EVALUATION OF INNOVATIVE NANO BIOMOLECULES IN MANAGEMENT OF BACTERIAL BLIGHT DISEASE OF POMEGRANATE

By

Mr. Rothe Anand Shivaji
(Reg. No. Ph.D. 2019/50)

A Thesis submitted to the
MAHATMA PHULE KRISHI VIDYAPEETH
RAHURI – 413 722, DIST. AHMEDNAGAR
MAHARASHTRA, INDIA

in partial fulfillment of the requirements for the degree

Of

DOCTOR OF PHILOSOPHY (AGRICULTURE)

in

PLANT PATHOLOGY

DEPARTMENT OF PLANT PATHOLOGY AND AGRICULTURAL MICROBIOLOGY

POST GRADUATE INSTITUTE
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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part
there of has not been submitted
by me or other person to any
other University or Institution
for a Degree or
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This is to certify that the thesis entitled, "**EVALUATION OF INNOVATIVE NANO BIOMOLECULES IN MANAGEMENT OF BACTERIAL BLIGHT DISEASE OF POMEGRANATE"** submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri Dist. Ahmednagar (M.S.) in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE)** in **PLANT PATHOLOGY,** embodies the results of a piece of *bona fide* research work carried out by **MR. ROTHE ANAND SHIVAJI,** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

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Place: M.P.K.V., Rahuri. **(Anand S. Rothe)**

Date : / /2022

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LIST OF ABBREVIATIONS/SYMBOLS

%	:	Per cent
/	:	Per
@	:	At the rate of
°C	:	Degree Celsius
Av.	:	Average
AgNPs	:	Silver nanoparticles
BOD	:	Bio Oxygen Demand
CD	:	Critical difference
Cm	:	Centimetre
CRD	:	Complete Randomized Design
Cv.	:	Cultivar
CuNPs	:	Copper nanoparticles
e.g.	:	For example,
et al	:	And other (et alli)
etc.	:	Et Cetra
Fig.	:	Figure(s)
Gm	:	Gram
GYCA	:	Glucose Yeast Chalk Agar
i.e.	:	That is / illustrated example
Kg	:	Kilogram
Lit.	:	Litter
Mm	:	Millimetre
Max.	:	Maximum
Min.	:	Minimum
M.S	:	Maharashtra State
No.	:	Number
NA	:	Nutrient Agar
NSA	:	Nutrient Sucrose Agar
Nm	:	Nanometre

PDC	:	Per cent disease control
PDI	:	Per cent disease intensity
PGI	:	Post Graduate Institute
pv.	:	Pathovar
Ppm	:	Parts per million
Rpm	:	Revolution per minute
SE	:	Standard error
TEM	:	Transmission Electron Microscope
UV	:	Ultra violet
viz.	:	Namely
YDCA	:	Yeast Extract Dextrose Calcium Carbonate Agar

ABSTRACT

EVALUATION OF INNOVATIVE NANO BIOMOLECULES IN MANAGEMENT OF BACTERIAL BLIGHT DISEASE OF POMEGRANATE

By

Mr. Anand Shivaji Rothe

A candidate for the degree

of
DOCTOR OF PHILOSOPHY (AGRICULTURE)
in
PLANT PATHOLOGY
2022

Research Guide **:** Dr. T. K. Narute

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Pomegranate (*Punica granatum* L.) is one of the ancient and highly priced favourite table fruits of tropical and sub-tropical regions. It belongs to the botanical family Punicaceae. This genus is composed of three species *viz. Punica protopunica, Punica nana* and *Punica granatum* (2n=16), of which *Punica granatum* is mainly cultivated for fruit production. It is commonly known as "Annar" in Hindi and "Dalimb" in Marathi.

The pomegranate is being cultivated around the world within the area lying between latitude 41^0N and 42^0S. In India, pomegranate is being cultivated on an area of 209 thousand ha with annual production of 2442 thousand metric tonnes. In India Maharashtra State, alone occupies an area of 136.75 thousand ha followed by Karnataka State (28.09 thousand ha.), Gujarat State (18.54 thousand ha.) and Andhra Pradesh State (7.71 thousand ha.). Among the major aspects liable for lower yields and reduced fruit quality, bacterial blight disease caused by *X. axonopodis* pv. *punicae,* plays a crucial role. Western part of Maharashtra is a leading zone under pomegranate cultivation area, production and productivity. Taking into account of economic importance of pomegranate and major hurdle of bacterial blight disease, present investigation was planned and carried out at Department of Plant Pathology and Agricultural Microbiology,

Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri, (M.S.) India for two years during 2020-2022 on detail study of isolation, pathogenicity, cultural and morphological characteristics of *X. axonopodis* pv. *punicae*. During the present investigation the major emphasis was concentrated towards the evaluation of new innovative eco-friendly molecules and alternative option to the routine chemical bactericides. Accordingly biosynthesized Copper ($CuSO_4$) and Silver ($AgNO_3$) nanoparticles were evaluated for their antibacterial potential under *in vitro* and *in vivo* conditions against bacteria.

The bacterial blight disease synonamely called as oily spot of pomegranate caused by *X. axonopodis* pv. *punicae,* produces first disease symptoms on leaves, stems and fruits. The isolation was made from diseased leaves and fruits samples collected from farmers' fields. The bacterium was successfully isolated on nutrient agar basal culture medium. It produced pale yellow coloured, convex, round, shiny colonies on cultural media.

The pathogenicity test was proved by pin-prick infiltration method. After inoculation of pathogen disease symptoms on leaves were developed within 10 to 18 days after inoculation. First symptoms were appeared on lower sides of the leaves as irregular water-soaked spots. Followed by raised spots on the upper side of the leaves with yellow haloes.

The biochemical characterization of bacteria under study showed positive reactions for potassium hydroxide (KOH) test, starch hydrolysis test and catalase test whereas, negative response for Gram staining test was showed.

Cultural characters like colony colour, growth appearance, elevations, texture and consistency were showed variations on different media *viz.* Nutrient Agar, Nutrient Sucrose Agar, Glucose Yeast Chalk Agar and Yeast Extract Dextrose Calcium Carbonate Agar.

Biosynthesis of Copper and Silver nanoparticles were carried out from fresh leaves of *Ocimum sanctum* (Tulsi) and *Azadirachta indica* (Neem), respectively. UV-Visible spectrophotometer characterization revealed that both Copper and Silver

nanoparticles showed absorption peak at 397.98 and 406.65 nm, respectively. Transmission Electron Microscopy (TEM) revealed that both Copper and Silver nanoparticles are spherical in shape and its size ranges from 8.97 to 10.89 nm and 10.33 to 12.21 nm, respectively.

Both *in vitro* and *in vivo* evaluation of antibacterial potential of bactericides, plant origin eco-friendly biosynthesis Copper and Silver nanoparticles were studied by paper disc method and under pot culture condition respectively. The results revealed that in both *in vitro* and *in vivo* studies the chemical bactericide treatment of 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm was found most effective for controlling *X. axonopodis* pv. *punicae,* with maximum inhibition zone, maximum per cent disease control and minimum per cent disease intensity, followed by eco-friendly nanoparticle treatments i.e. Copper and Silver nanoparticles. The pooled data revealed that treatment of bactericide 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm was found most effective with maximum per cent disease control by managing the disease up to 63.38 per cent over control. As per the objectives of present studies, the evaluation and comparison of chemicals and eco-friendly management practices, it is revealed and proved that the treatment of eco-friendly nanoparticles i.e. CuNPs and AgNPs both @100 ppm were found best treatments after chemical treatment in combating bacterial blight disease with maximum per cent disease control up to 47.97 and 41.86 per cent, respectively and sustainable alternative to chemicals management of bacterial blight disease of pomegranate produce quality and residue free pomegranate fruit yield.

1. INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the ancient and highly priced favorite table fruits of tropical and sub-tropical regions. This genus is composed of three species viz., *Punica protopunica, Punica nana* and *Punica granatum* (2n=16), of which *Punica granatum* is mainly cultivated for fruit production. It is commonly known as "Annar" in Hindi and "Dalimb" in Marathi. It belongs to the botanical family Punicaceae.

Pomegranate has long and exceptionally colourfull history, having been embraced by a number of different cultures, while at the same time it had been a normal horticultural fruit crop in different countries. Yet, despite its wide geographic distribution across several continents, very little information is available pertaining to its genetic origin and centers of diversity (Still, 2006). Wild pomegranate grows in Transcaucasia and Central Asia from Iran and Turkmenistan to Northern India (Levin, 2006; Holland *et al.,* 2009). According to Levin (2006), there are three mega-centers (Primary, Secondary and Tertiary) and five macro centers (Middle Eastern, Mediterranean, Eastern Asian, American and South African) of origin and genetic diversity of pomegranate. It is estimated that domestication process started some-where in the Neolithic era (Levin, 2006; Still, 2006). It was initially domesticated in Transcaucasia-Caspian region and northen Turkey (Zohary and Speigel-Roy, 1975).

It was reported in literature that pomegranate being cultivated around the world within the area lying between latitude 41^0 N and 42^0 S (Hodgson, 1917). Now the scenario of its cultivation has changed. Commercial orchards of pomegranate are now widely grown in Mediterranean basin and in Asia (Holland and Bar- Ya'akov, 2008) and thus India became the largest producer (Jadhav and Sharma, 2007). Even so, new orchards are tradionally cultivated in the southern hemisphere in South America, South Africa and Australia (Holland *et al*., 2009).

In India, pomegranate is being cultivated on an area of 209 thousand ha with annual production of 2442 thousand metric tonnes (Horticultural Statistics at a Glance, 2020). The Maharashtra state, alone occupies an area of 136.75 thousand ha followed by Karnataka (28.09 thousand ha), Gujarat (18.54 thousand ha.) and Andhra Pradesh (7.71 thousand ha.). Maharashtra is having largest area, production and the productivity are

1578.04 thousand metric tonnes and 11.02 metric tonnes per ha respectively (Horticultural Statistics at a Glance, 2020). Ganesh, Bhagwa, Ruby, Arakta and Mridula are the different varieties of pomegranates produced in Maharashtra. In India, pomegranate is commercially cultivated on large area in Solapur, Sangli, Nasik, Ahmednagar, Pune, Dhule, Aurangabad, Satara, Osmanabad and Latur districts of Maharashtra; Bijapur, Belgaum and Bagalkot districts of Karnataka and to a some extent in Gujarat, Andhra Pradesh and Tamil Nadu.

Pomegranate grows very well on the moderately alkaline soils as well as slightly acidic soils. It is common to the tropics, sub-tropics and sub-temperate regions and is well adapted to areas with hot and dry summers. Pomegranate has renowned interest as a commercial orchard crop because of the health benefits associated with its high level of antioxidants in the pulp and juice. The fruit has wide consumer preference for its attractive, juicy, sweet-acidic and refreshing arils. Therefore there is increasing demand for good quality fruits both for fresh fruits, processed juice syrup and wine. Seeds with fleshy portions of sour pomegranates are dried and marketed as “Anardana”, which is being used as a condiment and for souring curries. The fruits of pomegranate are known to possess pharmaceutical and therapeutic properties. In India, there is a common adage “Ek Anar Sau Bimar.” Its meaning is one fruit cures hundreds of diseases.

In the ancient Ayurveda system of medicine, the pomegranate has extensively been used as a source of traditional remedies for thousands of years. The rind of the fruit and the bark of the pomegranate tree are used as a traditional remedy against diarrhea, dysentery and intestinal parasites. The seeds and juice are considered a tonic for the heart, throat, eyes and for a variety of purposes, such as stopping nose bleeds and gum bleeds, toning skin, firming-up sagging breasts and treating hemorrhoids. In the past decade, numerous studies on the antioxidant, anticarcinogenic, and anti-inflammatory properties of pomegranate constituents have been studied and published, focusing on treatments and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance, and ultraviolet radiation-induced skin damage. Other potential applications include infant brain ischemia, male infertility, Alzheimer's disease, arthritis and obesity. Pomegranate is well known for antioxidant properties. It helps in preventing the formation of skin cancer by reducing the

frequency of lesions. It provides relief from minor skin irritations, such as dry skin, eczema and psoriasis (Bhowmik *et al.,* 2013).

Cultivation of high yielding varieties of pomegranate with intensive care and management in the recent past under irrigated condition with early stage exploitation of plants has lead to certain severe pest and disease problems. Among the major diseases, leaf spot, fruit spot and wilt results in drastic reduction of pomegranate fruit yield and put the growers in to hardship. Pomegranate is susceptible to many diseases which affect the fruit quality and yield. The major fungal diseases are *Cercospora* leaf and fruit spot, *Drechslera* fruit spots, *Colletotrichum* leaf spots, wilt (*Ceratocystis fimbriata*) and the bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae* (Madhukar and Reddy, 1989). However during recent years pomegranate cultivation in Maharashtra has been threatened due to wide spread incidence of bacterial blight (oily spot) disease. The disease has resulted in enormous losses to pomegranate orchards, as it renders fruits unfit for consumption and market value.

Leaf spot of pomegranate first time in India was reported by Hingorani and Mehta (1952). Later on in Hingorani and Singh (1959) took a thorough investigation on the disease and causal organism and designated the pathogen as *Xanthomonas punicae*. Chand and Kishun (1991) had isolated the causal organism of bacterial blight of pomegranate and based on its pathological, cultural, biochemical and physiological features, identified as a *Xanthomonas campestris* pv. *punicae*. Further in year 1995, Vauterin *et al*. had re-named this causal organism as *Xanthomonas axonopodis* pv. *punicae* keeping in view of activity of the presence or absence of metabolic activity on different carbon substrates.

In Maharashtra, the disease was first time reported in 2003 at Chickmahud village of Sangola Tahsil of Solapur district (Dhandhar *et al*., 2004). Now it has become a serious problem in pomegranate orchards of Solapur, Sangli, Ahmadnagar, Nashik, Satara, Latur and Osmanabad districts. This disease spread like wild fire in pomegranate plantation, resulting drastic reduction in fruit yield. Incidence of this disease is the major obstacle and limiting factor in Maharashtra State which alone accounts for complete loss of cultivated area, particularly in Nasik, Solapur, Sangli and Ahmednagar districts.

Since 2002, the disease has reached the alarming stage and hampering the Indian economy and export of quality fruits. The disease accounted up to 70–100 per cent losses during 2006 in Karnataka, Maharashtra, Andhra Pradesh and Tamil Nadu resulting in abandoning many pomegranate orchards. During the year 2007, the total output of pomegranate production in India was down by 60 per cent (Raghavan, 2007).

Bacterial blight infection results in appearance of water soaked oily spot symptoms on leaves, stems and fruits which consequently decreases fruit production and market value. The vital role of infected fallen leaves for the survival of pathogenic bacterium causing leaf spot of various crops is well established (Burkholder, 1948). Similarly, the infected plant residues of pomegranate, such as leaf, stem and fruit, which are left out in the field after the harvest of the crop also serve as a primary source of inoculum for initiation of the disease in next season. But, the information on longevity of the pathogen on infected plant parts is still not known. The permanent presence of the pathogen in the orchard throughout the season has led to many fold speculations on possible survival of the pathogen on some alternate hosts grown in and around the garden. Severity of incidence and losses varies from area to area and influenced by existing climatic conditions and geographical distribution (Mondal and Sharma, 2009; Petersen *et al*., 2007; Mondal and Mani 2012).

In recent year, due to the failure of earlier bactericides to manage bacterial blight disease of pomegranate, thus major pomegranate growing farmers suffers from huge losses and that's why pomegranate growing areas goes on decreasing. To take over these problems in recent years, nanoparticles are found suitable alternatives to manage many fungal and bacterial diseases as stated by earlier researchers (Shankar *et al*., 2004 and Panacek *et al*., 2006). Due to these it was decided to use nano materials for management of bacterial blight disease of pomegranate.

The idea of nanotechnology was first highlighted by Nobel laureate Richard Feynmen (1959). "Nanotechnology mainly consists of the processing, separation, deformation and consolidation of material by one atom or by one molecule" and another technologist promoted technological significance in nanoscale (Wang, 1991).

The term "nanoparticles" is used to describe a particle with size in the range of 1-100 nm, at least in one of the three possible dimensions. In this size range, the physical,

chemical and biological properties of the nanoparticles changes in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials. Nanoparticles can be made of materials of diverse chemical nature, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon and biomolecule (Khatun *et al.,* 2015).

Nanoparticles show properties of sharp contrast to their bulk in many respects which is utilized for its use in 'Nanotechnology' (Misra *et al.,* 2013). Properties of nano particles are, they are Small size (1-100nm), large surface to volume ratio, chemically alterable physical properties, change in the chemical and physical properties with respect to size and shape, structural sturdiness in spite of atomic granularity, enhanced or delayed particles aggregation depending on the type of the surface modification enhanced photoemission, high electrical and heat conductivity and improved surface catalytic activity.

