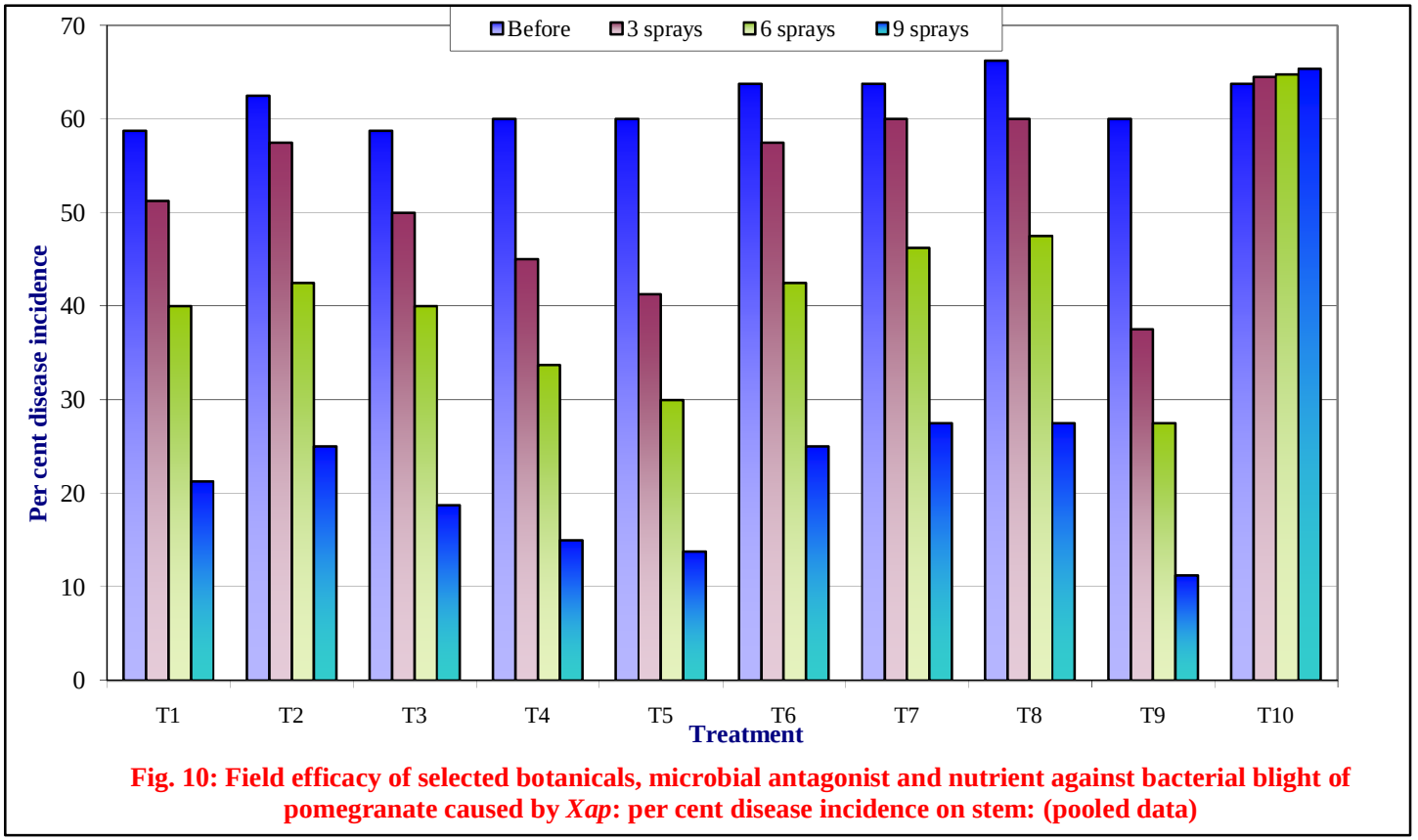

Fig. 10: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on stem: (pooled data)

Fig. 10: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap*: per cent disease incidence on stem: (pooled data

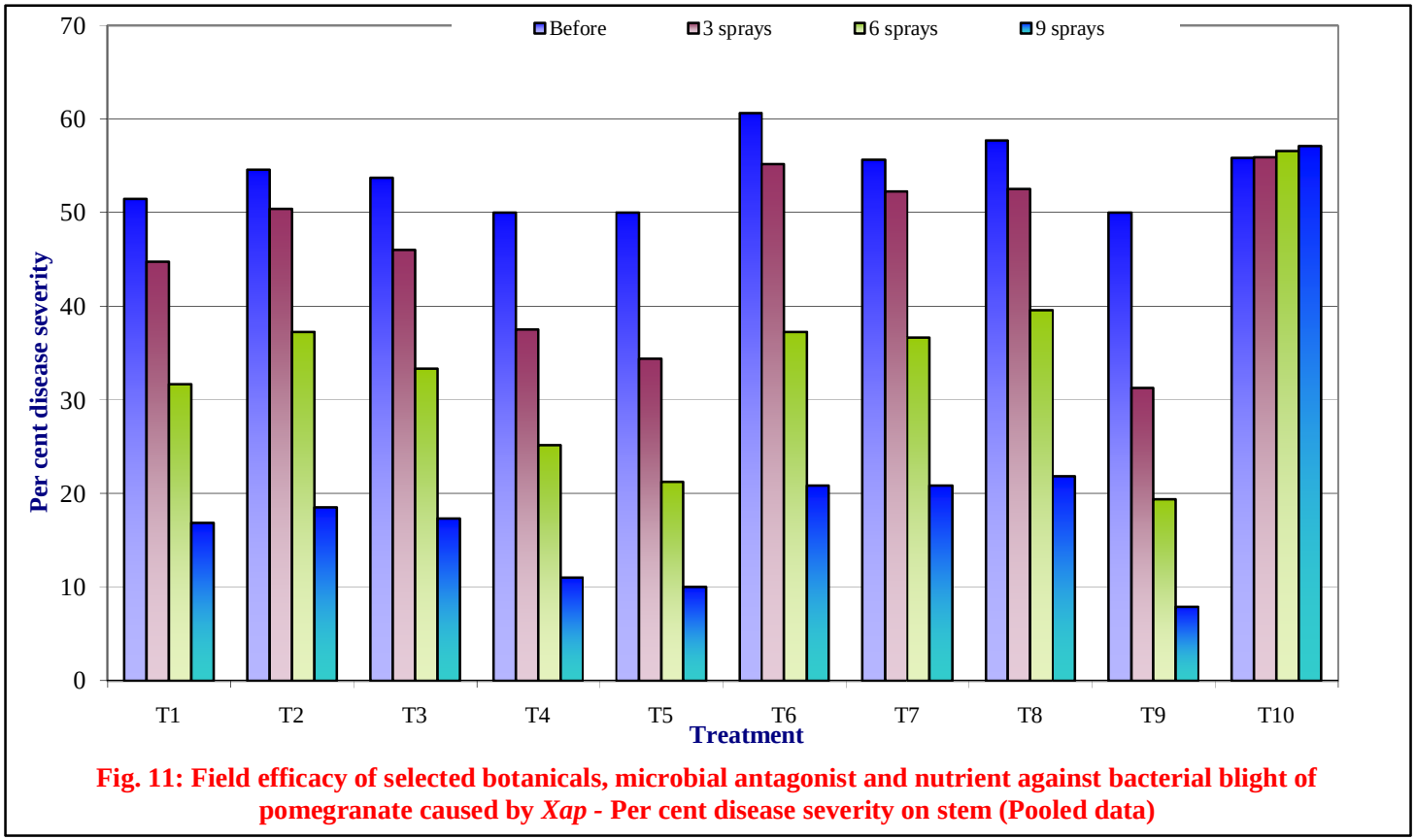

Fig. 11: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease severity on stem (Pooled data)

Fig. 11: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease severity on stem (Pooled data)

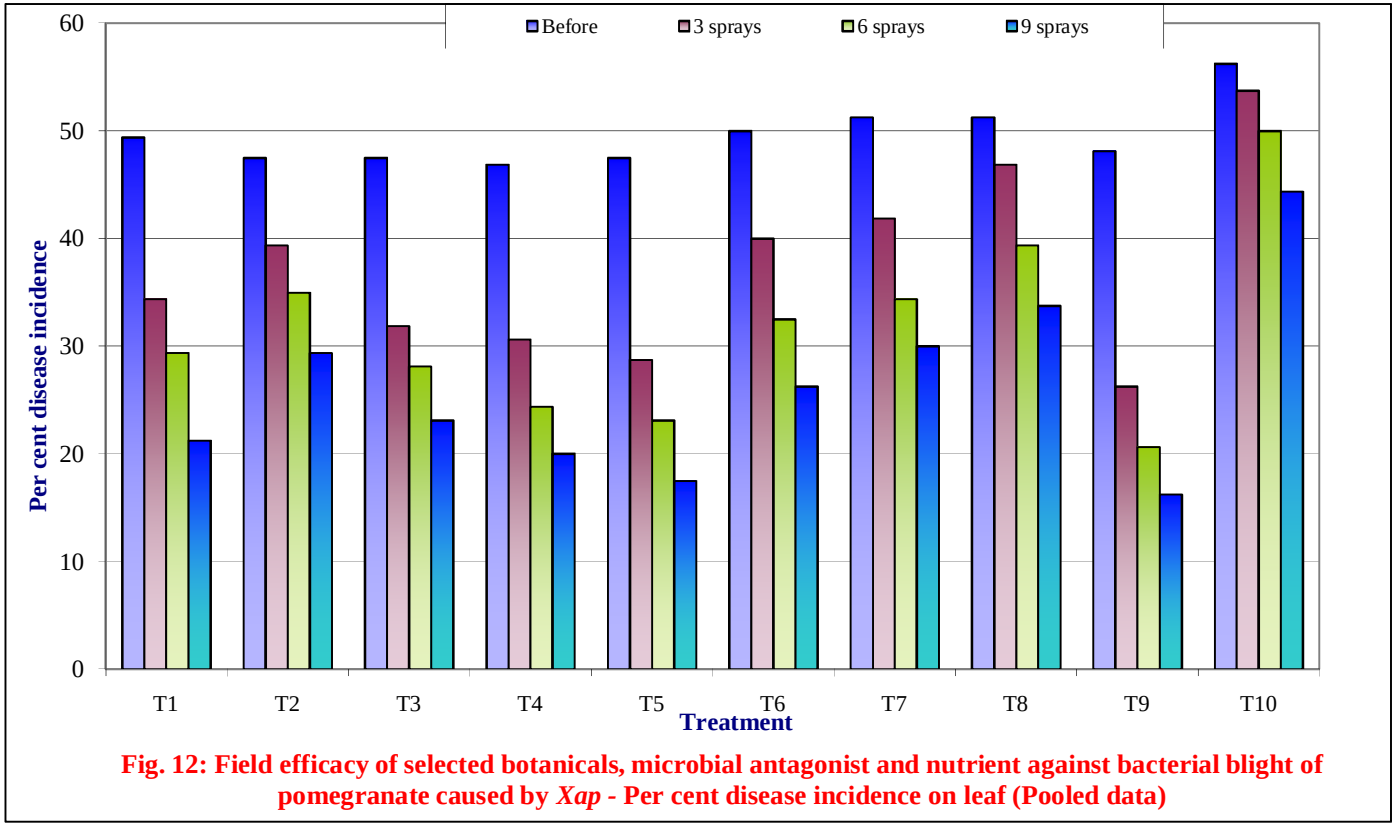

Fig. 12: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on leaf (Pooled data)