Nanoparticles can be broadly grouped into two, namely, Organic and inorganic nanoparticles which include carbon nanoparticles, while the inorganic nanoparticles include nobel metal nanoparticles (gold and silver) and semi-conductor nanoparticles (like titanium oxide, zinc oxide and copper). There is a increasing interest in nobel metal nanoparticles of gold and silver, as they provide superior material properties with functional versatility. Inorganic nanomaterial have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good compatibility, and capability of targeted drug delivery and controlled release of drugs (Xu *et al.,* 2006).

Nanoparticles have huge scope because of their small size, distinct properties compared to the bulk form of same material, thus offering many new developments in the fields of biosensors, biomedicine, and bio nanotechnology. Nanotechnology is rapidly gaining importance in a number of areas such as biopesticide, bioinsecticide, bioherbicide, health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug gene delivery, energy science, optoelectronics, catalysis, reorography, single electron transistors, light emitters, nonlinear optical devices, and photoelectron chemical applications (Wang, 1991 and Colvin and Alivisatos, 1994). Nanomaterials are seen as

solution to many technological and environmental challenges in the field of solar energy conversion, catalysis, medicine and water treatment. In context of global efforts to reduce hazardous waste, the continuously increasing demand of nanomaterials must be accompanied by green synthesis methods (Ingole *et al.,* 2010).

Currently, copper nanoparticles have received considerable attention because of their potential for use in nanomaterials, thermal conducting applications, lubrication, nano fluids, and catalysts (Lu *et al*., 2001). In recent years, copper nanoparticles have attracted much attention of researchers due to its application in wound dressing and biocidal properties, potential industrial use such as gas sensors, catalytic process, high temperature superconductors and solar cells (Kulkarni *et al.,* 2013).

Nano Silver is the most studied and utilized nano particle for biosystem. It has long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. Silver nanoparticles, which have high surface area and high fraction of surface atoms, have high antimicrobial effects as compared to the bulk silver. Double capsulized nano silver was prepared by chemical reaction of silver ion with aid of physical methods, reducing agents and stabilizers. They were highly stable and very well dispersive in aqueous solution. It eliminates unwanted microorganisms in soils and hydroponics systems. It is being used as foliar spray to stop fungi, moulds, rot and several other plant diseases. Moreover, silver is an excellent plant-growth stimulator (Singh *et al*., 2014).

Viruses, bacteria, fungi and nematodes are mainly responsible for plant diseases resulting in decreased yield and poor quality of plant products. Various approaches to manage crop disease are being used including genetic breeding, cultural practices with sanitation, host indexing, enhanced eradication protocols, use of new pesticide products and integrated pest management. Several studies have reported that nanoparticles can be used to suppress pathogens with increased crop growth. For the past few decades, there has been a considerable increment in research interest in the area of natural product delivery using particulate materials for controlling plant pathogens. The secondary metabolites in plants have been used in the formulation of nanoparticles through increasing the effectiveness of therapeutic compounds used to reduce the spread of plant diseases, while minimizing side effects for being rich source of bioactive chemicals,

biodegradable in nature and nonpolluting (eco-friendly). Particulate systems like nanoparticles have been used as physical approach to alter and improve the effectiveness to properties of some types of synthetic chemical pesticides or in the production of bio pesticides directly (Hameed and Alasmarrai, 2012).

Therefore, keeping in mind the above considerations, the present study is aimed at Evaluation of innovative nano biomolecules in management of bacterial blight disease of pomegranate.

Objectives:

1. To Isolate, identify and prove pathogenicity of *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate
2. To study the cultural and morphological characteristics of *Xanthomonas axonopodis* pv. *punicae*
3. To synthesize green copper nanoparticles (CuNPs) and silver nanoparticles (AgNPs) from botanical extract
4. To characterize the biosynthesized green copper nanoparticles (CuNPs) and silver nanoparticles (AgNPs) by using UV-Visible Spectroscopy and Transmission Electron Microscopy (TEM)
5. *In vitro* evaluation of antibacterial activity of biomolecules against *Xanthomonas axonopodis* pv. *punicae*
6. *In vivo* evaluation of antibacterial activity of biomolecules against *Xanthomonas axonopodis* pv. *punicae*

2. REVIEW OF LITERATURE

Pomegranate (*Punica granatum* L.) is one of the ancient and highly priced favorite table fruits of tropical and sub-tropical regions. Cultivation of high yielding varieties of pomegranate with intensive care and management in the recent past under irrigated condition has lead to certain severe pest and disease problems. Among the major diseases, leaf spot, fruit spot and wilt results in drastic reduction of pomegranate fruit yield and put the growers in to hardship. Pomegranate is susceptible to many diseases which affect the fruit quality and yield. The major fungal diseases are *Cercospora* leaf and fruit spot, *Drechslera* fruit spots, *Colletotrichum* leaf spots, wilt (*Ceratocystis fimbriata*) and the bacterial blight disease caused by *X. axonopodis* pv. *punicae* (Madhukar and Reddy, 1989). However, a bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* disease has been appearing in a devastating form in India since 1952 causing heavy losses. During recent years pomegranate cultivation in Maharashtra has been threatened due to wide spread incidence of bacterial blight (oily spot) disease. The disease has resulted in enormous losses to pomegranate orchards, as it renders fruits unfit for consumption and market value. Therefore, literature pertaining to present investigation on various aspects of pomegranate bacterial blight and its management by copper nanoparticles and silver nanoparticles synthesized from botanicals has been reviewed here under different aspects.

2.1 History, occurrence and yield losses of disease

2.2 Disease symptomatology

2.3 Isolation, identification and pathogenicity of *X. axonopodis* pv. *punicae*

2.4 Cultural and morphological studies of *X axonopodis* pv. *punicae*

2.5 Role of nanoparticles in plant disease management

2.6 Biosynthesis of green Copper nanoparticles from botanical extracts

2.7 Characterization of green Copper nanoparticles (CuNPs)

2.8 Antibacterial properties of green Copper nanoparticles (CuNPs)

2.9 Biosynthesis of green Silver nanoparticles from botanical extracts

2.10 Characterization of green Silver nanoparticles (AgNPs)

2.11 Antibacterial property studies of green Silver nanoparticles (AgNPs)

2.1 History, occurrence and yield losses of disease

For investigation on present impact of particular disease its important to review on past study of that disease and what is impact of that disease in farming community regarding the symptomatology, management and losses caused by that disease, so that keeping in mind this aspect following history is reviewed.

Hingorani and Mehta (1952) described bacterial leaf spot disease of pomegranate (*Punicae granatum* L.) for the first time in India at IARI, New Delhi. Later on, Hingorani and Singh (1959) took a thorough investigation on the disease and causal organism and they identified the pathogen as *Xanthononas axonopodis* pv. *punicae*. Then after Rangaswami (1962) observed the severe outbreak of disease at Annamalainagar, Tamil Nadu in 1958-60 and causal agent isolated was found to be identical with *X. axonopodis* pv. *punicae*. The disease was described and characterized by him with the appearance of irregular spots (primarily found on leaves), which were water soaked in the initial stage, became necrotic with brown centers and finally turned brown. Several spots coalesced, covered large areas, and were surrounded by chlorotic haloes with translucent and water soaked appearance. In severe infection, leaves were found distorted and easily shed off.

Sohi *et al.* (1964) observed pomegranate disease from Himachal Pradesh, North India and reported that the disease was caused due to *X. punicae*. Then after Kanwar (1976) reported the presence of *X. axonopodis* pv. *punicae* in Haryana State on *Punica granatum* L. Symptoms observed were faded green pin point spots, which later on get enlarged, changed its color from dark brown to black, surrounded by pale normal green zone, and resulting into a immature fruits. Further they observed pathogen and examined under the microscope, reported as *X. axonopodis* pv. *punicae.*

Chand and Kishun (1991) reported about 60-80 per cent losses caused by bacterial blight disease of pomegranate. Later on Chand and Kishun (1993) studied the systemic migration of *X. axonopodis* pv. *punicae* in pomegranate by artificial leaf inoculation in the greenhouse. The bacterium colonized the inter-cellular spaces in mesophyll cells before moving to the petiole and finally down to the node. They observed invaded cells were killed resulting in nodal blight. They recorded minimum and maximum incubation period in direct node inoculation and leaf infection after 12 and 33 days, and 48 and 90 days respectively. The extent of nodal blight resulting from leaf and node inoculation was

7.7 and 100 per cent, respectively. The average rate of bacterial migration was highest (0.6 cm/d) 18-22 days after infection. The population of *X. axonopodis* pv. *punicae* was $2x10^7$ and 9 x 106 c.f.u./g leaf at the 5^{th} and 32^{nd} days after inoculation, respectively. Defoliation was 82 per cent for infected leaves 14-21 days after inoculation. The leaf petioles were colonized, only 7-12 per cent facilitated the movement of the bacterium to the node.

Upasana and Verma (2001) observed that *X. axonopodis* pv. *punicae* produced typical black spot symptoms when inoculated with on different parts of pomegranate plants *viz.* flowers, fruits, leaves and twigs. Slightly injured surface of the host were preferred by the pathogen over uninjured parts of the plant for establishment within the tissues Cent per cent lesion formation was recorded both under artificial as well as field conditions in Punjab (India) in 1997. By the time Upasana and Verma (2002) observed black spot on pomegranate trees in plantations of Ludhiana, Punjab, India which appeared on flowers and developing fruits then on leaves and branches 7 initially as minute, water soaked spots that later become brown and necrotic, resulting in the formation of depressed, elongated or elliptical cankerous lesions. They reported disease severity ranged from 18.43 to 45.67 per cent in fruits and from 15.32 to 37.81 per cent in leaves. They also reported that *X. axonopodis* pv. *punicae* could survive on infected leaves kept protected under field conditions and at room temperature as well as in cankers formed on the fruits and branches. They finally concluded that gradual decline in temperature, sharp increase in relative humidity and moderate rainfall favored gradual increase in disease intensity.

Manjula and Khan (2003) reported bacterial blight disease on pomegranate in divesting form in Karnataka during late *Summer* and *Kharif* seasons. They observed that main symptoms of disease were minute water soaked lesions, which later turned brown, surrounded by diffused water soaked zone or yellow haloes on both fruits and leaves. L, Y and star shaped cracking on fruits within the spot. Further they found that affected fruits split open and were exposing arils. The lesions appeared on branches and the twigs leading to die back and death of the branches. However, in Maharashtra, Dhander *et al.* (2004) reported that, the bacterial leaf and fruit spot of pomegranate occurred as major

serious problem in villages of Sangli and Solapur district such as Jat, Sangola, Jadhavwadi, Chickmahood villages.

Patil (2007) reported that oily spot disease on pomegranate occurred as major threat in Solapur district. Several workers *viz.* Mondal and Sharma (2009), Mondal and Mani (2012), Atar (2011), Petersen *et al.* (2007) and Raut (2012) reported that the bacterial blight disease caused by *X. axonopodis* pv. *punicae* is a major threat for pomegranate cultivation in Maharashtra State. After that Arora *et al*. (2016) reported that bacterial blight of pomegranate caused by *X. axonopodis* pv. *punicae* is a wide spread disease affecting its successful production and every year and results into 50-100 per cent economic losses depending upon disease severity.

2.2 Disease symptomatology

For the first time the bacterial leaf spot of pomegranate was described by Hingorani and Mehta (1952) in India. They reported the symptoms primarily appeared on the leaves as irregular spots, varying from 2 mm to 5 mm in diameter. Number of adjacent spots may coalesce and covered larger areas. The initial light brown spots turn dark brown and were surrounded by prominent water soaked margin and the formation of several spots on a leaf induces shedding. The water soaked spots, when viewed against light, look translucent initially and later dark brown spots were surrounded by prominent water soaked margin. However, spots appear on twigs, branches and fruits (Hingorani and Singh, 1959).

Rangaswami (1962) described the symptoms of bacterial blight on leaves as necrotic spots which were also surrounded by chlorotic haloes with translucent and water soaked appearance. In severe infections, leaves become distorted and fall off from the tree. They also reported that at Annamalainagar, the disease also affected the fruits causing water soaked spots in the earlier stages and later the spots become dark brown coloured slightly raised from the surface with oily appearance. Similar symptoms were described both on leaves and fruits in Solan of Himachal Pradesh by Sohi *et al.* (1964). By the time Rangaswami (1962) reported that the bacteria infect through wounds and stomatal openings and cause water soaked lesions in 2-3 days, which later develop into characteristic irregular spots. The plant may be dwarfed, stunted with sickly appearance.

They also reported that pathogen spreads by air borne cells and can survive in soil for over 20 days and then cause fresh infections on new flush.

Kanwar (1976) observed small, brown, water soaked spots on leaves, flowers and fruits of pomegranate in different orchards of Haryana. He reported the appearance of symptoms on leaves similar to that of symptoms described by earlier workers except that in certain cases the spots were restricted by veins and therefore their enlargement resulted into linear stripes. In severe cases of infection, necrosis of tissues also occurred and ultimately the leaves became yellow in colour followed by defoliation from the infected twigs. He also reported the appearance of symptoms on flowers and fruits. On petals of the flowers small, brown, water soaked spots were observed. In advanced stages, they became black and bigger in size. Similar types of spots were also observed on immature fruits. In early stages, spots were very small, pin head like, circular with dull green colour which later on turned to deep brown or black with yellowish halo. In advanced stages, the spots coalesced and formed irregular lesions. The infected rind of fruits became rough. Under heavy infection fruits remained under developed and deformed.

Kishun (1993) noticed similar symptoms on leaves but on stem, the disease started as brown to black spots around the nodes. In advanced stages of nodal infection, girdling and cracking of nodes occur which finally lead to breakdown of branches. On fruits, brown to black spots were appeared on the pericarp with L or Y shaped cracks. Then after Manjula and Khan (2003) observed the symptoms on leaves as minute water soaked lesion which later turn brown to dark colour surrounded by a diffused yellow halo. The spots coalesce and in case of severe infection, the leaves turn yellow and defoliate. Initially, elongated, narrow brown lesions were observed on the stem and twigs and in severe cases leading to twig blight or nodal blight. Symptoms on fruits appeared as oily diffused spots initially and with the development of disease lead to necrotic with typical L or Y or star shaped cracking in the centre of the lesion ultimately leading to bursting of pericarp exposing the arils. These are new types of symptoms on fruits recorded by them for the first time.

Jalaraddi (2006) observed initially symptoms appeared on leaves as minute water soaked lesions which later turned brown to dark colour. He noticed spots coalesce and in case of severe infection the leaves turned yellow and defoliated but on fruits the increase

in the lesion size gave rise to star shaped or L or Y shaped cracking within the spots which eventually led to cracking of fruits longitudinally. Further he noticed stem and the twig lesions on bark at nodal regions were more prominent and lethal.

Kumar (2007) observed disease appearance in the form of minute water soaked lesions on leaves, which later turned brown surrounded by a diffused yellow halo. As the disease advanced, spots increased in size, coalesced and became dark brown. Finally, leaves turned yellow and defoliated. Narrow cankerous brown lesions were observed on the main stem, primary and secondary branches. Later the lesion turned purple black, which cracked and the infected branch dried up. Symptoms on the fruits appeared in the form of oily diffused lesions, which later on turned in to brown surrounded by diffused yellow zone. Several spots could be seen on the fruits and as the fruit increased in size, lesions cracked into L, Y or star shape.

Yenjerappa (2009) reported that, the disease manifested itself in the form of small water soaked brown to black coloured lesions on the upper surface of the leaves surrounded by a diffused water soaked margin on the lower leaf surface. As the disease progresses, the spots also grew, increased their size, coalesced and extended up to midrib by occupying major portion of the leaf lamina. Severely infected leaves turned yellow, became chlorotic and shed off finally. Stem infection was noticed in the form of long narrow, elongated brown to black colored lesions on the main stem and branches. The lesions later on became rough and cankerous, leading to stem girdling and breaking at the point of infection. On flower buds, symptoms appeared as brown to black coloured spots, which later on coalesced and lead to the dropping of flower buds in advanced stages of infection. On developing green fruits small pin head sized black coloured lesions were seen with diffused water soaked margin surrounding the spot. Later on these lesions were turned to black coloured, medium to big sized spots. Severely infected fruits split opened with L/Y/star shaped cracks. The infected fruits dried up and hanged in the plant itself.

Raju (2010) noticed initially small, water soaked, brown to black coloured lesions were noticed on the upper surface of the leaves. He described that the spots were round, angular to irregular in shape. As the disease progressed, these spots grew, increased their size (2.0-5.0 mm in diameter), coalesced and extended up to midrib in a week's time covering the major portion of the leaf lamina. Severely infected leaves turned yellow,

became chlorotic and finally fell off. Stem infection was manifested in the form of long, narrow and elongated light brown to black coloured lesion (1-4 cm long) on the main stem and branches. As the disease advanced, stem girdling and breaking was seen at the point of infection.