Fig. 12: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on leaf (Pooled data)

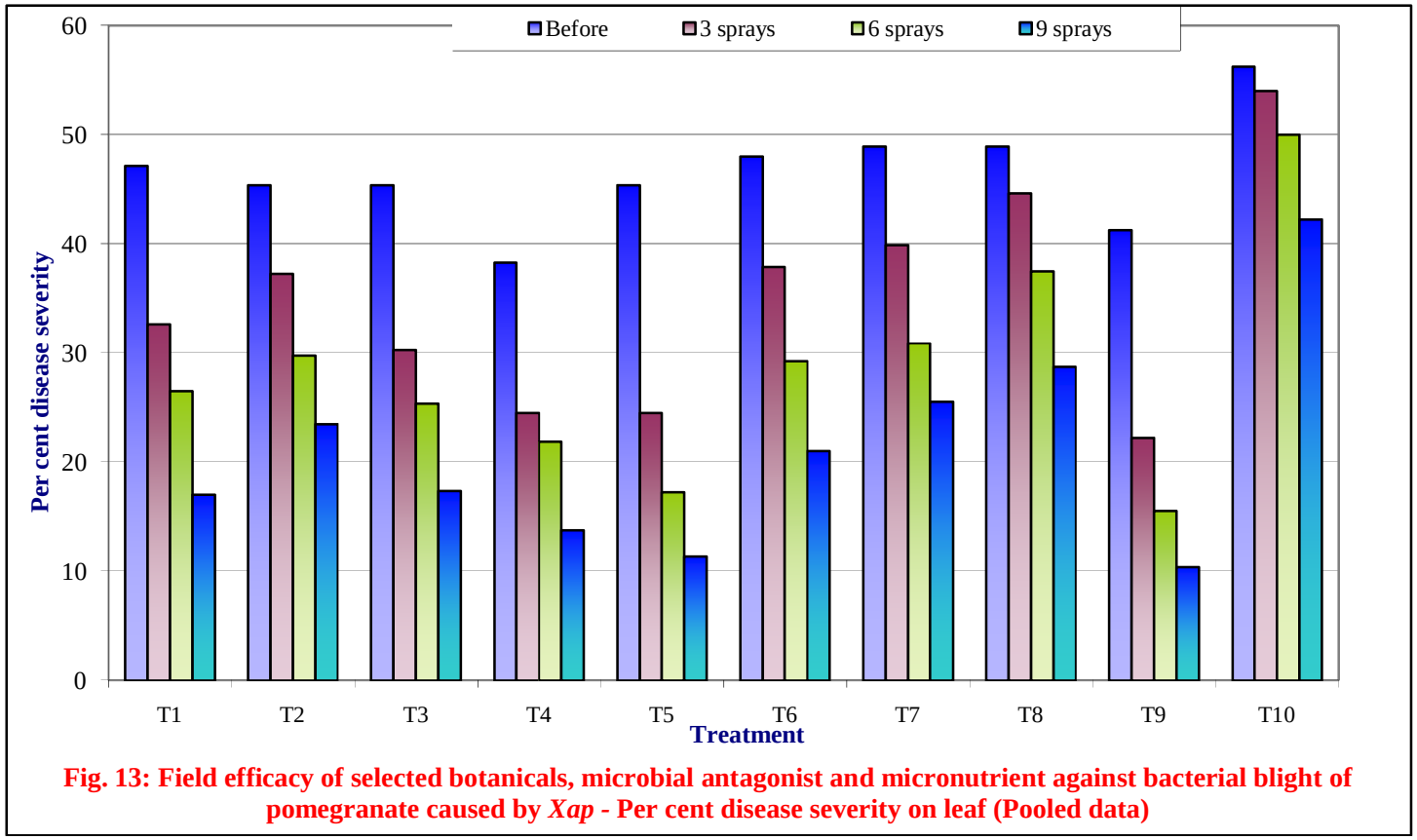

Fig. 13: Field efficacy of selected botanicals, microbial antagonist and micronutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease severity on leaf (Pooled data)

Fig. 13: Field efficacy of selected botanicals, microbial antagonist and micronutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease severity on leaf (Pooled data)

on stem significantly reduced in T_9, T_5 and T_4 with 8.75, 10.83 and 11.25 per cent respectively and they are on par with each other, followed by T_1 treatment (17.92%).

At the end of 9th spray, T_9 has minimum per cent leaf infection with 18.75, T_5 with 20.00 per cent, these two were significantly superior to rest of the treatments and on par with each other, followed by T_4 (22.5%) and T_3 (25.00%) and they were statistically differ with each other. However, maximum per cent stem infectionwas 47.50 per cent in untreated control (T_{10}). Before spray the initial per cent disease severity on leaves in the orchard was 58.75 to 45.00 per cent. After 9th spray lowest per cent disease severity was observed in T_9 (12.00%), T_5 (13.00%) and T_4 (15.50%). They were significantly superior to rest of the treatments and on par with each other. The per cent disease severity on leaves was maximum (45.25%) in untreated control.

The per cent disease incidence after 9th spray was lowest in T_9 (12.50%) which is significantly superior to rest of the treatments. Followed by T_5 (17.50%), T_4 (20.00%) T_3 (22.50%) and T_1 (22.50%) significantly they are on par with each other. The maximum per cent fruit infection was observed in untreated control plot (40%). At the end of 9^{th} spray, disease has reduced in all the treatment including untreated control. In untreated control, the per cent disease severity on fruits has attained maximum of (38.00%), it was observed minimum in T_9 (7.00%), T_5 (9.50%), T_4 (15.00%), followed by T_1 (15.5%), they were significantly superior to rest of the treatments and on par with each other.

The second field trial was conducted at Muddaballi during Mrigbahar (June-December, 2012) wherein the plants were pruned in the month of June.

The per cent stem infectionafter 9^{th} spray was maximum in untreated control (40.00%). T_9 (10.00), T_5 (12.50), T_4 (15.00), T_3 (17.50%), T_1 (20.00%), T_2 (22.50%), T_6 (22.50%), T_7 (25.00%) and T_8 (25.00%), were significantly superior to rest of the treatments and they are on par with each other. The per cent disease severity on stem after 9^{th} spray was significantly reduced in T_9, T_5 and T_4 with 7.08, 9.17 and 10.83 per cent respectively and they were on par with each other, followed by T_1 treatment (15.83%).

The per cent disease incidence on leaves after 9^{th} spray was minimum in T_9 (13.75) and T_5 with 15.00 per cent, these two were significantly superior to rest of the treatments and on par with each other, followed by T_4 (17.50%), T_1 (17.50%) and T_3 (21.25%). However, maximum per cent disease incidence on leaves was 41.25 per cent in untreated control (T_{10}). The per cent disease severity on leaves after 9^{th} spray was minimum in T_9 (8.75%), T_5 (9.75%), T_4 (12.00%), T_1 (14.00%) and T_3 (16.00%). They were significantly superior to rest of the treatments and on par with each other. The per cent disease severity on leaves was maximum (39.25%) in untreated control.

The per cent disease incidence after 9^{th} spray was lowest in T_9 (10.00%) and T_5 (12.50%) which were significantly superior to rest of the treatments and on par with each other, followed by T_4 (17.50%), T_3 (20.00%) and T_1 (20.00%). The maximum per cent fruit infection was observed in untreated control plot (42.50%). Per cent disease severity on the fruit after 9^{th} spray was lowest in T_9 (5.50%) and T_5 (7.00%) which is significantly superior to rest of the treatments and they were on par with each other. Next best treatments were T_4 (13.00%), T_1 (14.00%) and T_3 (19.00%).

Results pooled over the two locations revealed that after 9^{th} spray the disease incidence and severity on stem was significantly very less in the T_9, T_5 and T_4 and they were on par with each other (Fig. 10 and 11).

Per cent leaf infection after 9^{th} spray was minimum in T_9 and T_5 which were significantly superior to rest of the treatments followed by T_4. Per cent disease severity on leaf at the end of 9^{th} spray was minimum in T_9, T_5 and T_4 which were significantly superior to rest of the treatments and on par with each other followed by T_1 (Fig. 12, 13).

Per cent fruit infection after 9^{th} spray was minimum in T_9 which was significantly superior to rest of the treatments followed by T_5 and T_4. Per cent disease severity on fruit after 9^{th} spray was minimum in T_9 and T_5 were significantly superior to rest of the treatments followed by T_4 and T_1 (Fig. 14, 15).

Kishun and Sohi (1984) reported that bacterial canker of mango could be effectively controlled by four sprays of bavistin (0.1%) or bavistin (0.1%) + agrimycin-100 (100 ppm). Similarly, black rot of cabbage caused by *Xanthomonas campestris* pv. *campestris* was effectively controlled by spray application of agrimycin-100 (0.1%) or streptocycline @ 250 ppm. Present findings also indicated the efficacy of streptocycline in reducing the disease severity.