Benagi and Kumar (2011) characterized disease by the appearance of one to several small, water soaked, dark coloured irregular spots on leaves resulting in premature defoliation under severe cases. The pathogen also infects stem and branches causing girdling and cracking symptoms. Spots on fruits were dark brown, irregular, slightly raised with oily appearance, which split open with L-shaped cracks under severe cases.

2.3 Isolation, identification and pathogenicity of *X. axonopodis* pv. *punicae*

Hingorani and Singh (1959) observed the pomegranate pathogen as a *X. punicae* and reported that the pathogen was short rod with rounded end, single or in pairs, motile with single polar flagellum. They described bacterium was Gram -ve, non endospore former and non acid fast but capsule was present. They prved the pathogenicity of *X. punicae* on 60 days old healthy cuttings of pomegranate. Infection appeared after nine days of inoculation on injured leaves and after 12 days on uninjured leaves, as numerous, minute water soaked spots. Later on Kanwar (1976) noticed that pathogen occurred in single pairs and also in chains, rod shaped with rounded ends, measured 0.75 to 3.0 µm in length and 0.45 µm in width and Gram negative with single polar flagellum, neither capsule nor endospore was observed. Then after, Kamble (1990) proved the pathogenicity of bacterial leaf and fruit spot disease of pomegranate caused by *X. campestris* pv. *punicae*. By the time Chand and Kishun (1991) observed that the bacterium *X. punicae* was short with rounded end and bacterium was gram negative, motile with single polar flagellum and positive to catalase activity. They also proved the pathogenicity of *X. axonopodis* pv. *punicae* by inoculation of bacterial suspension (2x 10^8 cfu/ml) on leaves and fruits of cultivars of Kandhari by pinprick method. Upasana and Verma (2002) revealed that the size of the necrotic spots increased slowly during May and June, but increased by more than threefold during July. They also studied morphology of the bacterium isolated from infected fruits, leaves and twigs of pomegranate and identified the pathogen as *X. campestri* pv. *punicae*. Then after Manjula

and Khan (2003) isolated and proved the pathogenicity of *X. axonopodis* pv. *punicae* causing bacterial blight of pomegranate. Isolations from the infected leaves and fruits formed yellow coloured, convex, round and shiny colonies on nutrient agar medium. These isolates when inoculated pomegranate plants, produced typical symptoms of the disease as minute water soaked lesions which later turned brown in colour and surrounded by diffused water soaked zone or yellow halo on fruits and leaves. Jyoti *et al.,* (2005) isolated the bacterial blight pathogen of pomogranate on YDC (Yeast dextrose calcium) media and further studied the morphology of *X. axonopodis* pv. *punicae* and also proved pathogenicity.

Petersen *et al.* (2007) observed included leaf and fruit spots symptoms and cankers on stems, branches and trunks of pomegranate. Based on biochemical and molecular analyses and pathogenicity tests, the bacterium *X. axonopodis* pv. *punicae* was identified as the causal agent. During the period Yenjerappa (2009) found the bacterium by employing the serial dilution plating technique using nutrient agar medium and yielded well separated, typical, yellow, mucoid, colonies of bacterium on nutrient agar medium after 72 hours of incubation at 30^{o}C. For proving pathogenicity, the bacterial cell suspension ($5x10^{6}$ cfu/ml) of each of the isolates collected from different regions during survey were sprayed on to the susceptible pomegranate plants of Bhagwa variety. The characteristic symptoms were observed on pomegranate leaves after ten days of inoculation. Re-isolations were carried out from these lesions for each isolate and comparisons were made with the original culture to confirm the identity of the pathogen. Later on Mogle *et al.* (2010) proved the pathogenicity of *X. axonopodis* pv. *punicae* on young and healthy plants of highly susceptible pomegranate cultivar Ganesh. Forty days old healthy leaves of pomegranate were inoculated with sterilized thorny seeds of *Xanthium strumarium* and nodes by leaf cut method. They observed that typical symptoms of disease were appeared on the leaves within 9 to 13 days after inoculation with irregular water soaked spots encircled with yellow halo. Then after Petersen *et al.* (2010) observed the symptoms on leaf and fruit spots and canker on stems during spring season. Based on the biochemical, molecular and pathogenicity tests, the pathogen was identified as *X. campestris* pv. *punicae* as the causal pathogen of bacterial blight of pomegranate.

Raju (2010) isolated causal organism from the infected leaf, fruit and stem parts of pomegranate by following the serial dilution plating technique using nutrient agar medium. Culture of each isolate was purified by streaking suspected single colony on the yeast dextrose calcium carbonate agar medium. Pathogenic test revealed that the *X. axonopodis* pv. *punicae* was pathogenic to pomegranate and produced typical symptoms on pomegranate leaves after four days of inoculation as small water soaked lesions. After six days of inoculation it turned brown to black coloured lesions, which later developed into angular to irregular spots along the margins, veins and veinlets of leaf lamina. There after Atar (2011) isolated *X. axonopodis* pv. *punicae* from naturally affected fruits and leaves and reported that yellow, mucoid, shiny glistering bacterial colonies were developed on Nutrient Agar medium. He also proved the pathogenicity on cv. Bhagwa under controlled condition.

Gamangatti and Patil (2013) reported that bacterial blight caused by *X. axonopodis* pv. *punicae* is the devastating disease of pomegranate. Further they collected pathogen strains from predominant pomegranate growing areas of Karnataka State to understand the existence of biochemical variability among the different isolates. Among 3 strains (Xap1-Raichur, Xap2- Bellary, Xap3-Koppal) Xap1 showed maximum starch hydrolysis.

Raghuwanshi *et al.* (2013) isolated four different isolates of *X. axonopodis* pv. *punicae* from the highly infected plant materials collected during field survey. They detected *X. axonopodis* pv. *punicae* from infected plant and its identity was confirmed by morphological, physiological, hypersensitive and pathogenicity tests. Later on, Bora and Kataki (2014) established the pathogenicity test by spraying 48 hrs old bacterial culture suspension (2×10^8 cfu/ml) on pinpricked 40 days old healthy pomegranate leaves. Infection occurred within 23 days of inoculation and produced identical symptoms observed on original pomegranate plant. The organism was re-isolated from artificially inoculated plant which yielded an organism similar to one used in the inoculation experiments. This satisfied all the conditions required to establish that the disease was bacterial borne disease which was caused by the pathogen *X. axonopodis* pv. *punicae*.

Abhang (2015) observed that biochemical reaction such as Gram reaction, starch hydrolysis, gelatin liquefaction, indole production, acid and gas production, KOH,

catalase test. These test were performed and confirmed the identity of bacterium as *X. axonopodis* pv. *citri.* During the period Muswad and Chavan (2015) collected different isolates of *X. axonopodis* pv. *punicae* from different pomegranate growing regions of Maharashtra State and were purified by single spore method. The pathogenic potentials of all 10 isolates were proved on the pomegranate cv. Ganesh. Pure cultures of *X. axonopodis* pv. *punicae* were inoculated on pomegranate cv. Ganesh, by using pinprick method. The symptoms were recorded since 6 to 9 days after inoculation as irregular water soaked spots appeared on leaves with yellow halos on axial side. Then after Wayde *et al.* (2015) studied seven isolates of *X. axonopodis* collected from different pomegranate growing regions of Maharashtra. The pathogenic potentials of all seven isolates were proved on the pomegranate cv. Bhagwa by using pinprick method. The observations were recorded since 6 to 9 days after inoculation. Primarily, the symptoms appeared on leaves as irregular water soaked spots. Later on these spots appeared on the leaves with yellow halos on axial side through rough texture. Further re-isolation of pathogen form infected leaves through single colony method was accomplished and compared with original culture.

Katkar *et al*. (2016) reported that different 15 isolates were selected for studying morphological and biochemical characteristic all isolates were found rod shaped, gram negative with colony colour ranging from pale yellow to dark yellow. Later on, Chavan *et al.* (2017) studied 36 isolates causing oily spot disease of pomegranate. All the 36 strains of *X. axonopodis* were found to be positive during in vivo pathogenicity test on fresh and healthy fruits as the displayed the symptoms similar to oily spot disease. All the strains were successfully reisolated from the infected fruits and found to be matching their parent strains. The variations were observed in the infectivity and pathogenicity pattern during in vivo pathogenicity test. The time required for the development of disease was varied from 14 to 24 days with all the isolates.

2.4 Cultural and morphological studies of *X axonopodis* pv. *punicae*

The characteristics of the bacterium isolated from infected leaves of pomegranate were studied by different workers as follows.

Hingorani and Singh (1959) observed nutrient dextrose agar, yeast glucose chalk agar and potato cylinders are the best media for the cultivation of pomegranate bacterium,

because of luxuriant growth obtained on these media. The pathogen was found facultative anaerobic. They also described that colonies on nutrient dextrose agar were filiform, slightly raised, glistening, pale yellow and odourless. Similar characters were also found on yeast glucose chalk agar with an exception that colour of the colony was bright yellow in the beginning and gradually changed to quite dark brown with age and growth was very poor in nutrient agar.

Chand and Kishun (1991) recovered pathogen from leaf, fruit and node. They obtained mucoid, circular, convex, yellow, round, glistening and raised colonies. The bacterium was Gram negative with rounded ends, motile with single polar flagellum and measured 0.4 - 0.75 x 1.25 - 3 μm in size. Vauterin *et al.* (1995) obtained different isolates of *X. axonopodis* pv. *punicae* pomegranate gardens in Karnataka and Andhra Pradesh states, yielded yellow, slimy, glistening, mucoid, convex, small round to irregular colonies on nutrient agar medium and pale yellow to dark yellow colonies, convex with copious slime on YDCA medium. SX and BSCAA media supported luxuriant growth of all the seven isolates.

Manjula (2002) reported that media YDCA and BSCAA supported good growth of *X. axonopodis* pv. *punicae.* Then after Giri (2009) reported good growth of all the isolates on the YDCA, GYCA and YNA media. Except Uthnal (Xap- 06) and Hulihyder (Xap-13) isolates, all the remaining isolates showed good growth on SX agar, while many isolates failed to grow on the XPS medium and it supported the poor growth of few isolates. YDCA medium supported maximum colony diameter (2.24 mm) and least growth was observed in case of XPS agar medium. Later on Katkar *et al.* (2016) reported that different 15 isolates were selected for studying morphological and biochemical characteristic all isolates were found rod shaped, gram negative with colony colour ranging from pale yellow to dark yellow.

Patil *et al.* (2017) reported that cultural and morphological characters of different *X. axonopodis* pv. *punicae* (Xap) isolates were studied on nutrient agar media. Five isolates collected from different locations differed in respect of size of colony, shape of colony and colour of bacterial colony.

Hingorani and Mehta (1952) Hingorani and Singh (1959) described the bacteria as a short rods with rounded ends, occurred singly or in pairs, sometimes in chains,

measuring 1-2.5 x 0.5µ in size, motile with a single polar flagellum, Gram negative, non endospore forming, non capsulated, not acid fast. Growth on agar plates is slow, filiform with edges entire, glistening, colourless to pale yellow, and browning takes place at high temperatures on the medium, colonies on potato dextrose agar are round, raised, wet, shining, with entire edges, colourless to pale yellow and measured 1 to 2 mm in diameter on the average.

According to Kanwar (1976) pathogen was in single pairs and also in chains, rod shaped with rounded ends and measured 0.75 to 3.0 µ in length and 0.45 µ in width, Gram negative, with single polar flagellum, neither capsules nor endospores. Manjula (2002) noticed repeated isolations made from the infected parts of pomegranate plant yielded yellow, mucoid, glistening, slimy, convex and odourless colonies on nutrient agar. Then after Jyoti *et al.,* (2005) isolated the bacterial blight pathogen of pomegranate on YDC (yeast dextrose calcium) media and further studied the morphology of *X. axonopodis* pv. *Punicae*. Later on, Jalaraddi (2006) reported that the bacteria isolated from infected parts of pomegranate found to be aerobic, Gram negative, capsulated, non-spore forming and monotrichously flagellated. Kumar (2007) observed bacterium as small rods, Gram negative, non capsulated and non spore forming with monotrichous flagellation. The bacterial colonies of all the isolates were yellow coloured, slightly mucoid, circular, single, small in size on YDCA medium. Later on Yenjerappa (2009) reported bacterium as a rod shaped with rounded ends occurred singly or in pairs, Gram negative, capsulated, non-spore forming with single polar flagellum.

2.5 Role of nanoparticles in plant disease management

The definition of nanomaterials are still evolving and currently assumes that are insoluble or biopersistent materials (objects) that are produced intentionally and that have one or more external dimensions or an internal structure on a scale from 1 nm to 100 nm. Within this group of materials are NPs, have at least two dimensions on the nanoscale (Klaine *et al.*, 2008). The basis of the 100 nm limit is the fact that the novel properties that differentiate particles from bulk material typically develop at a critical length scale of under 100 nm (Ruzer, 2011). However, it must be taken into account that according to the researchers the current limit of 100 nanometers, which is the basis for the dimensions of NPs, is now out dated and that any new regulations that are created should be based on

the newer, more advanced systematics. This is because sometimes additives have almost identical characteristics as “normally” produced chemicals when reduced to the nanoscale and that the synthesized NPs of the same material have completely different properties depending on the size of the particles.

Due to the extensive use of fungicides and pesticides there is rapid increase in ecotoxicity and development of resistance in plant pathogenic microbes (Alghuthaymi *et al*., 2015). The possible ecofriendly solutions include biological control of plant pathogens by using extracts of the plants or microbes. Although, biological control methods for the management of phytopathogens have been useful, several inherent challenges need to be addressed. Therefore, there is a pressing need to search for alternatives for the management of phytopathogens. The emerging nanobiotechnology seems to be of paramount importance for the management of phytopathogens particularly in early detection of plant disease, as potential fungicides, in development of varieties resistant to fungal diseases, and also for smart delivery of fungicides to the plants. The fungi affecting agricultural production can be controlled by application of nano fungicides. The use of nanotechnology both in developing and developed countries will bring dramatic changes in agriculture. This would really be a journey from green revolution to green nano biorevolution.

The use of metals as antimicrobials against plant pathogens is well known since ancient times. The metals such as copper, silver, palladium, ruthenium and their compounds have been used against human and plant diseases (Medici *et al.,* 2015). With the advent of nano-biotehnology, different nano materials including nanoparticles have been evaluated for their potential for the management of plant pathogens. Viruses, bacteria and fungi are mainly responsible for plant diseases resulting in decreased yield and poor quality of plant products. Several studies have reported that NPs can be used to suppress pathogens which increased crop growth.

Jo *et al.* (2009) stated that AgNPs in 200 mg/l conc. reduced 50% colony formation of pathogenic fungi that caused disease in ryegrass. Lamsal *et al.* (2011) have also reported that application of AgNPs enhanced the disease suppression. Combined activities of AgNP with the fungicide fluconazole were found to be effective against *Candida albicans*, followed by *Phoma glomerata* and *Trichoderma spp.* (Gajbhiye *et al*.,

2009). ZnO NPs reduced growth by 26% of *Fusarium graminearum* grown in mungbean broth agar (Dimkpa *et al.,* 2013). MgO NPs exhibited significant antimicrobial activity due to strong interaction with a negative surface of bacterial membranes (Huang *et al.,* 2005). Chemically synthesized CuNPs demonstrated higher pathogenic fungal inhibition in comparison to the fungicide bavistin (Kanhed *et al.,* 2014).

Kasprowicz *et al.* (2009) observed that silver compounds inhibited colony formation of *B. sorokiniana* by 50 per cent at optimum concentration. Significant reduction in mycelial growth was observed from spores incubated with silver NPs. It greatly reduced the number of germinating fragments relative to the control at 24 hrs. incubation of spores with a 2.5 ppm solution of NPs. They also conducted field tests with silver NPs at various concentrations to determine antifungal activity. The highest inhibition rate for the growth of fungal pathogen on cucumber and pumpkins exposed to 100 ppm silver NPs (Lamsal *et al*., 2011). Au NPs synthesized by banana peel extract, exhibited antifungal activity against *C.albicans* (Bankar *et al*., 2010). Ag NP has been tested against few plant-pathogenic fungi due to its fungicidal activity and their impact was found to be significant in eliminating the fungi (Jo *et al*., 2009). Cioffi and his collaborators in 2004 studied antifungal activity of nanocopper against plant pathogenic fungi. Gul *et al*. (2014) presented an informative review on role of nanotechnology in crop protection.

Copper-based fungicides produce highly reactive hydroxyl radicals which can damage lipids, proteins, DNA, and other biomolecules. It plays an important role in disease prevention and treatment for large variety of plant diseases (Borkow and Gabbay, 2005). Complexation of copper with chitosan nanogels was shown to have strong synergistic effect between chitosan and copper in inhibiting the growth of phytopathogenic fungus *Fusarium graminearum*. Because of its bio-compatibility, these nanohydrogels are included as a new generation copper-based bio-pesticides and it could also be developed into an efficient delivery system for copper based fungicides for plant protection (Brunel *et al*., 2013). Low melting point soda-lime glass powder containing copper nanoparticles showed efficient antimicrobial activity against Gram-positive, Gram-negative bacteria, yeast and fungi. The key reason for the increased antimicrobial activity is because of inhibitory synergistic effect of the Ca^{2+} lixiviated from the glass.

Singh *et al.* (2013) reported that among nanoforms of 15 micronutrients, $CuSo_4$ and $Na_2B_4O_7$ are found most effective in controlling rust disease of field peas.

2.6 Biosynthesis of green Copper nanoparticles from botanical extracts

Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals (Ahmad and Sastry, 2004). Exploitation of various plant materials for biosynthesis of nanoparticles is considered a green technology because it does not involve any harmful chemicals. Later on, Lee *et al*. (2011) reported biologically synthesized of copper nanoparticles using plant leaf extract as reducing agent. Later on they also studied on treatment of aqueous solutions of $CuSO_4.5H_2O$ with leaf extract (Magnolia), stable copper nanoparticles were formed. They utilize UV-Visible spectroscopy to monitor the quantitative formation of copper nanoparticles and synthesized nanoparticles were characterized with TEM and SEM. Electron microscopy analysis of copper nanoparticles indicated that they ranged in size from 40 to 100 nm. After that Valodkar *et al*. (2011) stated extracellular synthesis of copper nanoparticles using stem latex of a medicinally important plant, *Euphorbia nivulia*. In the experiment of Valodkar *et al*. (2011) nanoparticles were stabilized and subsequently capped by peptides and terpenoids present within the latex. After that Harne *et al*. (2012) reported low cost, eco-friendly route for rapid biosynthesis of copper nanoparticles. Cysteine proteases present in the latex of *Calotropis procera* were used to fabricate copper nanoparticles from copper acetate. In the study of Harne *et al*. (2012) Copper nanoparticles were initially characterized by transmission electron microscopy and X-ray diffraction technique. This shows that copper nanoparticles synthesized by above method hold excellent biocompatibility and Present work deals with synthesis of copper nanoparticles at room temperature using 0.5 per cent aqueous extract prepared from *Calotropis procera* with average diameter of 15 ± 1.7 nm.

Gopalakrishna *et al*. (2012) reported exploitation of various plant materials for the biosynthesis of nanoparticles. In their studies water-soluble carbohydrates present in the plant materials were mainly responsible for the reduction of copper ions to nano-sized Cu_2O nanoparticles by surface coating with polyaniline by chemical polymerization

method using hydrogen peroxide as oxidizing agent. During the same period Majumdar *et al*. (2012) employed single step eco-friendly approach to synthesize copper nanoparticles. In their study, leaf extracts of the weed *Lantana camera* and microorganisms were screened for extracting copper from integrated circuits and obtaining it in nano form. Hence, this work gave a solution to bioremediation as well as the recovery of valuable metal nanoparticles.

Kulkarni and Kulkarni (2013) biosynthesized stable CuNPs using *Ocimum sanctum* leaf extract. These biosynthesized CuNPs were characterized with the help of X ray diffraction (XRD) pattern. In these experiment sizes of the NPs obtained were estimated to be 77 nm. By the time Subhankari and Nayak (2013) confirmed the morphology of copper nanoparticles by Transmission electron microscopy (TEM) and also reported a novel biological approach for the formation of copper nanoparticles using clove. After that Bhasker *et al*., (2014) synthesized copper nanoparticles from leaf extracts of *Ocimum sanctum* and standardized different parameters like metal ion concentration, reaction time, pH and ratio in different concentrations. Angrasan *et al*. (2014) synthesized copper nanoparticles by using plant leaf extract of *Vitis vinifera* (grape). In their studies reduction of copper sulphate to copper nanoparticle was confirmed by UV-Visible spectrophotometer in which absorption peak at 384 nm in UV Visible spectrum corresponds to the plasmon resonance of copper nanoparticles. During these period Sutradhar *et al.* (2014) reported the synthesis of copper oxide nanoparticles by irradiating metal salt and the extracts of tea and coffee in microwave at 540 W for 7-8 min. Ghorbani *et al.* (2015) synthesized CuNPs using *Salmonella typhimurium* by addition of culture supernatant with aqueous copper nitrate solution (1 mM) and CuNPs were formed having average diameter 49 nm and reaction mixture exhibit a peak at 565 nm corresponding to the Plasmon absorbance of CuNPs by UV–V is spectroscopy.

Patel *et al*. (2016) carried out study for synthesis of green copper nanoparticles in these experiment *O. sanctum* leaves were thoroughly washed in double distilled water. These leaves were fast dried at 110 C in an oven and crushed to make powder and 10 gm of the powder was added to 100 ml methanol and stirred at 250 rpm for 1 h to make pure extract. The extract was then filtered using Whatman's No. 1 filter paper. 100 ml solution of CuSO4 was slowly reduced by drop-wise addition of 15 ml methanolic extract of *O.*

sanctum leaves under atmospheric conditions. During the process of reaction, the solution mixture was stirred vigorously all process was carried out for 10 min at room temperature. After the incubation period, the solution was filtered thrice using Whatman's No.1 filter paper and filtered solution was stored for further antibiotic study.

Sumitha *et al*. (2016) reported the synthesis of copper oxide nanoparticles using copper acetate as precursor and *Ocimum tenuiflorum* leaf extract as a reducing agent. After that Hariprasad *et al*., (2016) prepared CuNPs by simple green method using valanata leaf extract. It is an effective method for the preparation of CuNPs at room temperature, UV-Visible spectrophotometer has the surface plasmon vibrations of CuNPs produced a peak at near 562 nm and SEM images indicated the spherical nature of CuNPs. During these period Kala *et al.* (2016) successfully synthesized copper bionanoparticles using leaf aqueous extract of *Datura innoxia* from copper sulphate. In the same year Mekala *et al*. (2016) synthesised and characterized CuNPs using Tulsi (*Ocimum sanctum*) leaf extract in these experiment leaf extract acts as both reducing and capping agent and SEM analysis confirmed the spherical shape of nanoclusters with size range from 150-200 nm. Then after Chung *et al.* (2017) synthesized CuNPs by mixing copper acetate solution with leaf extract of *Eclipta prostrate* without using any surfactant or external energy. *E. prostrata* leaves extract function as an excellent reducing agent of copper ions and in SEM micrographs of the CuNPs synthesized by the reduction of copper acetate revealed spherical, hexagonal and cubical NPs ranging from 28 to 105 nm, with an average size of 41 ± 0.8 nm.

Usha *et al.* (2017) conducted study for green synthesis copper nanoparticles. They utilized plant extract of tulasi leaves (25 ml) in 100 ml of 1mM aqueous copper sulphate pentahydrate ($CuSO_4.5H_2O$) solution under continuous string. After complete mixing of leaf extract with precursor the mixture was kept for incubation at 31^0C for 24 h. change in the colour from light green to dark green was observed and this indicated the formation of copper nanoparticles. Later on Ijjatdar *et al.* (2018) synthesized CuNP from *Ocimum sanctum* and *Ficus benghalenis* plants and investigated its antibacterial activity against different bacteria. In their study CuNP was synthesized by herbal method. Dagar *et al.* (2020) synthesized copper nanoparticles by using *Ocimum sanctum* extract. In the typical procedure, 20 mL of Tulsi extract was added to the 100ml of 0.01 M of copper sulphate

pentahydrate ($CuSO_4.5H_2O$) solution and stirred on the magnetic stirrer for 24 hours. The colour change was observed from dark green to sea green then solution was centrifuged at 6000 rpm for 15 min and copper nanoparticles were obtained.

Mishra *et al.* (2020) used single step, an eco-friendly, cost-effective method for the synthesis of copper nanoparticles (CuNPs) from 1.0 mM copper sulphate solution using extract of *Ocimum sanctum* (Tulsi) leaves as reducing and capping agent. In the same year Nirmala *et al.* (2020) prepared copper nanoparticles by using copper sulphate and tuisi leaves. They roughly wahshed leaves (100 gm) were cut and boiled with 100 ml of ionized water for 15 minutes using a heating mantle at 80^0C after that extract was filtered and stored in refrigerator. Then 10 ml of those tulsi leaf extract was added to 100 ml of the aqueous $CuSO_4 \cdot 5H_2O$ solution and finally flask was then kept overnight at room temperature later on copper nanoparticles solution thus obtained was purified by repeated centrifugation at 12,000 rotations per minute for 15 minutes. Then the obtained Cu nanoparticles are dried using hot air oven at 80^0C for 3 hours. After that Ayona and Neethu (2021) reported biosynthesis of copper nanoparticle by using the leaf extract of *Ocimum sanctum*. Ayona and Neethu (2021) observed color change in the *Ocimum* leaf extract when copper sulphate solution is added indicates the presence of copper nanoparticle at the same time effect of temperature and time of incubation on the biosynthesis of Cu NP were noted.

2.7 Characterization of green Copper nanoparticles (CuNPs)

Characterization refers to the study of material features such as surface morphology, composition, colour, size, structure, various properties like physical, chemical, and magnetic properties. Nanoparticles characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. Characterization can be done by using a variety of different techniques *viz.,* Visual observation (colour change) UV-Vis spectroscopy analysis and Transmission Electron Microscopy (TEM) etc.

Link and Sayed (1999) and Ramayadevi *et al*. (2011) reported that copper colloids exhibit strong absorption bands in the visible region and are therefore intensely colored, further they studied that plasmon bandwidth increases with decreasing size in the

intrinsic region (mean diameter smaller than 25 nm) and also increases with increasing size in the extrinsic size region (mean diameter larger than 25 nm).

Dang *et al*. (2011) synthesized CuNPs using a chemical reduction method involving the reduction of copper sulphate by sodium borohydride, ascorbic acid as antioxidant and the stabilizing agent being polyethylene glycol (PEG) 6000. They were characterized shape and size distribution of colloidal particles by transmission electron microscopy (TEM) and average crystal size of the particles were less than 10 nm, further they also observed that particles were large and widely dispersed with size range between 14 and 50 nm.

Patel *et al*. (2016) carried out synthesis of green copper nanoparticles. They recorded absorbance of nano colloidal solution using UV–vis spectrophotometer (Shimadzu UV-2450) in the wavelength range of 300 nm to 700 nm. Later on, Ebrahimi *et al.* (2017) used the aqueous extract of *Capparis spinosa* to synthesize the copper nanoparticles then UV-vis spectroscopy analyses and scanning electron microscopy (SEM) were used to identify the synthesized nanoparticles. After adding the *Capparis spinosa* extract to the copper sulfate solution, the color of the solution changed from light blue to yellowish green after that existence of a maximum peak at the wavelength of 414 nm confirmed the formation of the copper nanoparticles and Scanning electron microscopy demonstrated the particle size between 17 and 41 nm. After that Mohindru and Garg (2017) reports green synthesis of copper nanoparticles using plant extract (tea leaf extract) with water as the medium for reduction and identifies their main physical properties. They observed that formation of copper nanoparticles was indicated by change in colour from blue to yellowish black which is supported by the UV absorption at 570 nm and final analysis pertaining to the determination of size of nanoparticles is the TEM analysis and it was found that the particle size of copper nanoparticles falls in the range 70-90 nm.

Usha *et al.* (2017) conducted study for green synthesis copper nanoparticles. In their experiment biosynthesized copper nanoparticles were characterized by using UV-Visible spectrophotometer in which the absorbance of the sample recorded in wavelength ranged between 400-600 nm. After that Dagar *et al.* (2020) synthesized green copper nanoparticles by using *Ocimum sanctum* (Tulsi) leaves. In which Cu NPs exhibit a sea

green colour in aqueous solution. Absorption band with maximum absorbance at 600 nm indicating the presence of stable and well dispersed CuNPs. Later on Mishra *et al.* (2020) used single step, an eco- friendly, cost-effective method for the synthesis of copper nanoparticles (CuNPs) from 1.0 mM copper sulphate solution using extract of *Ocimum sanctum* (Tulsi) leaves as reducing and capping agent. They used UV-VIS, FESEM, FTIR and XRD to confirm and characterize the NPs in which the reduction of Cu ions to Cu particles was followed as a change in color from light yellow to black-brown. UV-Vis spectrum of colloidal solution of CuNPs has been recorded as a function of time. At 3 h, the CuNPs in the reaction mixture demonstrates a distinct peak at 563 nm.

Nirmala *et al.* (2020) prepared copper nanoparticles by using copper sulphate and tuisi leaves. In their study biosynthesized Cu nanoparticles were characterized with the help of Scanning Electron Microscopy (SEM). SEM images of copper nanoparticles showed that the cluster formation, presence of smaller and larger grains. Ayona and Neethu (2021) prepared copper nanoparticles by using tulsi leaves and characterized by using UV-Vis spectrophotometer. UV- visible spectral analyses of copper nanoparticles was done to characterize the Cu NP formed at a range of 350 nm to 700 nm and maximum absorption can be seen at a range between 550 nm and 600 nm at about 560 nm.

2.8 Antibacterial property studies of green Copper nanoparticles (CuNPs)

Stanic *et al.* (2010) synthesized copper and zinc doped hydroxyapatite nanopowders by neutralization method and evaluated effects of doped hydroxyl apatite nanopowders against pathogenic bacterial strains *Escherichia coli*, *Staphylococcus aureus* and pathogen yeast *Candida albicans* in solid and liquid media. Quantitative test in liquid media clearly showed that copper and zinc-doped samples had viable cells reduction ability for all tested strains. Then after Mondal *et al.* (2010) evaluated *in vitro* efficacy against *X. axonopodis* pv. *punicae* of nanocopper. They observed a very promising in vitro growth inhibition activity of nanocopper against *X. axonopodis* pv. *punicae.* Nanocopper was found to be effective even at very lower concentrations (0.2 ppm) as compared to normal recommended doses of antibiotics (200–500 ppm) and Cu-oxychloride (2,500–3,000 ppm). Nanocopper completely suppressed the bacterial population as indicated in CFU studies. This evidence confirmed that nanoparticles,

which have a larger surface-to-volume ratio, were more efficient for antibacterial activity against Gram negative bacteria, as previously reported for *E. coli* (Raffi *et al.,* 2010), *X. axonopodis* pv. *phaseoli*, and *X. oryzae* pv. *oryzae* (Mondal and Sharma, 2009 and Mondal *et al*., 2010).

Ramyadevi *et al.* (2011) synthesized CuNPs by using modified polyol method by the reduction of copper acetate hydrate in the presence of Tween 80 by refluxing between 190^0C and 200^0C. The antimicrobial activity was carried out against *Micrococcus luteus, Staphylococcus aureus, E. coli, Klebsiella pneumonia*, and *Pseudomonas aeruginosa* and fungus like *A. flavus, A. niger* and *Candida albicans*. In these experiments CuNPs showed more inhibitory activity of bacteria than the fungus and it also showed more zone of inhibition in *E. coli* (26 mm) than *C. albicans* (23 mm). After that Sawant *et al.,* (2011) prepared CuNPs by simple green method using copper sulphate, chitosan and sodium borohydrate. They revealed the activity of CuNPs encapsulated with Chitosan studied by simple Cork Borer antibacterial assy. The CuNPs solution have been tested for their antimicrobial activities and an interesting antimicrobial profile has been observed against Gram positive and Gram negative bacteria *B. subtilis*, *E. coli, P. aeruginosa, Staphylococcus aureus* and *K. pneumonia, Salmonella typhi, Proteus vulgaris* and *Methanococcus*. The average zone of inhibition observed is in between 6 mm to 30 mm of CuNPs. *Klebsiella pneumonia* showed maximum susceptibility to CuNPs at maximum dilution (1:500) with 16 mm zone of inhibition. *S. aureus* is more resistant than other bacteria showing 06 mm zone of inhibition at maximum dilution (1:500).