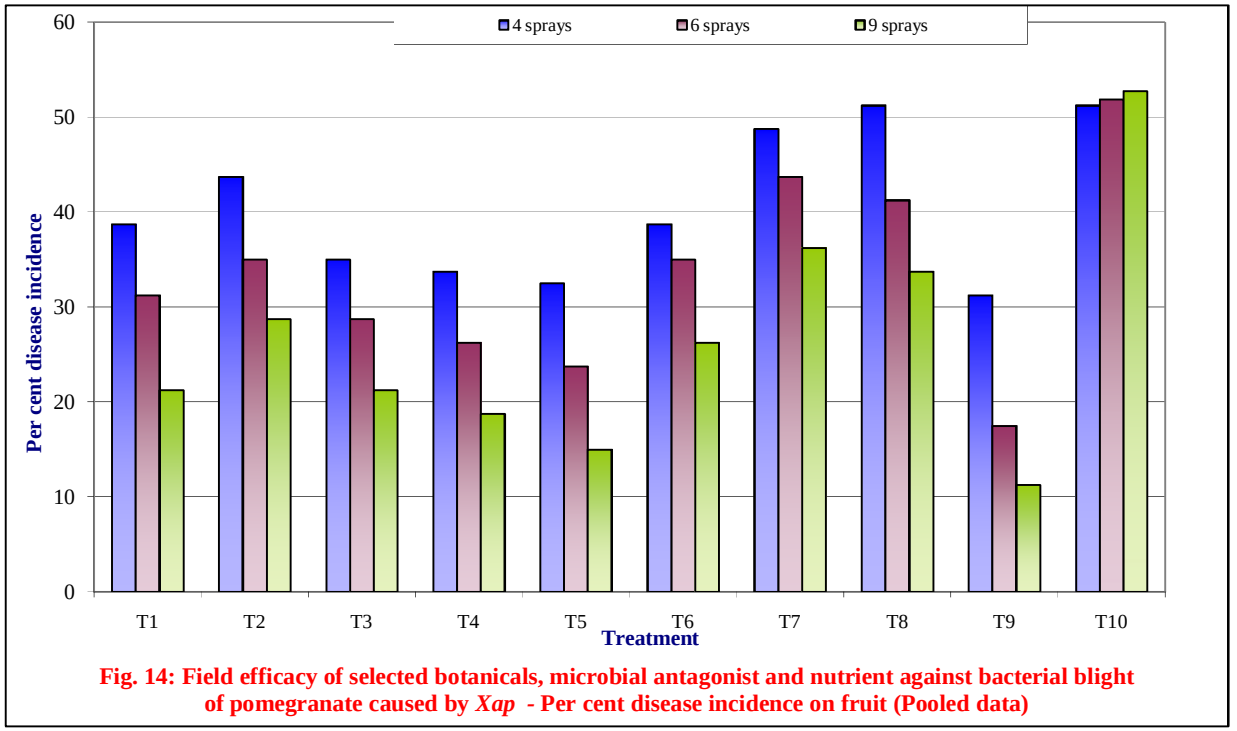

Fig. 14: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on fruit (Pooled data)

Fig. 14: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on fruit (Pooled data)

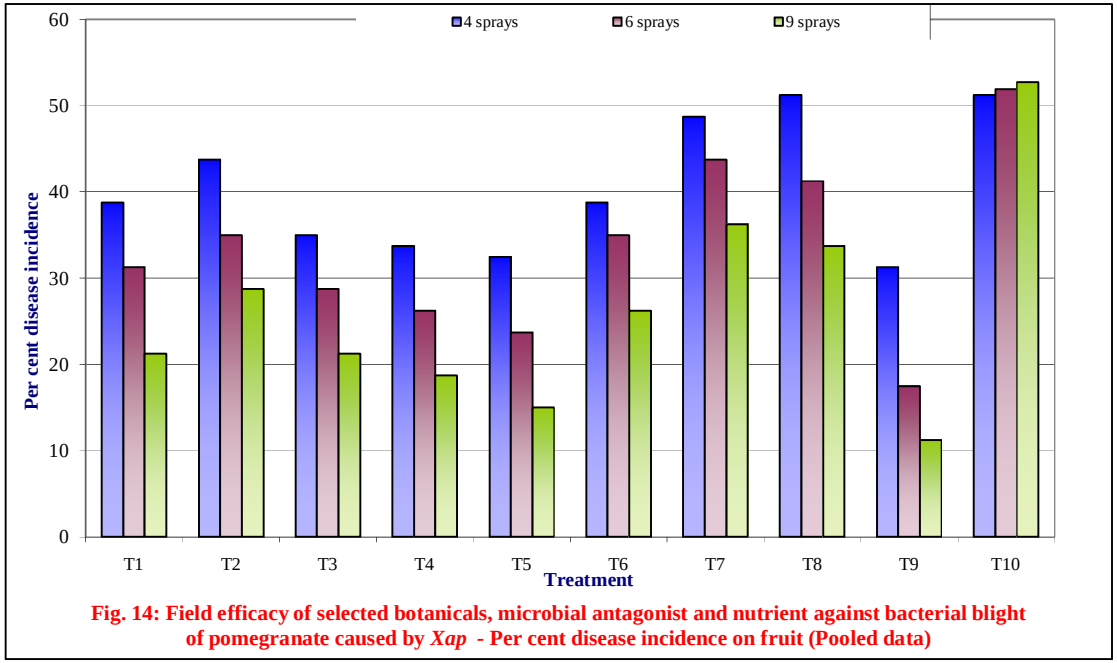

Fig. 14: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on fruit (Pooled data)

Fig. 14: Field efficacy of selected botanicals, microbial antagonist and nutrient against bacterial blight of pomegranate caused by *Xap* - Per cent disease incidence on fruit (Pooled data)

Yenjeerappa (2009) reported that lowest disease index of 22.3 per cent in streptocycline (0.05%) + copper oxychloride (0.2%) treated plots followed by bacterinol 100 (0.05%) + copper oxychloride (0.2%) and bacteriomycine (0.05%) + copper oxychloride (0.2%), which recorded 27.2 per cent disease index to two years experimentation against bacterial blight of pomegranate. All these three bactericides exhibited on par efficacy each other and significantly superior efficacy to *P. fluorescens* at 05% (bioagent) and garlic extract at 2% (botanical). Highest disease severity of 63.1 per cent was recorded in untreated check plot.

In the light of present day constraints with the use of chemical pesticides in plant disease management, the biological control as an alternate option is gaining importance and awareness as the approach is ecofriendly and cost effective. Under biological control of plant diseases, various antagonistic organisms have been identified, which fight against the pathogens by different mechanisms *viz.,* competition, lysis, antibiosis, siderophore production and hyper parasistism (Vidyasekaran, 1999). The presence of fluorescent pigments produced by *Pseudomonas* are sequester Fe^{3+} and are termed as siderophores, which act as a inhibitors for the growth of some phytopathogenic bacteria may be responsible for inhibiting the growth of *Xap.*

The results of present investigation derive support from the findings of Dutta *et al.* (2005), who reported that among the 21 rhizobacterial and 52 phylloplane bacterial isolates of mungbean, the isolates MRb (*Pseudomonas fluorescens*) and Plb-3 (*Bacillus* spp.) were effective in reducing the seedling and trifoliate leaf infection of mungbean induced *X. axonopodis* pv. *vignaradiatae.*

Use of biocontrol agents in the management of plant diseases is an age old practice and effectiveness of bacterial biocontrol agents against various crop diseases was reported by earlier workers. Kalita *et al.* (1996) reported the reduction of citrus canker by the sprays application of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Bacillus polymixa*. They recorded the least canker incidence with the treatment by *Bacillus subtilis*.

The efficacy of *Pseudomonas fluorescens* in reducing the bacterial blight intensity on rice was reported by Kaur and Thind (2002).

Least bacterial leaf spot incidence on grape vine was recorded in the plots treated with streptocycline or streptomycin sulphate each at 0.05% concentration (Ravikumar *et al.*, 2002). Pruning of infected parts along with spray of copper oxychloride followed by four sprays of streptocycline (100 ppm) + copper oxychloride (0.3%) was suggested by Gopal *et al.* (2004) for the effective control of bacterial canker of acid lime. Yenjerappa *et al.* (2004) reported the superior efficacy of streptocycline (0.05%) in combination with copper oxychloride (0.2%) in mitigating the bacterial blight menace on pomegranate. In the present investigation, the efficacy of streptocycline in combination with copper oxychloride was very much emphasized against bacterial blight control, but however on comparison, the efficacy of streptoclycine is non-significantly lower than other bactericides *viz.,* bronip/bactinash-200/bactrinashak in managing the disease effectively.

Jalaraddi (2006) reported that *Pseudomonas fluorescens*, was found moderately effective in inhibiting the pathogen *Xap* by producing inhibition zone of 10.8 mm, whereas, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Bacillus polymyxa* were not effective in inhibiting the pathogen.

Raju (2010) reported that two bio agents *Pseudomonas fluorescens* at 1.00 per cent and *Bacillus subtilis* at 1.00 per cent were moderately effective and they are significantly different from each other. Disease incidence of 56.65 per cent was recorded in case of *Pseudomonas flourescens* followed by *Bacillus subtilis* 57.26 per cent on stem but they were significantly differed from each other with 36.14 per cent PDI and 38.69 per cent PDI respectively.

Anon. (2009) recommended first spray should be given of Bordeaux mixture (1%) immediately after pruning, second spray at foliage initiation with streptocycline (250 ppm) + COC (0.25%), third spray with Bordeaux mixture (0.4%) and fourth spray with streptocycline (250 ppm) + carbendazim (0.1%) against bacterial blight of pomegranate.

Anon. (2011) reported that spray with Bordeaux mixture (1%), *Pseudomonas fluorescens* talc based formulation @10 g/l, Copper oxychloride 50WP (2.5g/l) + Bronopol (0.5g/l) at regular interval can manage the bacterial blight of pomegranate.

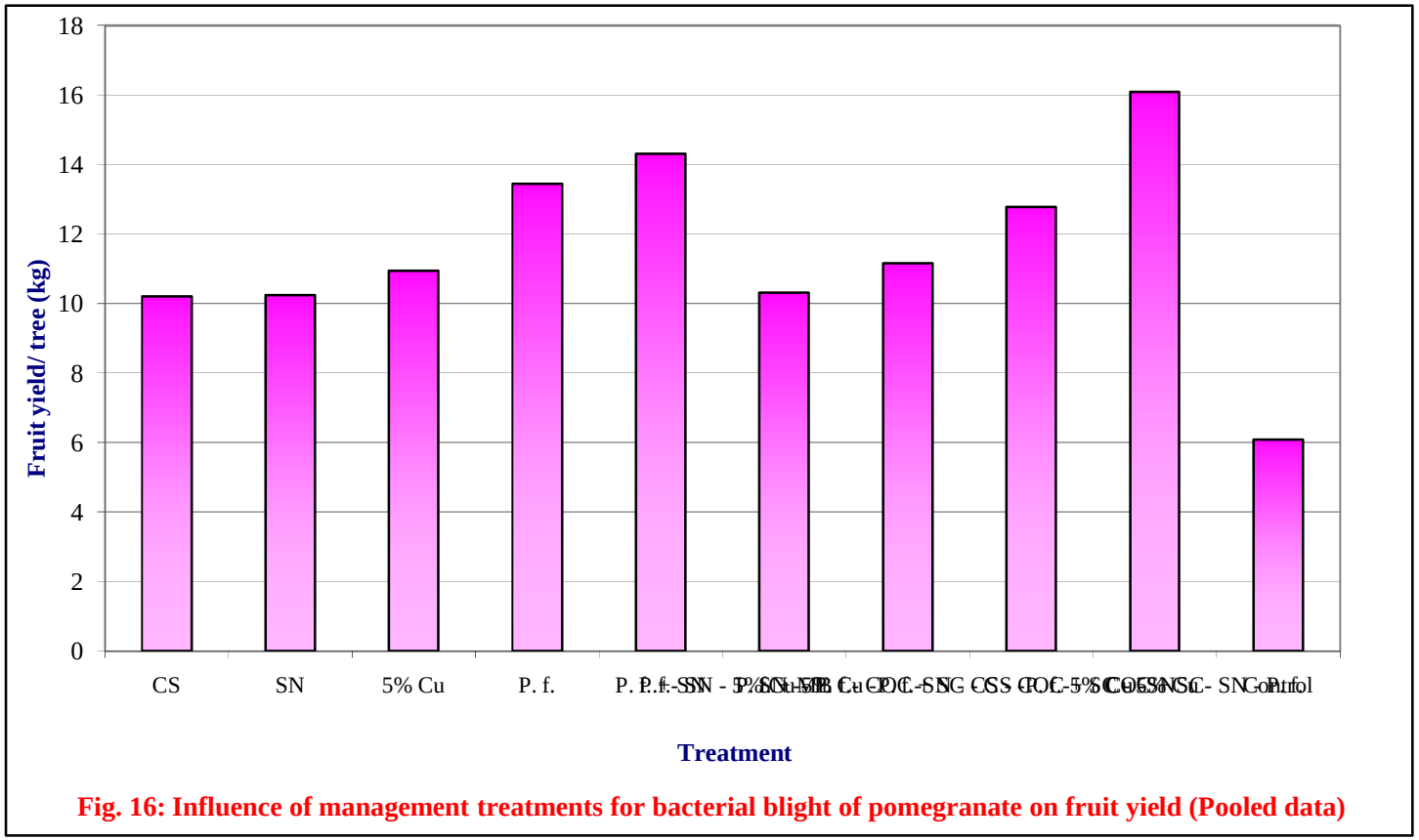

Fig. 16: Influence of management treatments for bacterial blight of pomegranate on fruit yield (Pooled data)

Fig. 16: Influence of management treatments for bacterial blight of pomegranate on fruit yield (Pooled data)

5.8.1 Effect of botanical, bioagents, bactericide, commercial formulation (5%) of copper and nutrient on severity of bacterial blight and yield of pomegranate

In first field the fruit yield per tree was maximum of 18.00 kg in T_9 (streptocycline + COC at 0.5% +3%) followed by 17.5 kg in T_5 (*Pseudomonas fluorescens* + soapnut at 1% +5%). However, it was least (8.5 kg) in case of untreated control during Mrigbahar season.

In second field the fruit yield per tree was maximum in T_9 (19 kg) followed by T_5 and T_4 they are significantly superior to rest of the treatments and on par with each other.

Results on pooled data of two locations revealed that significantly significantly highest fruit yield per tree was maximum in T_9 (17.13 kg) followed by T_5 (16.00 kg) and T_4 (15.00 kg). Least fruit yield per tree was observed in untreated control (8.75 kg) (Fig. 16).

Similar results were obtained by Yenjerappa *et al.* (2004) who reported the superior efficacy of Streptocycline (0.05%) in combination with Copper-oxychloride (0.2%) in mitigating the bacterial blight menace on pomegranate. In the present investigation, also it was found that treatments, Streptocycline + COC and Streptoclycine + Copper hydroxide were found superior in managing the bacterial blight disease.

Similar results obtained Manjula *et al.* (2002) as they achieved the effective control of bacterial blight of pomegranate with the sprays of Streptocycline or K-cycline or bactrinol-100.

Suriachandraselvan *et al.* (1993) suggested the application of Paushamycin (0.05%) in combination with copper oxychloride (0.2%) for the control of bacterial disease on pomegranate caused by *Xap*. Atulchandra *et al.* (1994) stated that same bacterium could be effectively controlled with the sprays of Bordeaux mixture (5:5:50) or any other copper fungicides at an interval of 15 days. Contradictory to this, performance of Bordeaux mixture was very poor in the present investigation against the disease probably due to the aggressive nature of the pathogen accompanied with favourable environmental conditions such as continuous/intermittent rainfall (in some weeks), maximum and minimum temperatures ranged between 27.7 to 35.8 and 12.1 to 24.9, relative humidity of 54.8 to 86.0 per cent which prevailed throughout mrigbahar season (2007), lead to the outbreak severity of the disease, which could not be mitigated easily with the sprays of Bordeaux mixture.

Further the findings are in line with findings of Ravikumar *et al.* (2002) reported that least bacterial leaf spot incidence on grape vine was recorded in the plots treated with Streptocycline or Streptomycin sulphate each at 0.05% concentration.

The disease management by COC+Streptocycline (0.3+0.05%) was reflected highest net profit and C:B ratio 1 :5.29 as maximum average yield of 16.10 tonnes per ha followed by *Pseudomona fluorescens* (1%) with net profit of Rs. 8,24466 and cost:benefit ratio of 1:4.76. Streptocycline which is used to cure many diseases in human being and animals. The use of streptocycline in curing the diseases of plants may not be a good option. As antibiotics are not used at the fruit maturity stage because it may cause residual effect on fruit. Hence management of bacterial blight with *Pseudomonas fluorescens* is the best option to reduce the residual effect and environmental pollution.

SUMMARY AND CONCLUSIONS

Pomegranate (*Punica granatum* L.) belongs to the family Punicaeae is an important fruit crop in India and in subtropical countries of the world. Among the different states cultivating pomegranate, Maharashtra is the largest producer followed by Karnataka, Andhra Pradesh, Gujrath and Rajasthan. This is subjected to various disease problems and bacterial blight caused by *Xap* (Hingorani and Singh,) Vauterin *et al.* is one of the important disease, which caused a great havoc in recent years.

The present investigation was undertaken to assess the diversity among different isolates collected from different agroclimatic regions and to manage this disease effectively. Initially the symptoms appeared as minute water soaked lesions which later turned brown to dark brown surrounded by diffused water soaked margin or yellow halo. Long, narrow and elongated light brown coloured lesions could be seen on the branches and also on the main stems with the advancements of disease, the lesions become rough, cankerous and appeared blackish. The rind of affected fruits cracks in the middle of the lesion in the form of L, Y or star shape.

Pathogenic test revealed that the *X. axonopodis* pv*. punicae* was pathogenic to pomegranate and produced typical symptoms on pomegranate leaves after six to nine days of inoculation as small water soaked lesions in *Xap*4, *Xap*7, *Xap*8, *Xap*10, *Xap*12, *Xap*14, *Xap*17. Ten to fifteen days incubation period required for *Xap*1, *Xap*2, *Xap*3, *Xap*5, *Xap*6, *Xap*9, *Xap*11, *Xap*15, *Xap*16, *Xap*18 and nineteen days incubation period required for *Xap*13, which was considered as less virulent isolate.

All the eighteen isolates of *Xap* injected into the intercellular spaces of *Nicotiana tabacum* leaves, produced characteristic water soaked lesions after 48 h of inoculation and produced desiccated confluent brown necrotic area in 90 h of inoculation.

The colony characters of eighteen *Xap* isolates varied from circular and entire colony shape; slightly mucoidness to highly mucoidness character; light yellow, dark yellow and medium yellow colour colony; convex, highly raised to slightly raised and flat and opaque, glittering colony. Glycerol stocks of the eighteen isolates were made by using 50% glycerol and stored at -80^0C for long term preservation of the culture.

The growth of bacteria starts after 40 hrs and maximum growth was observed at 128 and 136 hrs after inoculation *i.e.*, stationary phase, after 136 hrs there was gradual decrease in the growth of the bacterium *i.e.*, decline phase.

Among the varied temperature and pH levels tested for the growth of *X. axonopodis* pv. *punicae,* temperature of 28 ± 1^0C and pH of 6.0 to 7.0 were found optimum for the good growth of the pathogen. The too and below these levels, decreased the growth.

Among different NaCl concentrations, zero per cent and one per cent were found effective for the growth of all the isolates of *Xap*. All eighteen isolates of *Xap* produced fuscan pigment on LB agar after nine days of incubation.

Among the bactericides, K-Cyclin was found effective against all isolates of *Xap*, which is significantly superior to rest of the bactericides and is followed by streptocycline and plantomycine. Least inhibition was found in bronip and COC.

A 1,537 bp region of 16S rRNA gene was amplified (using primers forward-5′AGTGAAGAGTTTGATCCTGGC3′ and reverse-5′AGGAGGTGATCCAGCCGC3 ′). A 491 bp region of *gyr*B was targeted (using primers forward-5′GTGCGGCGCTGAT T C 3 ′ and reverse - 5 ′ CGTGTCGGGGTTGAC 3′) from *Xap* strains through polymerase chain reaction (PCR). The sequences obtained using forward primers were assembled using Vector NTI software. Nucleotide sequences of nine *Xap* isolates 16S rRNA region were analyzed using bioinformatics tools like NCBI (National Center for Biotechnology Information) BLAST programme. Based on sequence comparison, all the fragment of 16S rRNA gene sequences of nine isolates were confirmed as *Xap Xap*-85 strain 16S rRNA gene, partial sequence; ribosomal RNA. Phylogram results of 9 *Xap* isolates revealed that there are two major clusters, cluster A comprising all Xanthomonas species, whereas cluster B comprises *Pantoea agglumerans*. A glance towards dendrogram revealed that there is no much diversity among the nine isolates of *Xap*.

The multiplex polymerase chain reaction was performed using template containing DNA from *Xap* targeting 16S rRNA and *gyr*B gene by 16S rRNA and *gyr*B specific primers. High intensity of

amplification of the gene specific products of 1537 bp and 491 bp corresponding to 16S rRNA and *gyr*B were obtained at annealing temperature of 57.5º C for 1 minute as compared to other temperatures.

Among plant extracts tested, soapnut extract was found very effective in inhibiting the maximum growth of the pathogen followed by adahtoda + soapnut and miswak + soapnut.