Theivasanthi and Alagar (2011) discussed antibacterial activities of CuNPs on both Gram negative and Gram positive bacteria. They investigated the changes of surface area to volume ratio of CuNPs prepared in two different methods, its effects on antibacterial activities and proved that slight change of surface area to volume ratio results in the enhancement of its antibacterial activities. First time, they increased its antibacterial activities by using electrical power while on electrolysis synthesis and confirmed from its more antibacterial activities by using standard zone of inhibition (ZOI) microbiology assay which showed 15 mm diameter of inhibition zone against *E. coli* and 5 mm in *B. megaterium*. Later Kala *et al.* (2011) successfully synthesized CuNPs using leaf aqueous extract of *Datura innoxia* from copper sulphate. They studied

antibacterial activity of the synthesized CuNPs using well diffusion method. In which inhibitory effect of nanoparticles on bacterial growth was reflected by the zone of inhibition formed and comparison with known antibiotics. Biosynthesized CuNPs of 5-15 nm showed clear zone of inhibition against *X. oryzae* pv. *oryzae* (24 mm) as compared to 17 mm for *D. innoxia* leaf aqueous solution and 19 mm for plantomycin solution (antibiotic). The antimicrobial activity of CuNPs revealed that, they are effective growth inhibitors against *X. oryzae* pv. *oryzae*, the causative organism of bacterial leaf blight of paddy.

Bhasker *et al.* (2014) synthesized CuNPs from leaf extracts of *Ocimum sanctum* and analyzed antimicrobial activities against different pathogens on the basis of the zone of inhibition. They found that CuNPs exhibited strong antibacterial activity against human pathogens such as *Salmonella typhi, S. aureus, Vibrio cholerae, P. aeruginosa*. The CuNPs exhibited maximum effect against *Vibrio cholera* with a zone of inhibition 12 mm as compared to other pathogens. During the same period of time Kanhed *et al.* (2014) analyzed effect of chemically synthesized CuNPs and they studied antifungal activity of CuNPs against selected crop pathogenic fungi. They synthesized CuNPs by chemical reduction of Cu^{2+} in the presence of Cetyl Trimethyl Ammonium Bromide and isopropyl alcohol. Obtained zone of inhibition for antifungal activity of CuNPs against *Phoma destructiva* (22 ± 1 mm), *Curvularia lunata* (21 ± 0.5 mm), *Alternaria alternata* (18 ± 1.1 mm) and *Fusarium oxysporum* (24 ± 0.5 mm). The synthesis of CuNPs in present chemical method by using CTAB-IPA was found to be simple, economic and fast. Later on, Hariprasad *et al*. (2016) tested anti-bacterial activity of 50 nm CuNPS by disc diffusion method. In their experiment nano particles showed good bacterial activity against *E. coli, S. aureus, B. cereus* and *P. aeruginosa.* Then after Shende *et al.* (2016) carried out studies on the antimicrobial effect of biologically synthesized copper nanoparticles was on the basis of the zone of inhibition. Copper nanoparticles exhibited strong antimicrobial activity against plant bacterial pathogen such as *R. solani, X. axonopodis* pv. *citri, X. axonopodis* pv. *punicae* and plant fungal pathogens, such as *A. carthami, A. niger, C. gloeosporioides, C. lindemuthianum, Drechslera sorghicola, F. oxysporum* f. sp. *carthami, F. oxysporum* f. sp. *ciceri, F. oxysporum* f. sp. *udum, M. phaseolina, R. bataticola* and *R. stolonifer*. The maximum activity of copper

nanoparticles was found against bacterial pathogen *X. axonopodis* pv. *punicae* (17.25 ± 0.95) while minimum against *Rhizoctonia solani* (10 ± 0.81). The minimum inhibitory concentration of the copper nanoparticles against bacteria was found to be 0.01 mg/ml for *R. solani* and 0.03 mg/ml for *X. axonopodis* pv. *punicae* and *X. axonopodis* pv. *citri*.

Chormule (2017) evaluated the antibacterial properties of copper nanoparticles at different concentration against *X. axonopodis* pv. *punicae,* under *in vitro* condition. under *in vitro* condition he observed that at 70 ppm concentration of copper nanoparticles shown better antibacterial activity with 19.25 ± 0.95 mm imhibition zone, followed by 60 ppm (17.25 ± 0.55) and 50 ppm (16.25 ± 1.25).

Yilleng *et al.* (2020) evaluated antibacterial properties of copper nanoparticles against *S. aureus* and *E. coli*. The zone of inhibition by CuNP on Gram negative bacteria (*S. aureus*) is 20 mm ± 1 while on Gram positive bacteria (*E. Coli*) is 12.5 mm ± 1. Thus, the synthesized metal nanoparticles proved very high anti-bacterial activity on both *S. aureus* and *E. coli*.

2.9 Biosynthesis of green Silver nanoparticles from botanical extracts

Shankar *et al.* (2004) found that silver nanoparticles could be synthesized by using aqueous extract of *Azadiracta indica* leaves. Later on, Jha *et al.* (2009) reported that extract of *Cycas* (*Cycadaceos*), a common gymnospermic plant containing flavonoids broadly belonging to the class of phenolic compounds had been used to synthesize AgNPs with size ranging from 2-6 nm with an average of 3.29 ± 0.22 nm. Then after Philip (2011) found rapid biosynthesis of well dispersed silver nanoparticles by aqueous *Mangifera indica* leaf extract and they showed that the colloid consists of well dispersed triangular, hexagonal and nearly spherical nanoparticles having size ~20 nm. During the same period of time Prathna *et al.* (2011) reported that, silver nanoparticles were rapidly synthesized at room temperature by treating silver ions with the *Citrus limon* extract. Priyadarshini *et al.* (2012) synthesized AgNPs by using *Euphorbia hirta* plant leaf extract. By the time Zahir *et al.* (2012) reported that silver nanoparticles (AgNPs) were synthesized by using aqueous leaves extracts of *Euphorbia prostrate*.

Sharma *et al.* (2013) prepared silver nanoparticles by using *Azadirachta indica* (Neem) leaves. In their experiments, fresh and healthy leaves of *A. indica* were collected

from Yamunanagar (Haryana, India). After that 20 grams of fresh leaves of the plant were collected and washed with distilled water 3-4 times to removed the dust particles and were dried at 50^0C for 5-10 minutes in hot air oven. Then leaves were chopped into small pieces and mixed into 100 ml distilled water separately. Mixture was stirred at 60^0C for 10 minutes in water bath and it was allowed to cool and filtered with Whatman filter paper no. 1. The filtrate of the sample was stored at 4^0C for further experiments. 15 ml of the prepared extract was added to 45 ml of aqueous $AgNO_3$ (0.1M solution) at room temperature. This mixture was stirred continuously for 5-10 minutes. Then reduction was completed after 24 hours with the appearance of brownish-black colour which confirms the formation of silver nanoparticles. During the period Silva *et al.* (2013) prepared silver nanoparticles by using different concentrations (1, 2.5, 5 and 10 g/mL) of crude and modified leaf extracts (Neem and Kapparawalliya) were prepared. These extracts were mixed with 10 M AgN0 3 in four different ratios (1:1, 1:2, 1:3 and 1:9) without varying the other conditions. Agarwal *et al.* (2014) used the callus extract of *Capsicum annuum* L for the synthesis of silver nanoparticles.

Bindhani and Panigrahi (2015) carried synthesis of silver nanoparticles (SNPs), 5 ml of leaf extracts of *Ocimum sanctum* L. was added with 100 ml of 1×10^{-3} M aqueous $AgNO_3$ solution and it was stirred on the magnetic stirrer at room temperature. At 0 minute of reaction of the yellow colour mixture of $AgNO_3$ solution with leaf extract was changed black suspended mixture very rapidly at room temperature just after 2 minutes thus, nanoparticles were synthesized. Later on, Reenal and Selvam (2015) synthesized silver nanoparticles using *Oryza sativa* husk extract. Kumar *et al.* (2015) presented the works synthesis of silver nanoparticles from 1 mM $AgNO_3$ solution through various concentration of aqueous leaf extract of *A. indica* reducing as well as capping agent.

Arumugam *et al.* (2017) carried out synthesis of green silver nanoparticles from fresh leaf of *Musa barbisiana* (Banana), *Osmium tenuiflourum* (Black Tulsi), *A. indica* (Neem). 10 g of oven dried leaf was mixed with 100 ml of sterilized double distilled water. This mixture was boiled for 5 min in water bath for 70-80^0C for 6 hr. The extract was filtered through whatman filter paper no.1 and stored at -15^0C. Then 20 ml of each plant extract was added separately to 80 ml of silver nitrate solution keeping its concentration at 1 mM. As a result, a brown-yellow solution was formed, indicating the

formation of silver nanoparticles after the incubation of 12-15 hr at room temperature. During the same period of time Avinash *et al.* (2017) synthesized silver nanoparticles by leaf extract of *A. indica* 90 ml of 2 mM silver nitrate solution was mixed with the 10ml of neem leaf extract and maintained at 80^0C on a magnetic stirrer. After addition, the solution was kept at room temperature for 24 hours. Nanoparticles formation was confirmed by a distinct change of the hydrosol. Later on, Kuchekar *et al.* (2017) carried synthesis silver nanoparticles by the bioreduction method, in these method aqueous extract of *A. indica* (Neem) plant was used as reducing and stabilizing agent. On treating silver nitrate solution with *A. indica* (Neem) leaf extract, rapid reduction of silver ions was observed leading to the formation of stable silver nanoparticles in solution.

Shaikh *et al.* (2017) worked on synthesis of silver nanoparticles (AgNPs) from 0.1 gm silver nitrate ($AgNO_3$) aqueous extract of *A. indica* (neem) plant. By the same time Nzekekwu and Abosede (2019) studied green silver nanoparticles synthesis in which aqueous solution of silver nitrate ($AgNO_3$) at concentration of 0.1 M was prepared and used for the synthesis of AgNPs. For the reduction of Ag+ ions, 1 ml of *A. indica* extract was added into a clean test-tube and then 9 ml of 0.1 M aqueous $AgNO_3$ solution was added into the extract. On addition of aqueous $AgNO_3$ to the extract, colour change was noticed after about 30 minutes from yellow to brown.

Khan and Javed (2021) carried biological synthesis of Ag-NPs. Fresh leaves of neem were collected from the university campus. The leaves were washed thoroughly with distilled water and air-dried. These leaves were grinded, and a fine powder was obtained and 30 gm of dried powder was boiled in 100 ml of phosphate buffer, pH 8.0 for 30 min. After cooling at room temperature, it was centrifuged at 6000 rpm for 10 min and then filtered. The obtained filtrate was then stored at 4^0C for further experiments. In the first beaker, 9 ml extract of neem leaves was mixed with 36 ml of 1 mM $AgNO_3$ solution in the 1:4 ratios. In another beaker, 9 ml extract was mixed with 36 ml of 10 mM $AgNO_3$ solution in the same ratio under aseptic conditions. Both the beakers were kept in a shaking water bath at 37^0C in the dark for 5 h. A change in color intensities was observed after 5 h indicating NPs formation. After that Regmi *et al.* (2021) also the synthesized silver nanoparticles by using *A. indica* leaf extract as both reducing and stabilizing agent.

2.10 Characterization of green Silver nanoparticles (AgNPs)

Ramteke *et al.* (2013) worked on the synthesis of antibacterial silver nanoparticles (AgNPs) using leaf broth of medicinal herb, *O. sanctum* (Tulsi). In their experiments synthesized AgNPs had been characterized by UV-Vis spectroscopy, Transmission Electron Microscopy (TEM). The mean particle of synthesized NPs was found to be 18 nm, as confirmed by TEM and in UV-Vis absorption spectrum, it is observed that the silver surface plasmon resonance (SPR) occurs at 450 nm. After that Sharma *et al.,* (2013) prepared silver nanoparticles by using *A. indica* (Neem) leaves and UV-visible studies were conducted to confirm the formation of silver nanoparticles. The synthesized silver nanoparticles showed the absorption spectrum at 425 nm. Later on Silva *et al.* (2013) prepared silver nanoparticles by using different concentrations (1, 2.5, 5 and 10 g/ml) of crude and modified leaf extracts (Neem and Kapparawalliya) were prepared and ultraviolet-visible spectrum of the synthesized silver nanoparticles by neem leaf extract showed strong absorption peak ranged from 384 nm to 465 nm. During the same period of time Bindhani and Panigrahi (2015) studied synthesis of SNPs (Silver Nanoparticles) in aqueous medium using leaf extracts of *O. sanctum* L. and they reported that synthesis of silver nanoparticles were confirmed due to the SPR optical absorption band peak at ~440 nm by UV-vis spectrophotometer, nearly 15-45 nm in diameter with spherical in shape by TEM.

Kumar *et al.* (2015) synthesized silver nanoparticles from aqueous leaf extract of *A. indica* and characterized silver nanoparticles by using UV-Vis spectroscopy and SEM then, ultraviolet scanning spectroscopy peak values were observed at 435 nm and the silver nanoparticles formed were spherical in shape with diameter 46.67 nm as per the SEM analysis. Later on, Arumugam *et al.,* (2017) carried synthesis of green silver nanoparticles from fresh leaf of *Musa barbisiana* (banana), *Osmium tenuiflourum* (black tulsi), *A. indica* (neem) and absorption maxima of UV visible spectrum found in the range between 300 nm to 800 nm confirmed the formation of AgNPs. SEM images revealed relatively spherical shaped of AgNPs of biosynthesized AgNPs with mean diameter about 14.51 ± 1.5 nm in *O. tenuiflourum*, 09.10 ± 1.50 nm *M. barbisiana* and 11.00 ± 1.50 in *A. indica*. After that Avinash *et al.* (2017) synthesized silver nanoparticles by leaf extract of *A. indica* and UV–V is spectrum of the aqueous medium

containing silver nanoparticles exhibited absorption peak at around 405 nm and TEM analysis revealed AgNPs were spherical in shape and measured average size of AgNPs was 5-50 nm.

Kuchekar *et al.* (2017) carried silver nanoparticles synthesis by the bioreduction method and characteristics of silver nanoparticles were studied using UV-V is spectroscopy, in which appearance of a reddish-brown color confirms the formation of silver nanoparticles. The absorption spectrum of the synthesized nanoparticles was observed in the range of 450 nm. By the time Shaikh *et al.* (2017) investigated the synthesis of silver nanoparticles (AgNPs) from 0.1 gm silver nitrate ($AgNO_3$) aqueous extract of *A. indica* (Neem) plant. They characterized silver nanoparticles within 24 hours of incubation period have shown an absorption peak at around 400 nm in the UV-visible spectrum. Later on, Nzekekwu and Abosede (2019) conducted the study for green silver nanoparticles synthesis. The sharp bands of silver nanoparticles were observed around 455 nm in case of *A. indica* in UV-Vis spectroscopy.

Khan and Javed (2021) carried out biological synthesis of Ag-NPs from fresh leaves of neem. The synthesized nanoparticles were characterized using UV-visible spectrophotometry and TEM. Silver nanoparticles were analyzed by UV-Vis spectra and showed a peak at 220 nm and average particle sizes of Ag-NPs were found to be 8 and 13 nm in TEM analysis. Mustafa *et al.* (2021) prepared silver nanoparticles using neem and collagen of fish scales as a reducing and stabilizer agents and peak of absorbance for the synthesized AgNPs was at 454 nm, indicating conformed AgNPs and SEM image showed semi-evenly distributed rod shape particle. By the time Regmi *et al.* (2021) reported the synthesis of silver nanoparticles by using *A. indica* leaf extract and characterization of nanoparticles is done by using UV-VIS spectroscopy and TEM. The UV visible spectrum showed a peak at 455 nm and transmission electron microscopic (TEM) image shows agglomeration of nanoparticles may be due to surface forces: van der Waal forces, capillary forces, and electrostatic forces with sizes ranging between 2 nm to 12 nm.

2.11 Antibacterial property studies of green silver nanoparticles (AgNPs)

Yoon *et al.* (2007) evaluated antimicrobial characteristics of silver and CuNPs against *E. coli* and *B. subtilis* in their experiment, reaction of CuNPs of 100 nm with *B.*

subtilis showed the highest susceptibility (Z=0.0734 ml/µg) whereas the reaction of silver nanoparticles of 40 nm with *E. coli* showed the lowest one (Z=0.0236 ml/µg). This proved the superiority of Cu nanoparticles over silver. After that Rajesh *et al*. (2012) reported that, green synthesized AgNPs with *Ulva fasciata* inhibited the growth of *X. campestris* pv. *malvacearum* with zone of inhibition of 14.00 ± 0.58 mm and the minimum inhibitory concentration was fixed as 40.00 ± 5.77 µg/ml. Later on Sahayaraja *et al.* (2012) reported that plant based AgNPs inhibit the growth of *X. campestris* pv. *Malvacearum* with 10.33±0.33 mm zone of inhibition.