Among nine nutrients tested, $CuSO_4$ has recorded highest mean inhibitory zone followed by micron special (boron, $MgSO_4$ and $ZnSO_4$) at all concentrations tested.

Among five different strains of *P. fluorescens* tested strain No. 326(4) and PF1 has recorded highest inhibitory zone against *Xap* followed by strain No. 139, 134 and PF5. Among the eleven different strains of Pink Pigmented Facultative Methylotrophs (PPFM) tested, strain No. 10L has recorded highest inhibitory zone.

The detection of *Xap* from infected samples recovered on semi-selective media without DNA isolation. Single colony of *Xap* was picked up with a sterile pipette tip and added directly to the PCR mixture as a template for DNA amplification. Amplification of *gyr*B at 491 bp using a set of primers and annealing temperature for *gyr*B gene was 55°C for 1 minute.

Colony PCR using *gyr*B gene specific primer can be an efficient and economical method to monitor *Xap*. Colony PCR can be a quantitative tool for pathogen detection that may provide more information than standard detection protocols.

Among the botanicals soapnut, among bioagents *Pseudomonas fluorescens* strain (326(1)) and PPFM strain 10L, among nutrients $CuSO_4$ and $ZnSO_4$ and commercial formulation (5%) of copper, among bactericides COC + Streptocycline were tested in alone and in combination, COC + Streptocycline at 0.3% + 0.05% was given highest inhibition zone of 3.13 cm diameter followed by *P. f* strain 326(1) +soapnut, *P. f.* starin 326(1) + PPFM strain 10L and *P. f*. 326(4).

The per cent disease incidence and severity of disease on stem after 9^{th} spray was pooled over two locations revealed that stem infection was significantly very less in the T_9, T_5 and T_4. Disease severity was minimum in T_9, T_5 and T_4.

Per cent leaf infection after 9^{th} spray was minimum in T_9 and followed by T_4. Per cent disease severity was minimum in T_9, T_5 and T_4 followed by T_1.

Per cent fruit infection after 9^{th} spray was minimum in T_9 followed by T_5 and T_4. Per cent disease severity was minimum in T_9 and T_5 followed by T_4 and T_1.

Average fruit yield pooled over two lacations indicated that significantly highest fruit yield per tree was maximum in T_9, T_5 and T_4 were on par with each other.

The maximum C:B ratio was observed in COC+streptocycline (0.3+0.05%) followed by *Pseudomonas fluorescens* + soapnut extract (1% + 5%) with 1:4.59 and *P. fluorescens* (1%) with C:B ratio of 1:4.76

Future line of work

1. Sensitivity of different isolates of *Xap* may be carried out from time to time in order to recommend antibiotics based on location specific sensitivity. Such studies should be carried out continuously.
2. Streptocycline which is used to cure many diseases in human being and animals. The use of streptocycline in curing the diseases of plants may not be a good option. Hence further studies in mechanism of management of bacterial diseases may be continued with *P. fluorescens*, *Bacillus subtilis* and botanicals like soapnut, adahtoda, miswak.
3. There is a need to study the management of bacterial blight of pomegranate by means of organic way.
4. Residues analysis may be carried out in use of antibiotics.
5. The mechanism of action by use of nutrients in disease management needs further study.
6. Under changing environmental parameters, there is a need to re-schedule of integrated disease management of bacterial blight of pomegranate.

REFERENCES

Abd, E. L., Rahman, H. F., Skaug, N. and Whyatt, A., 2003, Volatile compounds in crude *Salvadora persica* extracts. *Pharmaceut. Biol*., **41** : 399-404.

Adachi, N. and Oku, T., 2000, PCR- mediated detection of *Xanthomonas oryzae* pv. *oryzae* by amplification of the 16S-23S rDNA spacer region sequence. *J. Gen. Plant Pathol*., **66** : 303-309.

Adhikari, T. B., Vera Cruz, C. M., Zhang, Q., Nelson, R. J., Skinner, D. Z., Mew, T. W. and Leach, J. E., 1995, Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. *Appl. Environ. Microbiol.,* **61** : 966–971.

Akhtar, M. A. and Bhatti, M. H. R., 1992, Occurrence of bacterial leaf spot of Pomegranate in Pakistan. *Pak. J. Agric. Res*., **13** : 95-97.

Al Sadhan, R. I. and Almas, K., 1999, Miswak (chewing stick) : A cultural and scientific heritage. *Saudi Dental J*., **11** : 80-88.

Anonymous, 1969, *Punica granatum* L. In : *Wealth of India, Raw Materials*, Publication and Information Directorate, CSIR, New Delhi, Vol. III, pp. 321-323.

Anonymous, 2002, Pomegranate growers are in dire straits. *The Hindu,* dt : 9-8-2008, p. 8.

Anonymous, 2005, Campaign against disease (oily spot) on pomegranate. *Dallimbavrutha Smaranika*, pp. 131-133.

Anonymous, 2008, *Half Yearly Report on Mitigating Bacterial Blight of Pomegranate Project,* Submitted by Department of Plant Pathology, U. A. S. Dharwad to Govt. of India.

Anonymous, 2009, *Training manual for Govt. and university officers on pomegranate orchard health management*, Submitted by National Research Centre on Pomegranate.

Anonymous, 2011, Indian Horticulture Database (2011), National Horticulture Board, Ministry of Agriculture, Govt. of India.

Anonymous, 2011, *Final report on advanced research on management of bacterial blight of pomegranate,* Submitted Submitted by Department of Plant Pathology, U. A. S. Dharwad to Govt. of India

Anonymous, 2012, *Copper : to be Redisctoed*, SUMI agro France.

Ansari, M. M. and Shridhar, R., 2001, Iron nutrition and virulence in *Xanthomonas oryzae* pv. *oryzae*. *Indian Phytopathol.,* **54**(3) : 279-283.

Ash, C., Farrow, J. A. E., Dorsch, M., Stackebrandt, E. and Collins, M. D., 1991, Comparative analysis of *Bacillus anthracis, Bacillus cereus* and related species based on the basis of reverse transcriptase sequencing of 16S rRNA. *Int. J. Syst. Bacteriol*., **41** : 343–346.

Atulchandra, Chandra, A. and Gupta, I. C., 1994, Pomegranate. In : *Arid Fruit Research*, Scientific Publishers, Jodhpur, p. 56,

Bariana, H. S., Shannon, A. L., Chu, P. W. G. and Waterhouse, P. M., 1994, Detection of five seed borne legume viruses in one sensitive multiplex polymerase chain reaction test. *Phytopathol.,* **84** : 1201-1205.

Benagi, V. I., Ravikumar, M. R. and Nargund V. B., 2011, Threat of bacterial blight on pomegranate in India : *Mitigation by Integrated Approach : II International Symposium on the Pomegranate*. Universitas Miguel Hernandez Elche, October; 19-21 : p. 51.

Bhat, V. V. and Patel, M. K., 1954, Comparative study of species of *Xanthomonas* parasitizing leguminous plants in India. *Indian Phytopathol*., **7** : 160-180.

Bogdanove, A. J., Koebnik, R., Lu, H., Furutani, A., Angiuoli, S. V., Patil, P. B., Van Sluys, M. A., Ryan, R.P., Meyer, D. F., Han, S. W., Aparna, G., Rajaram, M., Delcher, A. L., Phillippy, A. M., Puiu, D., Schatz, M. C., Shumway, M., Sommer, D. D., Trapnell, C., Benahmed, F., Dimitrov, G., Madupu, R., Radune, D., Sullivan, S., Jha, G., Ishihara, H., Lee, S. W., Pandey, A., Sharma, V., Sriariyanun, M., Szurek, B., Vera Cruz, C. M., Dorman, K. S., Ronald, P.C., Verdier, V., Dow, J. M., Sonti, R. V., Tsuge, S., Brendel, V. P., Rabinowicz, P. D., Leach, J. E., White, F. F. and Salzberg, S.L., 2011, Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic *Xanthomonas* spp. *J. Bacteriol,* **193** : 5450–5464.

Bose, T. K., 1985, Pomegranate. In : *Fruit of India – Tropical and Subtropical*, B. Mitra Naya Prakash Publications, Calcutta, p. 637.

Chadha, K. L., 2001, Pomegranate. ***In*: *Handbook of horticulture***, (Eds.) : Shashi, A. Verma and Somdutt, *In*:Directorate of Information and Publications of Agriculture, pp 297, ICAR, New Delhi, 1031 pp.

Chand, R. and Kishun, R., 1991, Studies on bacterial blight of pomegranate. *Indian Phytopathol*. **44**(3) : 370-372.

Chand, R. and Kishun, R., 1993, Systemic movement of *Xanthomonas campestris* pv. *punicae* (Hingorani and Singh) Dye from leaf to node in pomegranate. *Int. J. Tropical Plant Dis.,* **11(1)** : 85-90.

Chand, R., Kishun, R. and Singh, S. J., 1991a, Characterization of bacteriophage parasitizing on *Xanthomonas campestris* pv. *Viticola*. *Indian J. Exptl. Biol.,* **29 :** 180-181.

Darout, I., Christy, A. and Skaug, N., 2000, Identification and quantification of some potentially antimicrobial anionic components in miswak extract. *Indian J. Pharmacol*., **32** : 11-14.

Darsonval, A., Darrasse, A., Durand, K., Bureau, C., Cesbron, S., and Jacques, M. -A. 2009. Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*. *Mol. Plant Microbe Interact*., **22 :**747-757.

Das, A. K. and Shyam S., 2003, Integration of chemicals and cultural practices for management of bacterial canker (*Xanthomonas axonopodis* pv. *citri*) in acid lime (*Citrus aurantifolia*). *Indian J. Agric. Sci.,* **73**(10) : 570.

David, H. G., 2005, Management of *Xanthomonas* leaf blight of onion with a plant activator, biological control agents and copper bactericides. *Plant Dis.,* **89**(6) : 631-639.

Dordas, C., 2008, Role of nutrients in controlling plant diseases in sustainable agriculture- A review. *Agron. Sustain. Dev.,* **28** : 33-46.

Dutta, S., Singh, R. P. and Jinda, J. K., 2005, Effect of antagonistic bacteria and plant defense activators on management of bacterial leaf spot of mungbean. *Indian Phytopathol.,* **58**(3) : 269-275.

Ezmirly, S. T. and El-Nasr, M. S., 1981, Isolation of gluco-tropaeolin from *Salvadora persica*. *J. Chem. Soc. Pak.,* **3** : 9-12.

Falico, De and Alcaraz, L. M., 1973, *Xanthomonas vesicatoria* (Doidge) Dowson of tomato and capsicum currents province Argentina. *Hort. Abstr*., **46** : 4694.