Aksar *et al*. (2013) evaluated antibacterial activity of bio-synthesized silver nanoparticles against various plant pathogenic bacteria with different levels of zone of inhibitions viz., 20.0 mm for *E. cartovra*, 14.0 mm for *E. amylovra*, 10.0 mm for *Dickya chransanthemi*, 18.0 mm for *Dickya dianthicale*, for *Pectobacterium wasaibiae* it was 18.0 mm, 19.0 mm for *Pectobacterium atrosepticum*, depicting antibacterial activity of silver nanoparticles. After that Chahardooli *et al*. (2014) found the antibacterial activity of green synthesized AgNPs against the plant pathogenic bacteria viz., *Pectobacterium carotovorum, R. solanacearum, E. amylovora* and *X. citri.* During the same period of time Ramteke *et al.* (2013) found antimicrobial activity of AgNPs against well-known pathogenic strains, namely *S. aureus* and *E. coli*. Zones of 11 mm and 10 mm were observed for *E. coli* and *S. aureus*, respectively. Divya (2013) evaluated effectiveness of silver and silica nanoparticles against *X. axonopodis* pv. *punicae in vitro* condition. Amomg the nanoparticles tested, silver nanoparticles were found to be effective with maximum inhibition with of 23.3 mm at 100 ppm and the least inhibition zone of 12.30 mm at 20 ppm.

Kumar *et al.* (2015) studied the effectiveness and antimicrobial activity of silver nanoparticles against Gram positive (*Micrococcus, Bacillus and Staphylococcus species*) and Gram negative (*Klebsiella* species and *E.coli*) bacteria. For *A. indica* the zone of inhibition was found to be 9-11 mm for *Klebsiella* species, 13-14 mm for *Bacillus* species, 10-11mm for *E.coli*, 13-15 mm for *Staphylococcus* species and 10-14 mm for *Micrococcus* species. During the same period Sherkhane *et al.* (2018) synthesized silver nanoparticles from leaf extracts of Tulsi, Neem, Tridax and Drumstick at variable concentrations and studied antimicrobial activity against *X. axonopodis* pv. *punicae.*

Their results indicated that silver nanoparticles found statistically effective with the inhibition zone of 15 mm showed highest antimicrobial activity and minimum at 5 mm inhibition zone. This study showed that synthesized silver nanoparticles having bactericidal potential and successfully controlled the growth of *X. axonopodis* pv. *punicae*. Therefore, silver nanoparticles would be used as powerful weapons against *X. axonopodis* pv. *punicae* even at very lower concentrations.

3. MATERIAL AND METHODS

The investigation was undertaken to isolate, identify, prove pathogenicity of *Xanthomonas axonopodis* pv. *punicae* and to synthesize copper and silver nanoparticles to evaluate its antimicrobial potential against *X. axonopodis* pv. *punicae* causing bacterial blight of pomegranate. This study was conducted at Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri (M.S), India and NRC on Grape, Manjari, Pune during 2021-22. The material and methods employed during the course of investigation are described in this chapter.

3.1 Materials

3.1.1 Diseased samples

Diseased samples of oily spot/ bacterial blight caused by *X. axonopodis* pv. *punicae* of pomegranate were collected during July to October 2021, from pomegranate orchards of Mahatma Phule Krishi Vidyapeeth, Rahuri, (M.S) India and also from farmer's fields. The leaf samples were collected from infected plants and the photographs of leaves and fruits sample were taken in the field. The infected leaf samples were kept in paper envelope and carried out to laboratory for further studies. The infected samples were stored in refrigerator and used for further isolation. Infected portion of the leaf was selected for isolation.

3.1.2 Culture media

The nutrient agar (NA) was used for culturing and maintaining the bacterial culture in the laboratory. Nutrient Sucrose Agar Medium (NSA), Yeast Extract Dextrose Calcium Carbonate Agar (YDCA) and Glucose Yeast Chalk Agar (GYCA) were used to conduct the cultural and morphological studies of test pathogen.

3.1.3 Chemicals

Chemicals of Analytical Reagent (AR) and Guaranteed Reagent (GR) grades of standard make were used during the study. The pH of the media was adjusted using either 0.1 N HCl or 0.1 N NaOH, Gram staining kit, Potassium hydroxide, Hydrogen paraoxide (H_2O_2), Lugol's iodine solution, copper sulphate, Silver nitrate, Captan, Streptocycline and 2-Bromo-2-Nitro -1,3-propanediol etc. available at department were used for laboratory research work.

3.1.4 Equipments

The following equipments like Hot air oven, autoclave, BOD Incubators, Refrigerator, Electronic balance, pH meter, magnetic stirrer, UV-Visible Spectrophotometer, Transmission Electron Microscope (TEM) and the other miscellaneous materials like camel brush, inoculation needles, cork borer, spirit lamp, Whatman No. 1 filter paper and cotton etc. available at department were used for laboratory research work.

3.1.5 Glass-wares

The glasswares like Petri plates (90 mm diameter), conical flasks (100, 250, 500 and 1000 ml), measuring cylinders (10, 50 and 100 ml), test tubes, sprader, beakers (150 and 250 ml), Separating funnel (500 ml) etc. available at department were used for laboratory research work.

3.1.6 Sterilization

The glassware's were sterilized in an autoclave at 1.1 kg/cm^2 pressure for 20 minutes and then they were kept in hot air oven at 160^{0}C for one hour. The media was sterilized at 1.1 kg/cm^2 pressure for 20 minutes. Soil used for pot culture experiments was sterilized in an autoclave at 1.1 kg/cm^2 for 2 hrs for two consecutive days.

3.2 Methods

3.2.1 Isolation and purification of *X. axonopodis* pv. *punicae*

Pomegranate plants showing typical bacterial blight symptoms on fruits, leaves and twigs were collected for isolation from the field. The infected plant parts were washed in running tap water to remove the other particles before isolation to avoid contamination. The infected part were cut into small bits of the size 2.5 mm with sterilized scalpel. These bits were then surface sterilized with 0.1 per cent sodium hypochlorite solution for two minutes and washed with three successive changes of sterilized water to remove the traces of sodium hypochlorite. The bits were blot dried and four bits each were placed on the solidified nutrient agar media (NA media) plates. These plates were then incubate at 27 ± 1^{0}C for four days. The pure bacterial growth was transferred to the NA plates for further studies.

3.2.2 Pathogenicity test

The pathogenicity test was carried out in insect proof glass house at Department of Plant Pathology and Agril. Microbiology on healthy pomegranate seedling of *cv.* Super Bhagwa by pin-prick infiltration method. For the pathogenicity test healthy seedlings were procured from Central nursery of Department of Horticulture, MPKV, Rahuri (M.S.). These seedlings were transferred earthen pots in glass house at Department of Plant Pathology. The culture of test pathogen was inoculated by pin-prick infiltration method in leaf and stem of seedling. After inoculation of pathogen in seedlings, seedlings were covered with plastic bags for maintain humidity for 3-4 days. The disease symptoms on leaves were developed within 10 to 18 days after inoculation. First symptoms were appeared on lower sides of the leaves as irregular water soaked spots. Later on such spots appeared in raised from on the upper side of the leaves and were noticed rough to touch with yellow haloes. Subsequently these spots became brown in colour and coalesced to form blighting appearance on the leaves. The uninoculated control plants were found free from infection. The re-isolation attempted from artificially infected plant tissues on nutrient agar consistently yielded the growth of *X. axonopodis* pv. *punicae,* these fulfillied Koch's postulates and association of *X. axonopodis* pv. *punicae* with pomegranate.

3.2.3 Biochemical test

3.2.3.1 Gram staining

A loopful of bacterial suspension was transferred at the center of slide with the help of wire loop. The drop was smeared over slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with crystal violet for 30 seconds, washed in the tap water. Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 seconds and washed under tap water then decolorized slide with 95 per cent alcohol and rinsed with water. Slide was counter stained with safranin for 10 second and washed with tap water and air dried. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lens.

3.2.3.2 Potassium hydroxide (KOH) test

Two drops of Potassium hydroxide was placed on a glass slide. A colony of culture was picked up from the medium with the help of inoculating needle and mixed

with KOH drops for 10 seconds and raised the needle for 0.5 to 2 cm to from thread to check the test.

3.2.3.3 Starch hydrolysis test

Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylose, a straight chain polymer of 200- 300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. The positive test indicates by the presence of amalyse enzyme, an exoenzyme that hydrolyses (cleaves) starch, into maltose (disaccharide) and some monosaccharides such as glucose. Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis was observed for colorless zone.

3.2.3.4 Catalase test

Using a sterile inoculating loop, small amount of organism was collected from a well isolated 18- to 24-hours colony and placed it on the slide. Using a droppler, placed 1 drop of 3 % H_2O_2 on the organism over the slide. Observed for immediate bubble formation (O_2 + water = bubbles). Observed for the formation of bubbles against a dark background enhances readability.

3.2.4 Cultural and morphological characteristics of *X. axonopodis* pv. *punicae*

The isolated bacterial pathogen was grown on different media *viz.* Nutrient Agar (NA), Nutrient Sucrose Agar (NSA), Glucose Yeast Chalk Agar (GYCA) and Yeast Extract Dextrose Calcium Carbonate Agar (YDCA) and incubated at 27 ± 2°C temperature. The observations *viz.* colour of colony, growth on the media, growth appearance, elevations and texture and consistency on different were recorded after 72 hrs. of incubation.

3.2.5 Biosynthesis of green Copper nanoparticles from botanical extracts

Fresh leaves of *Ocimum sanctum* (Tulsi) were collected from Central campus area of Mahatma Phule Krishi Vidyapeeth, Rahuri. Leaves were washed and then air dried under shade for ten days. Air dried leaves were chopped into fine pieces and powdered. Out of this powder, 200 g of powder was taken and equal amount of water i.e. 200 ml was mixed with plant extract powder. This mixture was boiled for 20 minutes and filtered through Whatman No. 1 filter paper and stored in refrigerator for further studies.

For the synthesis of Cu nanoparticles the precursor *Ocimum sanctum* (Tulsi) leaf extract 20 ml and the reducing agent 1mM aqueous $CuSO_4.5H_2O$ solution 180 ml were mixed in a clean sterilized flask in 1:10 proportion and kept under aseptic condition for bioreduction of copper ions in the solution. After mixing, final solution of 200 ml was prepared and kept in 250 ml flask and this flask was covered with the aluminum foil for overnight and observed for colour changes of mixture. The copper nanoparticles solution was centrifuged at 13000 rpm for 20 minutes to separate the CuNPs. The resultant pellet was kept in hot air oven for 20 minutes at 180^0C to make a fine crystal of nanoparticles.

3.2.6 Biosynthesis of green Silver nanoparticles from botanical extracts

Fresh leaves of neem *Azadirachta indica* (Neem) were collected from Central campus area of Mahatma Phule Krishi Vidyapeeth, Rahuri. Leaves were washed and then air dried under shade for ten days. Air dried leaves were chopped into fine pieces and powdered. Out of this powder, 200 gm of powder was taken and equal amount of water i.e., 200 ml was mixed with plant extract powder. The mixture was boiled for 20 minutes and filtered through Whatman No. 1 filter paper and store in refrigerator for further studies.

The 20 ml botanical extract of *Azadirachta indica* (Neem) was mixed with 180 ml of 1mM silver nitrate (AgNO3) solution in 1: 10 prpportion, in conical flask under aseptic condition for bioreduction of Ag+ ions in the solution. After mixing, final solution of 200 ml was poured in 250 ml flask and the flask was covered with the aluminum foil for overnight and observed for colour changes of mixture. The silver nanoparticles solution was centrifuged at 13000 rpm for 20 minutes to separate the AgNPs. The resultant pellet was kept in hot air oven for 20 minutes at 180^0C to make a fine crystal of nanoparticles.

3.2.7 Characterization of biosynthesized green Copper and Silver nanoparticles

3.2.7.1 UV-Visible spectroscopy

UV-Visible spectroscopy analysis was done by using UV-Visible Hitachi U-2900 spectrophotometer available at Department of Soil science, NRC on Grape, Pune. The reduction of pure copper and silver ions were monitored after 24 hrs. by recording the UV-Vis spectrum of the reaction medium after diluting a small aliquot of the sample into deionized water.

3.2.7.2 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) analysis of the sample was carried out at Eco-friendly Disease Management and Beneficial Microbes Research Laboratory, MPKV, Rahuri.

3.2.8 *In vitro* evaluation ecofriendly antimicrobial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles against *X. axonopodis* pv. *punicae*

Both copper and silver nanoparticles synthesized by botanical extract were evaluated under *in vitro* condition against the bacterial plant pathogens by inhibition zone method (Paper disc method) using NA as basal medium to observe the inhibitory effect of both CuNPs and AgNPs on test bacterial plant pathogens. The agar plate surface was inoculated by spreading a volume of the microbial inoculums over the entire nutrient agar surface. Then, a sterilized paper disc with a diameter of 5 mm was dipped in nano solution then aseptically transferred it at the centre of nutrient agar plate inoculated with test microorganism. In control plate sterilized paper disc dipped in distilled water. Then nutrient agar plates were incubated under suitable conditions depending upon the test microorganisms. The nanoparticles diffuses in the agar medium and inhibits the growth of the test pathogen. The zones of inhibition were measured with measuring scale in mm.

Experimental details (*In vitro* studies)

i. Design : Completely Randomized Design (CRD)
ii. Replications : Three
iii. Treatments : Twelve (12)

Test pathogens - *Xanthomonas axonopodis* pv. *punicae*

Treatment details :-

T_1 : CuNPs @ 25 ppm
T_2 : CuNPs @ 50 ppm
T_3 : CuNPs @ 75 ppm
T_4 : CuNPs @ 100 ppm
T_5 : AgNPs @ 25 ppm
T_6 : AgNPs @ 50 ppm
T_7 : AgNPs @ 75 ppm
T_8 : AgNPs @ 100 ppm

T_9 : Captan @ 2.4 g/lit water

T_{10} : Streptocycline @ 250 ppm

T_{11} : 2-Bromo-2-Nitro-1, 3-propanediol @ 250 ppm

T_{12} : Control (Untreated)

Observations on inhibition zone of the test pathogen were recorded after 72 hrs and 96 hrs of incubation and the inhibition zone was measured with measuring scale in mm.

3.2.9 *In vivo* evaluation ecofriendly antimicrobial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against *X. axonopodis* pv. *punicae*

Pot culture experiment were conducted in two successive years during 2020-21 and 2021-2022. Six months old 36 healthy pomegranate plants were inoculated using spray method with pure culture of *X. axonopodis* pv. *punicae* under glass house conditions at glass house of Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri. The pathogenic bacterium was multiplied by inoculating the loopful of culture in 200 ml of nutrient broth and incubated at 28^0C for 48 hours in shaker. The broth was diluted 5 times before spaying. The plants were provided with water spray and covered with polythene cover for 24 hrs (pre-incubation). The leaves were slightly injured with sterilized needles. The bacterial suspension (after incubation) was sprayed onto the surface of injured leaves of different plants with hand sprayer. The plants after inoculation were covered with a polythene sheet and observed regularly for the development of symptoms.

After development of typical bacterial blight symptoms initial Per cent Disease intensity (PDI) was recorded and calculated then biologically synthesized Copper (CuNPs) and Silver nanoparticles (AgNPs) at different concentrations were applied to infected pomegranate plants in four spray at 15 days intervals as given below,

Experimental details (Pot studies):-

i. Design : Completely Randomized Design (CRD)

ii. Replications : Three

iii. Treatments : Twelve (12)

Test pathogens - *Xanthomonas axonopodis* pv. *punicae*

Treatment details :-

T_1 : CuNPs @ 25 ppm

T_2 : CuNPs @ 50 ppm

T_3 : CuNPs @ 75 ppm

T_4 : CuNPs @ 100 ppm

T_5 : AgNPs @ 25 ppm

T_6 : AgNPs @ 50 ppm

T_7 : AgNPs @ 75 ppm

T_8 : AgNPs @ 100 ppm

T_9 : Captan @ 2.4 g/lit water

T_{10} : Streptocycline @ 250 ppm

T_{11} : 2-Bromo-2-Nitro-1, 3-propanediol @ 250 ppm

T_{12} : Control (Untreated)

Per cent disease intensity (PDI) was calculated after each spray for evaluation of *in vivo* CuNPs and AgNPs effect on *X. axonopodis* pv. *punicae*. At the end of 60^{th} days (After fourth spray), the fallen leaves were randomly picked up and disease intensity were calculated.

Per cent disease intensity was calculated by following formula given below by Wheeler (1969).

$$\text{Disease intensity (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{Total numbers of leaves observed x Maximum grade}} \times 100$$

The effect of nanoparticles on disease intensity was calculated on the basis of scale given by Sharma *et al.* (2017) which is as below.

Grade	Percent infection
0	No disease infection
1	1- 10 Percent area infected
2	11 - 25 Percent area infected
3	26 - 50 Percent area infected
4	51 - 75 Percent area infected
5	76 - 100 Percent area infected

Per cent disease control was calculated by following formula,

$$\text{Per cent disease control} = \frac{C - T}{C} \times 100$$

Where,

C = Per cent Disease intensity in control

T = Per cent Disease intensity in treatment

3.2.10 Statistical data analysis

The data recorded in all experiments (*in vitro* and *in vivo*) were statistically analyzed by using statistical techniques. The per cent values were transformed into arcsine values before data analysis. The standard error (SE) and critical difference (CD) at 5% level of significance were worked out and results obtained were compared statistically.