Figueiredo, D. V. and Torres Brioso, P. S., 2007, Mulitplex PCR for detection of banana streak virus and cucumber mosaic virus from micropropagated banana. *Summa Phytopathol.,* **33**(3) : 229-232.

Gambino G. and Gribaudo, I., 2006, Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. *Phytopathol*, **96** : 1223-1229.

Garg, S. C. and Kesara, H. L., 1984, Antimicrobial activity of the essential oil of *Anacardium occidentale* Linn. *Indian Perfumer,* **28 :** 95**-**97**.**

Garg, S. C. and Kesara, H. L., 1998, Antimicrobial activity of the essential oil of *Callistemon viminalis* Cheel. *Indian Perfumer,* **42 :** 138**-**141**.**

Garg, S. C. and Oswal, V. B., 1981, *In vitro* antimicrobial activity of the essential oil of *'Buddleia asiatica'* Lour. *Indian Perfumer,* **25 :** 7**-**9**.**

Gent, D. H., A. Al-Saadi, Gabriel, D. W., Louws, F. J., Ishimaru, C. A. and Schwartz, H. F., 2005, Pathogenic and genetic relatedness among *Xanthomonas axonopodis pv. allii* and other pathovars of *X. axonopodis*. *Phytopathol*, **95** : 918–925

Gent, D. H., Schwartz H. F., Ishimaru, C. A., Louws F. J., Cramer, R. A., and Lawrence, C. B., 2004, Polyphasic characterization of *Xanthomonas* strains from onion. *Phytopathol*, **94** : 184–195

Gopal, K., Reddy, M. R. S. and Prasad Babu, G., 2004, Management of bacterial canker in acid lime (*Citrus aurantifolia* Swingle) in Andhra Pradesh. *Indian J. Pl. Prot.,* **32**(1) : 111-113.

Gour, H. N., Ashiya, J., Mali, B. L. and Ranjan Nath, 2000, Influence of temperature and pH on the growth and toxin production by *Xanthomonas axonopodis pv. vignicola* inciting leaf blight of cowpea. *J. Mycol. Pl. Pathol*., **30**(3) : 389-392.

Grainage, M., Berger, L. and Ahmed, S., 1985, Effect of extracts of *Atrabotrys uncinatus* and *Allium sativum* on *Xanthomonas campestris* pv. *Oryzae. Curr. Sci*., **54 :** 90.

Hauben, L., Vauterin L., Swings, J. and Moore, E R B., 1997, Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.,* **47** : 328–335.

Hayashi, K., 1991, PCR-SSCP: A simple and sensitive method for detection of mutation in the genomic DNA, *PCR Methods Application*, **1** : 34-38.

Hingorani, M. and Mehta, P. P., 1952, Bacterial leaf spot of pomegranate. *Indian Phytopathol.,* **5** : 55-56.

Hingorani, M. K. and Singh, N. J., 1959, *Xanthomonas punicae* sp. Nov. on pomegranate (*Punica granatum* L.). *Indian J. Agric. Sci*., **29** : 45-48.

http : //www. ncbi. nlm. nih. gov.

Jabeen, R., Iftikhar, T. and Batool, H., 2012, Preservation and pathogenicity test of *Xanthomonas oryzae pv. oryzae* causing BLB disease in rice. *Pak. J. Bot*., **44**(1) : 261-265.

Jadeja, K. B., Mayani, N. G., Patel, V. A. and Ghodasara, M. T., 2000, Chemical control of canker and gummosis of citrus in Gujarat. *J. Mycol. Pl. Pathol.,* **30**(1) : 87-88.

Jalaraddi, J. N., 2006, Biological control of Bacterial blight of pomegranate caused by *Xap*. *M. Sc. (Agri) Thesis,* Univ. Agric. Sci., Bangalore.

Jindal, J. K. and Patel, P. N., 1984, Variability in *Xanthomonas* of grain legume IV. Variation in bacteriological properties of 83 isolates and pathogenic behavior of cultural variants. *Phytopathol.,* **110** : 63-68.

Jones Nirmalnath, P., 2010**,** Molecular diversity of arbuscular mycorrhizal fungi and pink pigmented facultative methylotrophic bacteria and their influence on grapevine (*Vitis vinifera). Ph. D. Thesis,* Univ. Agric. Sci. Dharwad (India).

Jones Nirmalnath, P., Benagi, V. I., Basamma, Madhu, S. G., Shivananda, J., Jagadeesh, K. S., Alagawadi, A. R. and Shripad K., 2011a, Bioassay of fluorescent pseudomonas against *Xap* causing bacterial blight of pomegranate. In : *National Symposium on Integrated Disease Management Strategies in Relation to Climate Change in South India.* Univ. Agric. Sci. Dharwad and Indian Phytopathol. Soc. (South Zone) from 14-15, October 2011.

Jones Nirmalnath, P., Benagi, V. I., Shivananda, J., Madhu, S. G., Basamma, Nargund, V. B., and Krishnaraj, P. U., 2011b, *In vitro* screening of native pink pigmented facultative methylotrophs against bacterial blight of pomegranate. In : *National Symposium on Integrated Disease Management Strategies in Relation to Climate Change in South India*. Univ. Agric. Sci., Dharwad and Indian Phytopathological Society (South Zone) from 14-15, October 2011.

Kalita, P., Bora, L. C. and Bhagabati, K. N., 1996, *Phylloplane microflora* of citrus and their role in management of citrus canker. *Indian Phytopathol.,* **49**(3) : 234-237.

Kamel, M. S., Ohtani, K. and Assaf, M. H., 1992, Lignan glycosides from stems of *Salvadora persica*. *Phytochemistry*, **31** : 2469-2471.

Kanthaswamy, A., Subramanian, S. and Govindasamy, S., 1989, Bactericidal and fungicidal effects of *Prosopis juliflera* alkaloidal fraction. *Indian Drugs*, **26** : 380-394.

Kanwar, Z. S., 1976, A note on bacterial disease of pomegranate (*Punica granatum* L.) in Haryana. *Haryana J. Hort. Sci*., **5** : 177-180.

Karuna, K. and Khan, A. N. A., 1994, Effect of plant extracts on *Pseudomonas solanacearum* causing wilt of tomato plants. *Indian Phytopathol*., **47** : 326.

Kaur, M. and Thind, B. S., 2002, Effectiveness of *Pseudomonas fluorescens* against *Xanthomonas oryzae* pv. *oryzae* and its mass culturing. *Proc. Ann. Meet. Sym. - Integrated Plant Disease Management through Ecofriendly Strategies, Indian Phytopathol. Soc.* (Northern zone), pp. 98-105.

Khan, A. N. A., 1989, Studies on the methods of *Xanthomonas campestris* pv. *vignicola* (Burkh.) Dye in cowpea seeds and its survival nature. *Ph. D., Thesis*, Univ. Agric. Sci., Bangalore.

Kim, J. H., Choi, G. S., Kim, J. S., Lee, S. H., Choi, J. K. and Ryu, K. H., 2006, Development of single-tube multiplex immunocapture RT-PCR assay for simultaneous detection of two pepper tobamoviruses. *Plant Pathol. J*., **22**(2) : 164-167.

King, E. K, Ward, M. K. and Raney, D. E., 1954, Two simple media for the demonstration of pyconyacin and fluorescin. *J. Clin. Med*., **44** : 301-307.

Kiran, K., 2000, Studies on the effect of botanicals on important plant pathogenic bacteria, *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Kishun, R., 1993, Bacterial disease of fruits. In : *Advances in Horticulture*, Vol. 3 (Eds.) : Chand K. L. and Pareek, O. P., Malhotra Publishing House, New Delhi, pp. 1398-1400.

Kishun, R. and Sohi, H. S., 1984, Control of bacterial canker of mango by chemicals. *Pesticides*, **18 :** 32-33.

Klement, A. and Goodman, R., 1967, The hypersensitivity reaction to infection by bacterial plant pathogens. *Ann. Rev. Phytopathol.,* **5 :** 17-44.

Kong, P., Richardson, P. A. and Hong, C., 2005, Direct colony PCR-SSCP for detection of multiple pythiaceous oomycetes in environmental samples. *J. Microbiol methods*, **61** : 25-32.

Krishnan, K. J. and Madhumeeta, 1988, Chemical control of bacterial leaf and flower spot of Zinnia by *X. campestris.* pv. *zinnia*. *Indian Phytopathol*., **41** : 271.

Kumar, S. and Kotur, S. C., 1989, Effect of boron on susceptibility of cauliflower to black rot. *Indian Phytopathol.,* **44**(2) : 153-157.

Laha, G. S., Singh, R. P. and Verma, V. P., 1992, Biocontrol of *Rhizoctonia solani* in cotton by fluorescent pseudomonads. *Indian Phytopathol*., **45 :** 412-415.

Lehrer, R. I., Barton, A., Daher, K. A., Harwig, S. S., Ganz, T., 1989, Interaction of human defensins with *Escherichia coli.* Mechanism of bactericidal activity. *J. Clin. Invest*. **84** : 553–561.

Madhiazhagan, K., Ramadoss, N. and Anuradha, R., 2002, Effect of botanicals on bacterial blight of rice. *J. Mycol. Pl. Pathol.,* **32**(1) : 68-69.

Madhu S. G., 2009, Morphological and molecular variability of *Xap* causing bacterial blight of pomegranate. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Dharwad, Karnataka (India).

Malhotra, N. K., Khajuria, H. N. and Jawanda., 1983, Studies on physico-chemical characters of pomegranate cultivars II. Chemical characters. *Punjab Horti. J.,* **23** : 158.

Mangamma, P. and Sreeramulu., 1991, Garlic extract inhibitory to the growth of *Xanthomonas campestris* pv. *vesicatoria*. *Indian Phytopathol.,* **44** : 372-374.

Manjula, C. P. and Khan, A. N. A., 2002, Incidence of bacterial blight of pomegranate (*Punica granatum* L.) in Karnataka. Paper presented In : the *Ann. Meet. Symp. Plant Disease Scenario in Southern* India, Bangalore (India). December, 19-21, pp. 51-52.

Manjula, C. P. and Khan, A. N. A., 2003, Studies on symptomatology and etiology of bacterial blight of pomegranate (*Punica granatum* L) caused by *Xap*. *In : Nat. Seminar on Integrated Plant Disease Management and Sustainable Agriculture* held at Annamalai University during Mar. 20-21, 2003.

Manjula, C. P., Khan, A. N. A., Jalikop, S. H. and Ravikuar, M. R., 2003, Chemical and biological management of pomegranate caused by *Xanthomonas axonopodis pv. punicae. In: Nat. seminar on integrated plant disease management and sustainable agriculture* held at Annamalai University during Mar. 20-21, 2003.