4. RESULT AND DISCUSSION

The study of "Evaluation of innovative nano biomolecules in management of bacterial blight disease of pomegranate" was carried out during 2020-21 and 2021-22 at Department of Plant Pathology and Agril. Microbiology Post Graduation Institute MPKV Rahuri, (M.S) India. The results and its interpretation of the present studies are given in this chapter.

Pomegranate is susceptible to different diseases i.e., *Cercospora* leaf and fruit spot, *Drechslera* fruit spot, *Colletotrichum* leaf spot, wilt (*Ceratocystis fimbriata*) and bacterial blight disease cause by *Xanthomonas axonopodis* pv. *punicae* (Madhukar and Reddy, 1989). Among this bacterial blight disease of pomegranate is one of the most destructive disease infecting considerable quantitative and qualitative losses. The disease occurred mostly on leaves, stems and fruits. Considering the economic importance of the fruit crop in Maharashtra State and occurrence of bacterial blight disease, present investigation was carried out for isolation, pathogenicity, cultural and morphological characteristics of *X. axonopodis* pv. *punicae*. Biosynthesis of Copper ($CuSO_4$) and Silver ($AgNO_3$) nanoparticles and evaluation of their antibacterial potential under *in vitro* and *in vivo* conditions against *X. axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate was carried out during present study. Results of this experiment are compiled, statistically analysed and presented under following headings.

4.1 Collection of diseased samples

Diseased samples of oily spot/ bacterial blight caused by *X. axonopodis* pv. *punicae* of pomegranate were collected during July to October 2021, from pomegranate orchards of Mahatma Phule Krishi Vidyapeeth, Rahuri, (M.S) India and also from farmer's fields. The leaf samples were collected from infected plants and the photographs of leaves and fruits sample were taken in the field. The infected leaf samples were kept in paper envelope and carried out to laboratory for further studies. The infected samples were stored in refrigerator and used for further isolation. Infected portion of the leaf was selected for isolation.

4.2 Disease symptomatology

Pomegranate orchards were frequently visited and critically observed for the occurrence and incidence of bacterial blight (oily spot) disease of pomegranate and

typical symptoms of the disease were observed and recorded. The symptoms of the disease like minute, water-soaked lesions which later turned brown surrounded by diffused water-soaked zone or yellow halo on the leaves as shown in photograph of Plate 1 were recorded. The lesions also appeared on flowers as water-soaked spots as shown in photograph of Plate 2A. Besides leaves and flowers lesions were also observed on branches and the twigs leading to die back and death of branches (Photograph shown in Plate 2B). Affected plants appeared unthrifty, weak and died later on necrotic lesions also increased leading to L and Y shaped cracking within spots. Severally affected fruits split open, partially exposing the arils as shown in photograph Plate 3. Similar symptoms have been reported by the earlier workers from New Delhi by Hingorani and Singh (1959); in Haryana by Kanwar (1976); in Tamil Nadu by Rangaswami (1962); in Himachal Pradesh by Sohi *et al*. (1964) and in Karnataka by Chand and Kishun (1991). In Karnataka, Manjula and Khan (2003) and Jalaraddi (2006) also reported similar symptoms on leaves, twigs and fruits. Manjula and Khan (2003) noticed that the disease as bacterial blight on the basis of extensive necrosis on fruits, twigs and leaves. Benagi and Kumar (2011) characterized the disease by the appearance of one to several, small, water soaked and dark coloured irregular spots on leaves resulting in premature defoliation under severe cases. They noticed that pathogen was also found to infect stem and branches causing girdling and cracking symptoms, further they observed that spots on fruits were dark brown, irregular, slightly raised with oily appearance, which split open with L-shaped cracks under severe cases.

Hingorani and Mehta (1952) described symptoms as irregular leaf spots, varying from 2.0 mm to 5.0 mm in diameter and later adjacent spots coalesce and cover larger areas. Rangaswamy (1962) observed the symptoms of bacterial blight on the leaves as necrotic spots surrounded by chlorotic halos with translucent water-soaked appearance. Kanwar (1976) observed small, brown, water-soaked spots on leaves, flowers and fruits of pomegranate in different orchards of Haryana. Initially, spots on leaves were small, circular with yellowish border and brown centre. Similar observations of bacterial blight disease on pomegranate were also reported by Kishun (1993) and Manjula and Khan (2003). During the present studies, the natural symptoms observed in bacterial blight of pomegranate plants are similar to those symptoms reported earlier by several workers.

4.3 Isolation, identification and pathogenicity of *X. axonopodis* pv. *punicae*

4.3.1 Isolation and identification

The isolation of *X. axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate was carried out on nutrient agar media. The diseased leaves and fruits samples were rinsed in running tap water and used for isolation. Samples were disinfected with 0.1 per cent aqueous solution of sodium hypochlorite for two minutes. Then surface sterilized bits were washed by giving three successive washings of sterile distilled water to remove traces of sodium hypochlorite. Then these bits were placed in sterilised test tube containing 2.5 ml sterile distilled water and crushed with the help of sterilized glass rod. The loopful of bacterial suspension was streaked on solidified nutrient agar medium in zigzag manner by streaking method under aseptic condition in Laminar air chamber. After three to four days yellow coloured, convex, round, shiny colonies were observed on nutrient agar medium as shown in photograph of Plate 4. Then after the bacterial colonies were transferred on fresh nutrient agar plates by streak plate method. The pathogen was purified through frequent sub culturing and the purified pure growth of culture was maintained on fresh nutrient broth as shown in photograph of Plate 4. This pure culture was then preserved in refrigerator for further studies.

Similar work on isolation of *X. axonopodis* pv. *punicae* bacterium pathogenic to several host plants including pomegranate were reported earlier by several workers. Upasana and Verma (2002) observed the size of the necrotic spots increased during May and June, but increased by more than threefold during July. Based on the morphology of the bacterium isolated from infected fruits, leaves and twigs of pomegranate, the pathogen was identified as *X. axonopodis* pv. *punicae*. Similar observations regarding isolation of *X. axonopodis* pv. *punicae* were also reported by Hingorani and Singh (1959); Kamble (1990); Chand and Kishun (1991); Jyoti *et al*. (2005); Petersen *et al.* (2010); Atar (2011) and Muswad and Chavan (2015). The observations recorded during present study are similar to that of earlier reports.

4.3.2 Pathogenicity

The pathogenicity test was carried out in insect proof glass house at Department of Plant Pathology and Agril. Microbiology, Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S) on healthy pomegranate seedling of *cv.* Super Bhagwa by pin-prick infiltration

method as shown in photograph of Plate 5. The seedlings were procured from Central nursery of Department of Horticulture, Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S). These seedlings were transferred to earthen pots in glass house at Department of Plant Pathology. The test pathogen was inoculated by pin-prick infiltration method in leaf and stem of seedling. After inoculation of pathogen in seedlings, seedlings were covered with plastic bags to maintain the humidity for 3-4 days. The disease symptoms on leaves were developed within 10 to 18 days after inoculation. First symptoms were appeared on lower sides of the leaves as irregular water-soaked spots. Later on, such spots appeared in raised from on the upper side of the leaves and they were noticed rough to touch with yellow haloes. Subsequently these spots became brown in colour and coalesced to form blighting appearance on the leaves. The uninoculated control plants were found free from infection. The re-isolation attempted from artificially infected plant tissues on nutrient agar consistently yielded the growth of *X. axonopodis* pv. *punicae*, these fulfilled Koch's postulates and association of *X. axonopodis* pv. *punicae* with pomegranate.

4.3.3 Biochemical test

Different biochemical test were conducted for identification and characterization of the pathogen i.e., Gram staining test, potassium hydroxide test, starch hydrolysis and catalase test and the photographs are shown in Plate 6 and presented in Table 1.

4.3.3.1 Gram staining

A loopful of bacterial suspension was transferred at the center of slide with the help of wire loop. The drop was smeared over slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with crystal violet for 30 seconds, washed in the tap water. Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 seconds and washed under tap water then decolorized slide with 95 per cent alcohol and rinsed with water. Slide was counter stained with safranin for 10 second and washed with tap water and air dried. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lens. The bacterial pathogen showed Gram negative reaction i.e. rod shape red/ pink cell were observed under oil immersion lens.

Table 1. Biochemical characterization of *X. axonopodis* pv. *punicae*

Sr. No.	Biochemical test	Reaction
1.	Gram staining test	Negative
2.	Potassium hydroxide (KOH) test	Positive
3.	Catalase test	Positive
4.	Starch hydrolysis test	Positive

4.3.3.2 Potassium hydroxide (KOH) test

Formation of slime threads or loop is an indication of being gram-negative because gram negative bacteria have relatively fragile cell walls, bounded by an outer membrane. This is readily disrupted by exposure to 3 % KOH releasing the viscous DNA. The *X. axonopodis* pv. *punicae* were showed to form mucoid thread after added KOH and found positive test.

4.3.3.3 Starch hydrolysis test

X. axonopodis pv. *punicae* produced colourless zone around bacterial growth on starch agar medium flooded with Lugol's iodine and showed positive for starch hydrolysis test. The *X. axonopodis* pv. *punicae* were showed that hydrolyzed starch by exoenzyme amylase and broken down to dextrins, maltose, and glucose/alpha amylase.

4.3.3.4 Catalase test

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. A small inoculum of bacterial suspension was mixed into hydrogen peroxide solution (3 %) and is observed for the rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production. Catalytic activities of *X. axonopodis* pv. *punicae* were found positive, when culture was produced bubbles of oxygen within one minute after addition of H_2O_2.

4.4 Cultural and morphological studies of *X. axonopodis* pv. *punicae*

The isolated bacterial pathogen was grown on different media *viz.* Nutrient Agar (NA), Nutrient Sucrose Agar (NSA), Glucose Yeast Chalk Agar (GYCA) and Yeast Extract Dextrose Calcium Carbonate Agar (YDCA) and incubated at 27 ± 2^0C temperature. The observations *viz.* colour of colony, growth on the media, growth

appearance, elevations and texture and consistency on different media were recorded after 72 hrs of incubation and revealed in Table 2 and photograph were shown in Plate 7.

Morphologically the bacterium *X. axonopodis* pv. *punicae* was rod shaped found in single pairs and also in chain, 0.75 to 3.0 µ in length and 0.45 µ in width, Gram negative, with single polar flagellum, neither capsules nor endospores. The results presented in Table 2 and Plate 7 revealed that colony colour of bacterium shows variation on different media i.e., light yellow on NA media, dark yellow on NSA media, dark yellow on GYCA media and whitish yellow on YDCA media. Then growth of bacterium on different media showed variation from excellent to good growth. Excellent growth was observed in NA media followed by NSA media and GYCA media while good growth in YDCA media. Growth appearances were observed slightly raised on NA media and YDCA media, whereas highly raised and glistering on NSA and GYCA media. Growth elevations convex were observed on all media. Later on texture and consistency showed differentiation on different media i.e., mucoid on YDCA media, slightly mucoid on NA media and highly mucoid on GYCA media and NSA media.

Similar results were observed by Patil *et al.,* (2017) and they reported that cultural and morphological characters of different *X. axonopodis* pv. *punicae* isolates on nutrient agar media. Further they noticed that five isolates collected from different locations were differed in respect of size of colony, shape of colony and colour of bacterial colony. Vauterin *et al.* (1995) obtained different isolates of *X. axonopodis* pv. *punicae* pomegranate gardens from Karnataka and Andhra Pradesh States. These isolates yielded yellow, slimy, glistening, mucoid, convex, small round to irregular colonies on nutrient agar medium and pale yellow to dark yellow colonies, convex with copious slime on YDCA medium, while SX and BSCAA media supported luxuriant growth of all the seven isolates.

Table 2. Cultural characteristics of *X axonopodis* pv. *punicae* on different growth media

Sr. No.	Colony character	NA media	NSA media	GYCA media	YDCA media
1.	Colour	Light Yellow	Dark Yellow	Dark Yellow	Whitish Yellow
2.	Growth on the media	Excellent	Excellent	Excellent	Good
3.	Growth appearance	Slightly raised	Highly raised, glistering	Highly raised, glistering	Slightly raised
4.	Elevation	Convex	Convex	Convex	Convex
5.	Texture and consistency	Slightly mucoid	Highly Mucoid	Highly Mucoid	Mucoid

*NA=Nutrient Agar, NSA= Nutrient Sucrose Agar, GYCA= Glucose Yeast Chalk Agar and YDCA= Yeast Extract Dextrose Calcium Carbonate Agar

4.5 Biosynthesis of green Copper nanoparticles from botanical extracts

Fresh leaves of *Ocimum sanctum* (Tulsi) were collected from Central campus area of Mahatma Phule Krishi Vidyapeeth, Rahuri. Leaves were washed and then air dried under shade for ten days. Air dried leaves were chopped into fine pieces and powdered. Out of this powder, 200 gm powder was taken and equal amount of water i.e., 200 ml mixed with plant extract powder. This mixture was boiled for 20 minutes and filtered through Whatman No. 1 filter paper and stored in refrigerator for further studies.

For synthesis of Cu nanoparticles the precursor *Ocimum sanctum* leaf extract 20 ml and the reducing agent 1mM aqueous $CuSO_4.5H_2O$ solution 180 ml were mixed in a clean sterilized flask in 1:10 proportion and kept under aseptic condition for bioreduction of Copper ions in the solution. After mixing, final solution of 200 ml was prepared and kept put in 250 ml flask. This flask was covered with the aluminium foil for overnight and observed for colour changes of mixture. Synthesis of the nanoparticles were noticed in the solution. Light brown to dark brown colour was observed as shown in photograph of Plate 8. To separate the copper nanoparticles, solution was centrifuged at 13000 rpm for 20 minutes. Then after, the resultant pellet was kept in hot air oven for 20 minutes at 180^0C to make a fine crystal of nanoparticles as shown in photograph of Plate 8.

4.6 Characterization of biosynthesized green Copper nanoparticles

The synthesized nanoparticles were characterized by using UV-Visible spectroscopy and Transmission Electron Microscopy (TEM) available at Eco-friendly Disease Management and Beneficial Microbes Research Laboratory, MPKV, Rahuri.

4.6.1 UV-Visible spectroscopy

The most common technique for characterization of green copper nanoparticle is UV-Visible spectroscopy, which is used for analysis of intensely colored colloidal dispersions having surface plasmon absorption. Copper colloids exhibit strong absorption bands in the visible region and are therefore intensely colored. The plasmon bandwidth increases with decreasing size in the intrinsic region (mean diameter smaller than 25 nm) and also increases with increasing size in the extrinsic size region (mean diameter larger than 25 nm) (Link and Sayed, 1999; Ramayadevi *et al*., 2012).

Reduction of copper ion to obtain CuNPs on exposure to *Ocimum sanctum* (Tulsi) leaf extracts was observed by colour change and detected by UV-Visible spectroscopy. The biologically synthesized green copper nanoparticles formed in the reaction had sharp absorbance peak at 397.98 nm which can be attributed to absorption by nano size copper particle confirming the presence of copper nanoparticles. It is graphically showed in Figure 1, and shown in Plate 8. The synthesized nanoparticles were characterized and showed maximum absorbance 1.372 at 397.98 nm by using tulsi leaf extract as reducing agent. The results are presented in Table 4. Similar absorbance of CuNPs using plant extracts as reducing agent was also recorded between 300-600 nm by other scientists (Patel *et al*., 2016; Ebrahimi *et al*., 2017; Mohindru and Garg, 2017; Usha *et al*., 2017; Mishra *et al*., 2017). Ayona and Neethu (2021) prepared copper nanoparticles by using tulsi leaves and characterized by using UV-Vis spectrophotometer. UV- visible spectral analyses of Copper nanoparticles was done to characterize the Cu NP formed at a range of 350 nm to 700 nm and maximum absorption can be seen at a range between 550 nm and 600 nm at about 560 nm. The observations recorded during present study are similar to that of earlier reports.

4.6.2 Transmission Electron Microscopy (TEM)

Biologically synthesized CuNPs were found spherical in shape with 8.97 nm to 10.89 nm size and well dispersed which were confirmed under TEM studies as shown in

photographs of Plate 9 and presented in Table 4. The shape and size distribution of colloidal particles were characterized by transmission electron microscopy (TEM) (Dang *et al.,* 2011). Mohindru and Garg (2017) reported green synthesis of Copper nanoparticles using plant extract (tea leaf extract) with water as the medium for reduction and identified their main physical properties. They observed formation of copper nanoparticles indicated by change in colour from blue to yellowish black which was supported by the UV absorption at 570 nm. The final analysis pertaining to the determination of size of nanoparticles was done under TEM and it was found that the particle size of Copper nanoparticles in the range of 70-90 nm.