Manjula, C. P., 2002, Studies on bacterial blight of pomegranate (*Punica granatum* L.) caused by *Xap*. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Manjula, C. P., Khan, A. N. A. and Ravikumar, M. R., 2002, Management of bacterial blight of pomegranate (*Punica granatum* L.) caused by *Xap*. Paper presented In : *Ann. Meet. Symp. Plant Disease Scenario in Southern India,* held at Bangalore (India), December 19-21, p. 52.

Meng, X. Q., Umesh. K. C., Davis, R. M. and Gilbertson R. L., 2004, Development of PCR-based assays for detecting *Xanthomonas campestris pv. carotae*, the carrot bacterial leaf blight pathogen from different substrates. *Plant Dis*., **88** : 1226–1234.

Mogle, T. R., Magar, S. J., Suryawanshi, A. P. and Somwanshi, S. D., 2009, Pathogenicity, morphology and cultural characteristics of *Xap*, causing oily spot of pomegranate. In : *II International Symposium on Pomegranate and Minor Including Mediterranean fruits*. From 23-27 June, Univ. Agric. Sci., Dharwad, p. 137.

Mohammad, A. and Turner, J. E., 1983, *In vitro* evaluation of Saudi Arabian toothbrush tree (*Salvadora persica*). *Odontostomatol Trop*., **53** : 145-148.

Mondal, K. K. and Kumar, A., 2011, Phenotype based identification of *Xap*. In : *Practical Manual on Advances in Phenomics, Genomics and Diagnostics of Xap Causing Bacterial Blight of Pomegranate,* pp. 5-11.

Mondal, K. K. and Mani, C., 2009, ERIC-PCR generated genomic fingerprints and their correlation with pathogenic variability of *Xanthomonas campestris* pv. *punicae*, the incitant of bacterial blight of pomegranate. *Curr. Microbiol.*, **59** : 616-620.

Mondal, K. K. and Mani, C., 2012, Investigation of the antibacterial properties of nanocopper against *Xap*, the incitant of pomegranate bacterial blight. *Ann. Microbiol.*, **62**(2) : 889-893.

Mondal, K. K. and Singh, D., 2008, *Bacterial Blight of Pomegranate-A Technical Bulletin,* Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, p. 8.

Mondal, K. K. and Singh, D., 2009, Bacterial blight-an emerging disease of pomegranate, In : *5th International Conference on Plant Pathology in the Globalized Era*. Indian Phytopathlogical Society (IPS). 10-13rd Nov '09, IARI, New Delhi (Abstr. No. 058A/S-11), p. 44.

Mondal, K. K., Rajendran, T. P., Phaneendra, C., Mani, C., Sharma, J., Shukla, R., Pooja, Verma, G., Kumar, R., Singh, D., Kumar, A., Saxena, A. K. and Jain, R. K., 2011, The reliable and rapid polymerase chain reaction (PCR) diagnosis for *Xap* in pomegranate. *Afr. J. Microbiol. Res.,* **6**(30) : 5950-5956.

Mondal, K. K. and Sharma, J., 2009 Bacterial blight: An emerging threat to pomegranate export. Indian Farming **59**:22– 23

Mondal, K. K., Verma, G. and Mani, C., 2012, Phylogenetic relatedness of *Xap*, the causal agent of bacterial blight of pomegranate based on two loci, 16S rRNA and *gyr*B, Ann. Microbiol.

Monod, J., 1949, The growth of bacterial cultures, *Annu. Rev. Microbiol.,* **3** : 371-394.

Motoshima, M., Yanagihara, K., Fukushima, K., Matsuda, J., Sugahara, K., Hirakata, Y., Yamada, Y., Kohno, S. and Kamihira, S., 2007, Rapid and accurate detection of *Pseudomonas aeruginosa* by real-time polymerase chain reaction with melting curve analysis targeting *gyr*B gene. *Diagnostic Microbiol. Infect. Dis.*, **58** : 53-58.

Nagaraj, M. S., 1997, Ecology, transmission and control of bacterial blight of Mulberry (*Morus alba* L.) caused by *Xanthomonas campestris* pv. *Mori. M. Sc. (Agri) Thesis,* Univ. Agric. Sci., Bangalore.

Nakamura, Y., Kawakami, M., Yoshihiro, A., Miyoshi, N., Ohigashi, H., 2002, Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. *J. Biol. Chem.,* **277** : 8492–8499.

Neilands, J. B. and Leong, S. A., 1986, Siderophores in relation to plant growth and disease, *Annu. Rev. Plant Physiol.,* **37** : 187-208.

Nesme, X., Vaneechoutte, M., Orso, S., Hoste, B. and Swings, J., 1995, Diversity and genetic relatedness within genera *Xanthomonas* and *Stenotrophomonus* using restriction endonuclease site differences of PCR-amplified 16S rRNA gene. *Syst. Appl. Microbiol.*, **18** : 127–135.

Opina, N. and Tavner, F., 1997, A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia Pacific Mol. Biol. and Biotech.,* **5**(1) : 19-30.

Ozdemir, Z., 2005, Development of a multiplex PCR assay for concurrent detection of *Clavibacter michiganensis* ssp. *Michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria*. *Plant Pathol J.,* **4**(2) : 133-137.

Parkinson, N., Cowie, C., Heeney, J. and Stead, D., 2009, Phylogenetic structure of *Xanthomonas* determined by comparison of *gyr*B sequences. *J. Syst. Evol. Microbiol.,* **5**9 : 264–274.

Patel, M. K., Srinivasan, M. C. and Thirumalachar, M. J., 1965, Evaluation of host specificity character in the differentiation of *Xanthomonas* species. *Indian Phytopathol*., **18** : 174-180.

Petersen Y, Mansvelt E. L., Venter E, Langenhoven W. E., 2010, Detection of *Xap* causing bacterial blight on pomegranate in South Africa*. J. Aust. Plant Pathol.,* **39** : 544–546

Picard, Y., P. Roumagnac, D., Legrand, L. H., Robe`ne-Soustrade, I., Chiroleu, F., Gagnevin, L. and Pruvost, O., 2008, Polyphasic characterization of *Xanthomonas axonopodis pv. allii* associated with outbreaks of bacterial blight on three *Allium* species in the Mascarene archipelago. *Phytopathol.,* **98** : 919–925.

Pooler, M. R., Ritchie, D. F. and Hartung, J. S., 1996, Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified Polymorphic DNA PCR, Repetitive Extragenic Palindromic PCR and Enterobacterial Repetitive Intergenic Consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Appl. and Environ. Microbiol.,* pp. 3121-3127.

Raghavan, R., 2007, Oily spot of pomegranate in India (Maharashtra). *Express India,* dt : **18**. 11. 2007.

Rajendra, P. and Alankara R, 1987, *In vitro* antimicrobial screening of Indian essential oils. Part I : *Ocimum* spp. *J. Scientific Res*., **9** : 7-9.

Raju, J., 2010, Management of bacterial blight of pomegranate caused by *Xap. M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Dharwad, Karnataka (India).

Ramesh, B., 2009, Partial genome sequence analysis of banana streak, banana bract mosaic and cucumber mosaic virus isolates naturally infecting *Musa* spp. in Andhra Pradesh and development of diagnosis for their detection. *Ph. D. Thesis,* Sri Venkateswara University, Tirupati, p. 125.

Ramesh, C. and Ram, K., 1991, Studies on bacterial blight (*Xap)* of pomegranate. *Indian Phytopathol.,* **44 :** 51-54.

Ramos, R. A., Vidaver, A. K. and Flynn, P., 1991, Characterization of epiphytic *Xanthomonas campestris pv. phaseoli* and pectolytic Xanthomonads recovered from symptomless weeds in the Dominican Republic. *Ecol. Epidemiol*., **81**(6) : 677-681.

Rangaswamy, G., 1962, *Bacterial Plant Diseases in India*. Edn. II., pp 179, Asia Publications House, Bombay, p. 830.

Ravikumar, M. R., 1994, Studies on bacterial spot of tomato (*Lycopersicon esculentum* Mill.) caused by *Xanthomonas campestris* pv. *vesicatoria* (Dodge) Dye. *M. Sc. (Agri) Thesis,* Univ. of Agri. Sci., Bangalore.

Ravikumar, M. R., Jahagirdar, S. and Khan, A. N. A., 2002, Management of bacterial leaf spot of grape through chemicals and antibiotics in Northern Karnataka. Paper presented. In : *Ann. Meet. Symp. Plant Disease Scenario in Southern India,* Bangalore (India), December 19-21, p. 50.

Ravikumar, M. R., Jahagirdar, S., Yenjerappa, S. T. and Patil, H. B., 2006, Epidemiology and management of bacterial blight of pomegranate caused by *Xap*. In : *International Symposium on Pomegranate and Minor Mediterranean Fruits*, Adana, Turkey, from 16-19, October 2006.

Rodrigues, J. L. M., Silva-Stenico, M. E., Gomes, J. E., Lopes, J. R. S. and Tsai, S. M., 2003, Detection and diversity assessment of *Xylella fastidiosa* in field collected plant and insect samples by using 16S rRNA and *gyr*B sequences. *Appl. Environ. Microbiol.,* **69**(7) : 4249–4255.

Roland, K., Rolf, K., Beat, B. and Franco W., 2006, Genetic diversity and pathogenicity of the grass pathogen *Xanthomonas translucens* pv. *graminis*. *Sys. Appl. Microbiol.,* **29** : 109–119.

Roumagnac, P., Gagnevin, L., Gardan, L., Sutra, L., C. Manceau., Dickstein, E. R., Jones, J. B., Rott, P. and Pruvost. O., 2004, Polyphasic characterization of *xanthomonas* isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. *Int. J. Syst. Evol. Microbiol*., **54** : 15–24

Safiyazov. J. S., Mannhov, R. N. and Saturna, R. K., 1995, The use of bacterial antagonists for the control of cotton diseases. *Field Crops Res.,* **43 :** 51-54.

Saktivel, N., Sivamani, E., Unnamalai, N. and Gnanamanickam, S. S., 1986, Plant growth promoting rhizobacteria in enhancing plant growth and suppressing plant pathogens. *Curr. Sci.,* **55** : 22-25.

Salle, A. J., 1961, Fermentation of carbohydrates and related compounds. In : *Laboratory Manual on Fundamental Principles of Bacteriology*. V Ed. McGraw Hill Book Company, *Inc.,* New York, pp. 94-98.

Schaad, N. W., 1992, Laboratory guide for the identification of plant pathogenic bacteria. 2nd ed. *Am. Phytopathol. Soc.*, p. 138.

Schaad, N. W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I., Stromberg, P. E., Stromberg, V. K., Vidaver, A. K., 2007, *Xanthomonas alfalfae* sp. nov., *Xanthomonas citri* sp. nov. and *Xanthomonas fuscans* sp. nov. In : *List of New Names and New Combinations Previously Effectively, but not Validly,* Published, Validation list no. 115. *Int. J. Syst. Evol. Microbiol.*, **57** : 893-897.

Sharma, V., Midha, S., Ranjan, M. K., A. Pinnaka and Prabhu, B. P., 2012, Genome Sequence of *Xap* Strain LMG 859. *J. Bacteriol.,* **194**(9) : 2395.

Sher, H., Nasser, M., Al-yemeni and Wijaya, L., 2011, Ethnobotanical and antibacterial potential of *Salvadora persica* l : A well known medicinal plant in Arab and Unani system of medicine. *J. Med. Pl. Res.,* **5(7) :** 1224-1229.

Shobha, G., 1998, Detection, survival and control of *Xanthomonas axonopodis* pv. *glycines* (Nakano) Vauterin causing bacterial pustule of soybean [*Glycine max* (L.) Merrill]. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Sivamani, E., Anaratha, C. S. and Gnanamanickam, S., 1987, Toxicity of *Pseudomonmas fluorescens* towards bacterial plant pathogens of banana (*Pseudomonas solanacearum*) and rice (*Xanthomonas campestris* pv. *oryzae*). *Curr. Sci.,* **56 :** 547-548.

Sofrata, A., Santangelo, E. M., Azeem, M., Borg-Karlson, A. K., Gustafsson, A., 2011, Benzyl Isothiocyanate, a major component from the roots of *Salvadora Persica* is highly active against Gram-negative bacteria, *PLoS ONE.*, **6(8**) : e23045. doi : 10. 1371/journal. pone. 0023045

Sohi, H. S., Jain, S. S., Sharma, S. L. and Verma, B. R., 1964, New records of plant disease from Himachal Pradesh. *Indian Phytopathol.,* **17 :** 35-41.

Soustrade, I. R., Legrand, D., Gagnevin, L., Chiroleu, F., Laurent, A. and Pruvost. O., 2010, Multiplex Nested PCR for Detection of *Xanthomonas axonopodis pv. allii* from Onion Seeds. *Appl. Environ. Microbiol.*, **76**(9) : 2697.

Soustrade, R. I., Laurent, P. L., Jouen, G. E. and Pruvost. O., 2006, Specific detection of *Xanthomonas axonopodis pv. dieffenbachia* in anthurium (*Anthurium andreanum*) tissues by nested PCR. *Appl. Environ. Microbiol.*, **72** : 1072–1078.

Srinivasachary, 1995, Etiology and ecology of the causal organism of bacterial blight of mulberry (*Morus alba* L.). *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Sukhatme, P. V. and Amble, V. N., 1985, *Statistical Methods for Agricultural Workers,* Publication and Information Division, New Delhi, p. 553.

Suriachandraselvan, M., Jayasekar, M. and Anbu, S., 1993, Chemical control of bacterial leaf spot and fruit spot of pomegranate. *S. Indian Hort.,* **41**(4) : 228-229.

Sushma, J., 1997, Etiology, survival and chemical control of bacterial blight of *anthurium*. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Takao, I., Hiroyuki, I. and Katsumi, O., 2002, Simultaneous detection of six citrus viroids and Apple stem grooving virus from citrus plants by multiplex reverse transcription polymerase chain reaction. *J. Virol. Methods.,* **106** : 235-239.

Thammaiah, N., 1991, Studies on the detection of *Xanthomonas campestris* pv. *vignicola* (Burkh.) Dye in cowpea seeds and its control. *M. Sc. (Agric.). Thesis*, Univ. Agirc. Sci. Bangalore (India).

Thammaiah, N., Khan, A. N. A. and Elizabeth J., 1998, Effect of extract from *Adhatoda zeylanica* on *Xanthomonas campestris* pv. *vignicola* causing bacterial blight of cowpea. *Indian Phytopathol.*, **46** : 337.

Thirumalachar, M. J. and Patel, M. K., 1956, Effects of in vitro evaluation of some antibiotics on thirty two *Xanthomonas* species occurring in India. *Phytopathol.*, **46** : 486-488.

Thirumalachar, M. J. and Patel, M. K., 1966, Notes on some *Xanthomonas* spp. Described from South India. *Rev. Appl. Mycol.*, **45** : 12.

Thirumalesh, B. V., Thippeswamy B. and Krishnappa M., 2012, *In vitro* efficacy of eight plant extracts on *Xanthomonas campestris* pv. *mangiferaeindicae* affecting bacterial black spot of mango. *J. Crop Sci.,* **3**(2) : 78-80.

Trebaol, G., Manceau, C., Tirilly, Y. and Boury. S., 2001, Assessment of the genetic diversity among strains of *Xanthomonas cynaraeby* randomly amplified polymorphic DNA analysis and development of specific characterized amplified regions for the rapid identification of *X. cynarae*. *Appl. Environ. Microbiol.,* **67** : 3379–3384.

Tzeng, K. C., Lin, Y. C. and Hso, S. T., 1994, Foliar fluorescent pseudomonas from crops in Taiwan and their antagonism to phytopathogenic bacteria. *Plant Pathol. Bull.,* **3 :** 24-33.

Umesha, S., Chandan, S. and Nanjunda Swamy, L., 2012, Colony PCR- single strand confirmation polymorphism for the detection of *Ralstonia solanacearum* in tomato. *Int. J. Integrative biol.*, **13**(1) : 45-51.

Unnamalai, N. and Gnanamanickam, S. S., 1984, *Pseudomonas fluorescens* is an antagonistic to *Xanthomonas citri* (Hasse) Dye. The incitant of citrus canker. *Curr. Sci.,* **53 :** 703-704.

Valasubramanian, R., Chatterjee, A. and Gnanamanickam, S. S., 1994, Biological control of rice blast : Mediation of disease suppression antibiotics produced by *Pseudomonas fluorescens*. *Indian Phytopathol.,* **47 :** 281.

Vauterin, L., Haste, B., Kersters, K. and Swings, J., 1995, Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.*, **45** : 475-489.

Venugopal, M., 1983, Studies on variation in *X. campestris* pv. *mangiferaeindicae* the causal organism of bacterial canker of mango. *M. Sc. (Agri.) Thesis*, Univ. Agric. Sci., Bangalore, Karnataka (India).

Vidyasekaran, P., 1999, Biotechnological approaches to suppress seed to plant transmission of seed borne pathogens. In : *Nat. Symp. Seed Sci. Tech.,* University of Mysore, manasagangotri, Mysore, India, 5-7 August, p. 41.

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., Woodward, D. L. and Rodgers, F. G., 2002, Colony Multiplex PCR Assay for Identification and Differentiation of *Campylobacter jejuni*, *C. coli, C. lari, C. upsaliensis*, and *C. fetus* subsp. *fetus. J. Clin. Microbiol.,* **40**(12) : 4744–4747.

Wheeler, B. E. J., 1969, *An Introduction to Plant Diseases,* John Wiley and Sons Limited, London, p. 301. www. soapnut. pro, 2011.

Yenjerappa, S. T., 2009, Epidemiology and management of bacterial blight of pomegranate caused by *Xap*, *Ph. D. Thesis,* Univ. Agric. Sci. Dharwad (India).

Yenjerappa, S. T., Ravikumar, M. R., Jawadagi, R. S. and Nazir A. K., 2004, *In vitro* and *in vivo* efficacy of bactericides against bacterial blight of pomegranate. Paper presented In : *Nat. Symp. Crop Surv. : Disease Forecasting and Management*, IARI, New Delhi (India), February 19-21, p. 84.

Appendix I: Composition of various media used in different tests

Nutrient Glucose Agar (NGA)

Beef extract	: 3.0 g
Peptone	: 5.0 g
Glucose	: 10.0 g
Agar	: 20 g
Water	: 1000 ml

Luria Bertani's Agar (LBA)

Tryptone	: 10.0 g
Yeast extract	: 5.0 g
NaCl	: 10.0 g
Agar	: 20 g
pH	: 7.0
Water	: 1000 ml

MOLECULAR PHYLOGENETIC ANALYSIS OF *Xanthomonas axonopodis* pv. *punicae* ISOLATES AND BIO-PROSPECTING OF SELECTED BOTANICALS AND BIO-AGENTS AGAINST BACTERIAL BLIGHT OF POMEGRANATE

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ABSTRACT

Pomegranate, *Punica granatum* L., regarded as the "Fruit of Paradise" is subjected to various disease problems. Among diseases, bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh, 1959) Vauterin *et al.* is one of the important diseases, which caused a great havoc in recent years. The present investigation was undertaken to assess the diversity among different isolates collected from different agroclimatic regions and to manage this disease effectively.

Water soaked lesions on leaves were produced after six days of inoculation. All the eighteen isolates of *Xap* showed hypersensitive symptoms in *Nicotiana tabacum* leaves. Colony characters of eighteen *Xap* isolates varied from circular and entire colony shape; slightly to highly mucoid character. Among the varied temperature and pH levels tested, 28 ± 1^{0}C and pH of 6.0 to 7.0 were found optimum. All eighteen isolates of *Xap* produced fuscan pigment on LB agar after nine days of incubation. Among the bactericides, K-Cycline was found effective against all isolates of *Xap*, which was significantly superior to rest of the bactericides.

The multiplex polymerase chain reaction was performed using template containing DNA from *Xap* targeting 16S rRNA and *gyr*B gene by 16S rRNA and *gyr*B specific primers. High intensity of amplification of the gene specific products of 1537 bp and 491 bp corresponding to 16S rRNA and *gyr*B were obtained at annealing temperature of 57.5º C for 1 minute as compared to other temperatures.

Aqueous soapnut extract, *Pseudomonas fluorescens* strain [326(4)] and Pink Pigmented Facultative Methylotroph (PPFM) strain 10L, $CuSO_4$, $ZnSO_4$, COC, Streptocycline showed inhibition zone. Average fruit yield of two locations recorded significantly higher fruit yield per tree in T_9 (COC 0.3% + streptocycline 0.05%) and T_5 (*P. fluorescens* strain 326 (4) 1.0% + soapnut 5.0%) which were on par with each other. The maximum C:B ratio was observed in COC + streptocycline (0.3+0.05%).