Similar results of size of nanoparticles were recorded between 10-41nm by other workers (Ebrahimi *et al.,* 2017 and Dang *et al.,* 2011). Thus, the present study in respect of colour change and TEM analysis are matched with the earlier research.

Table 3. Colour changes in botanical extracts and capping agent during biosynthesis of Copper and Silver nanoparticles

Sr. No.	Botanical extract /Capping agent	Before reduction	After Bioreduction	Colour intensity	Time (hrs.)
1.	*Oscimum sanctum* (Tulsi) leaf extract	Light brown Colour	Dark brown	++	24
2.	*Azadirachta indica* (Neem) leaf extract	Light yellow green	Dark yellow green	++	24
3.	Copper sulphate ($CuSO_4$)	Blue colour	Dark brown	++	24
4.	Silver nitrate ($AgNO_3$)	Dull white	Dark yellow green	++	24

Colour intensity : Light : +, Dark : ++, High dark : +++

Table 4. Characterization of biosynthesized Copper and Silver nanoparticles

Sr. No.	Solution	UV-Spectrophotometer reading after 24 hrs. of reduction (Peak at nm)	Absorbance	Size range of particle in TEM analysis (nm)
1.	*Oscimum sanctum* (Tulsi) leaf extract + Copper sulphate	397.98	1.372	8.97 – 10.89
2.	*Azadirachta indica* (Neem) leaf extract + Silver nitrate	406.65	1.392	10.33 – 12.21

4.7 Biosynthesis of green Silver nanoparticles from botanical extracts

Fresh leaves of *Azadirachta indica* (Neem) were collected from Central campus area of Mahatma Phule Krishi Vidyapeeth, Rahuri. Leaves were washed and then air dried under shade for ten days. Air dried leaves were chopped into fine pieces and powdered. Out of this powder, 200 gm of powder was taken and equal amount of water i.e., 200 ml was mixed with plant extract powder. The mixture was boiled for 20 minutes and filtered through Whatman No. 1 filter paper and store in refrigerator for further studies.

Later on, 20 ml water extract of *Azadirachta indica* (Neem) leaves was mixed with 180 ml of 1mM Silver nitrate ($AgNO_3$) solution in 1:10 proportion in conical flask under aseptic condition for bioreduction of Ag+ ions in the solution. After mixing, final solution of 200 ml was poured in 250 ml flask and the flask was covered with the aluminium foil for overnight. Synthesis of the nanoparticles was observed with change in the colour of solution i.e., light yellow green to dark yellow green. The colour change is shown in photograph of Plate 10. Then after the silver nanoparticles solution was centrifuged at 13000 rpm for 20 minutes to separate the AgNPs. The resultant pellet was obtained and kept in hot air oven for 20 minutes at 180^0C to make a fine crystal of nanoparticles as shown in photograph of Plate 10.

4.8 Characterization of biosynthesized green Silver nanoparticles

Characterization of biosynthesized green silver nanoparticles were done by using UV-Visible spectroscopy and Transmission Electron Microscopy (TEM) available at Eco-friendly Disease Management and Beneficial Microbes Research Laboratory, MPKV, Rahuri.

4.8.1 UV-Visible spectroscopy

UV-Vis spectroscopy is the common technique for characterization of green Silver nanoparticle which is used for analysis of intensely coloured colloidal dispersions having surface plasmon absorption. In present study the absorption spectra of AgNPs formed in the reaction synthesized by using biological reduction had sharp absorbance peak at 406.65 nm in UV-Visible spectrophotometer which could be attributed to absorption by nano size silver particle confirming the presence of AgNPs. The sharp peak at 406.65 nm in UV-Visible spectrophotometer was reflected which is shown in graph

Figure 2. Similar absorbance was also reported by Sharma *et al.* (2013) prepared silver nanoparticles by using *A. indica* (Neem) leaves and UV-visible studies were conducted to confirm the formation of Silver nanoparticles and they observed that synthesized silver nanoparticles showed the absorption spectrum at 425 nm.

The above findings also matched with the work of Ramteke *et al.* (2013); Silva *et al.* (2013); Bindhani and Panigrahi (2015); Kumar *et al*. (2015); Arumugam *et al*. (2017); Kuchekar *et al*. (2017); Shaikh *et al*. (2017); Khan and Javed (2021) and Regmi *et al*. (2021).

4.8.2 Transmission Electron Microscopy (TEM)

In the present study the nanoparticles were characterized by using Transmission Electron Microscopy (TEM) to determine their size and morphology from drop-coated films of the silver nanoparticles synthesized by botanical extract. TEM micrograph revealed that the nanoparticles were formed in the size range of 10.33 nm to 12.21 nm with spherical morphology as shown in photograph of Plate 11 and presented in Table 4. TEM micrograph indicates the particles relatively uniform in nature and also showed that the particles were well separated from each other having no accumulation. Avinash *et al.* (2017) synthesizes silver nanoparticles by leaf extract of *A. indica* and UV–Vis spectrum of the aqueous medium containing silver nanoparticles exhibited absorption peak at around 405 nm and TEM analysis revealed AgNPs spherical shape which measured with average size of AgNPs was 5- 50 nm. Similar results were also reported by Ramteke *et al.* (2013); Bindhani and Panigrahi (2015); Kumar *et al*. (2015); Arumugam *et al*. (2017); Khan and Javed (2021) and Regmi *et al*. (2021). Thus, the present study in respect of colour change and TEM analysis are matched with the earlier research.

4.9 *In vitro* evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles against *X. axonopodis* pv. *punicae*

Very meager information is available on antimicrobial activity of copper (CuNPs) and silver (AgNPs) nanoparticles. The present research in subject is at basic level in case of agricultural plant pathogens for Copper and Silver nanoparticles.

Total three bactericide *viz,* 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm, Streptocycline @ 250 ppm and Captan 50 % WP @ 0.24 % and two nanoparticles *viz,*

Copper (CuNPs) and Silver (AgNPs) nanoparticles each at the concentration of 100 ppm, 75 ppm, 50 ppm and 25 ppm were evaluated under *in vitro* condition against *X. axonopodis* pv. *punicae,* as shown in photograph of Plate 12. Observations on inhibition zone of the test pathogen were recorded after 72 hrs and 96 hrs of incubation. The results are presented in Table 5 and graphically showed in the Fig. 3. The primary screening of antimicrobial activities of the biologically synthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles were analyzed on the basis of inhibition zone obtained in paper disc method. The Copper (CuNPs) and Silver (AgNPs) nanoparticles showed antimicrobial activity against bacteria *X. axonopodis* pv. *punicae* at different concentrations. The result of the present finding are presented in Table 5 and it is revealed that, bacteria *X. axonopodis* pv. *punicae*. is highly sensitive to the action of CuNPs and AgNPs at higher concentration i.e., 100 ppm.

The observations on inhibition zone of test bacterium were recorded after 72 hrs. and presented in Table 5. It is revealed that in treatment T_{11} 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm was found statistically significant over the untreated control with 20.83 mm inhibition zone. The next best treatment was T_{10} (Streptocycline @ 250 ppm) which was found statistically at par with T_9 (Captan 50 % WP @ 0.24 %) and recorded 18.43 mm and 17.66 mm inhibition zone respectively, over untreated control (0.0 mm). In eco-friendly treatments the maximum inhibition zone was noticed in treatment T_4 (CuNPs @ 100 ppm) with 16.56 mm inhibition zone of test bacterium which was statistically at par with T_8 (AgNPs @ 100 ppm) which recorded 16.16 mm inhibition zone. The next best treatment was T_3 (CuNPs @ 75 ppm) (15.40 mm) followed by T_7 (AgNPs @ 75 ppm) (15.00 mm), T_2 (CuNPs @ 50 ppm) (12.36 mm) and T_6 (AgNPs @ 50 ppm) (11.66 mm) whereas treatment T_1(CuNPs @ 25 ppm) and T_5 (AgNPs @ 25 ppm) were found less effective and recorded 8.16 mm and 7.96 mm inhibition zone respectively, over untreated control (0.0 mm). In untreated control inhibition zone was not noticed i.e., 0.0 mm inhibition zone.

Data presented in Table 5, revealed that after 96 hrs of incubation there is no significant growth of test bacterium. Photograph in the Plate 12 showed that with increase in concentration of nanoparticles, zone of inhibition of pathogen also increases, it means concentration of nanoparticles were directly proportional to the inhibition zone. From the

graphical presentation of Figure 3 it was observed that bactericides show highest zone of inhibition than the innovative nanoparticles. It was observed that after 96 hrs. of incubation treatment T_{11} (2-Bromo-2-Nitro-1,3-Propanediol) at concentration of 250 ppm was found to be statistically superior over the rest of the treatments and recorded 20.90 mm inhibition zone, over untreated control (0.0 mm). However after 96 hrs of incubation, the treatment T_{11} recorded 20.83 mm inhibition zone and it was increased by 0.07 mm within 72 hrs. The next best treatment T_{10} (Streptocycline @ 250 ppm) found statistically at par with T_9 (Captan 50% WP @ 0.24 %) recorded 18.46 mm and 17.70 mm zone of inhibition respectively. After 72 hrs. the inhibition zone was 18.43 mm and 17.66 mm recorded in treatment T_{10} (Streptocycline @ 250 ppm) and treatment T_9 (Captan 50% WP @ 0.24 %) respectively. In concerned with the innovative biomolecule's treatments, the maximum zone of inhibition was observed in treatment T_4 i.e., CuNPs @ 100 ppm with 16.60 mm zone of inhibition which was statistically at par with T_8 (AgNPs @ 100 ppm) (16.20 mm zone of inhibition). The next superior treatment was T_3 (CuNPs @ 75 ppm) (15.46 mm) followed by T_7 (AgNPs @ 75 ppm) (15.03 mm), T_2 (CuNPs @ 50 ppm) (12.36 mm) and T_6 (AgNPs @ 50 ppm) (11.66 mm). Treatment T_1(CuNPs @ 25 ppm) and T_5 (AgNPs @ 25 ppm) were found less effective with 8.16 mm and 7.96 mm inhibition zone respectively, over untreated control. In untreated control there was no inhibition zone recorded after 96 hrs. i.e., 0.0 mm inhibition zone. Mean inhibition zone was ranged from 7.96 mm to 20.86 mm were presented in Table 5 and graphically shown in Figure 3. Significantly highest inhibition zone of 20.86 mm was recorded in treatment T_{11} (2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm). However, it was significantly least in treatment T_5 i.e., AgNPs @ 25 ppm (7.96 mm inhibition zone) over untreated control. The next best treatment was T_{10} (Streptocycline @ 250 ppm) with 18.44 mm inhibition zone of test bacterium was statistically at par with T_9 (Captan 50 % WP @ 0.24 %) with 17.68 mm inhibition zone of test bacterium. From the innovative biomolecule's treatments, the maximum zone of inhibition was found in T_4 i.e., CuNPs @ 100 ppm with 16.58 mm inhibition zone, which was statistically at par with T_8 (AgNPs @ 100 ppm) with 16.18 mm inhibition zone. The next best treatment from biomolecules are T_3 (CuNPs @ 75 ppm) (15.43 mm) followed by T_7 (AgNPs @ 75 ppm) (15.01 mm), T_2 (CuNPs @ 50 ppm) (12.36 mm) and T_6 (AgNPs @ 50 ppm) (11.66 mm).

Table 5. ***In vitro*** **evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles against *X. axonopodis* pv. *punicae***

Tr. No.	Treatment details	Inhibition zone (mm) (Average of three replication)		Mean inhibition zone (mm)
		After 72 hrs.	After 96 hrs.	
T_1	CuNPs @ 25 ppm	8.16^{i}	8.16^{i}	8.16
T_2	CuNPs @ 50 ppm	12.36^{g}	12.36^{g}	12.36
T_3	CuNPs @ 75 ppm	15.40^{de}	15.46^{de}	15.43
T_4	CuNPs @ 100 ppm	16.56^{bc}	16.60^{bc}	16.58
T_5	AgNPs @ 25 ppm	7.96^{i}	7.96^{i}	7.96
T_6	AgNPs @ 50 ppm	11.66^{gh}	11.66^{gh}	11.66
T_7	AgNPs @ 75 ppm	15.00^{ef}	15.03^{ef}	15.01
T_8	AgNPs @ 100 ppm	16.16^{cd}	16.20^{cd}	16.18
T_9	Captan 50% WP @ 0.24 %	17.66^{b}	17.70^{b}	17.68
T_{10}	Streptocycline @ 250 ppm	18.43^{b}	18.46^{b}	18.44
T_{11}	2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm	20.83^{a}	20.90^{a}	20.86
T_{12}	Control	0.00	0.00	0.00
	SE±	0.9766	0.9233	-
	CD at 5 %	2.9033	2.7435	-

In above findings the chemicals i.e., 2-Bromo-2-Nitro-1, 3-Propanediol, Streptocycline and Captan were found statistically significant to control growth of *X. axonopodis* pv. *punicae,* causing bacterial blight disease of pomegranate, whereas among biologically synthesized green nanoparticles i.e., Copper (CuNPs) and Silver (AgNPs) nanoparticles at concentration of 100 ppm were found effective against *X. axonopodis* pv. *punicae.* Effective chemical i.e. 2-Bromo-2-Nitro-1, 3-Propanediol has 20.86 mm inhibition zone, followed by green nanoparticles i.e., CuNPs @ 100 ppm and AgNPs @ 100 ppm have 16.58 mm and 16.18 inhibition zone, over untreated control. Thus, difference between inhibition zone observed between effective chemical and effective green nanoparticles are not much more i.e., 4.68 mm. therefore, by considering the harmful effect of chemical on the environment, residual effect of chemical in fruit and hampering in export- import its better to use green nanoparticles than chemical for management of bacterial blight disease of pomegranate, because this nanoparticles are eco-friendly and haven't bad environmental effect and barriers in export- import.

The results obtained by using paper disc method shown that copper and silver nanoparticles possess antibacterial activity. In comparative study, it was found significant as compared to control. This evidence confirmed that nanoparticles, which have a larger surface-to volume ratio, are more efficient for antibacterial activity against Gram negative bacteria, as previously reported for Escherichia coli (Raffi *et al*., 2010), *X. axonopodis* pv. *phaseoli*, and *X. oryzae* pv. *oryzae* (Mondal, 2009; Mondal *et al*., 2010). Shende *et al.* (2016) carried out studies on the antimicrobial effect of biologically synthesized copper nanoparticles was analyzed on the basis of the zone of inhibition. They noticed that copper nanoparticles exhibited strong antimicrobial activity against plant bacterial pathogen such as *Rhizoctonia solani, X. axonopodis* pv. *citri, X. axonopodis* pv. *punicae* and plant fungal pathogens, such as *Alternaria carthami, Aspergillus niger, Colletotrichum gloeosporioides, C. lindemuthianum, Drechslera sorghicola, Fusarium oxysporum* f. sp. *carthami, F. oxysporum* f. sp. *ciceri, F. oxysporum* f. sp. *udum, Macrophomina phaseolina, R. bataticola* and *Rhizopus stolonifer*. The maximum activity of copper nanoparticles was found against bacterial pathogen *X. axonopodis* pv. *punicae* (17.25 ± 0.95) while the minimum against *R. solani* (10 ± 0.81). Similar results were obtained in the study of Chormule (2017) evaluated the antibacterial properties of copper nanoparticles at different concentration against *X. axonopodis* pv. *punicae,* under *in vitro* condition. He observed that at 70 ppm concentration of copper nanoparticles showed better antibacterial activity with 19.25 ± 0.95 mm inhibition zone, followed by 60 ppm (17.25 ± 0.55) and 50 ppm (16.25 ± 1.25). Divya (2013) evaluated effectiveness of silver and silica nanoparticles against *X. axonopodis* pv. *punicae* under *in vitro* condition. Amomg the nanoparticles tested, silver nanoparticles were found effective with maximum inhibition with of 23.3 mm at 100 ppm and the least inhibition zone of 12.30 mm at 20 ppm. These findings also matched with the works of Mandal *et al*. (2010); Sawant *et al*. (2010); Rajesh *et al*. (2012); Sahayaraj *et al*. (2012); Sherkhane *et al*. (2018) and Yilleng *et al*. (2020). Thus, the present studies in respect of inhibition zone obtained by using paper disc method are matched with the earlier research.

4.10 *In vivo* evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against *X. axonopodis* pv. *punicae*

Pot culture experiments were conducted in two successive years during 2020-21 and 2021-2022. Six months old 36 healthy pomegranate plants were inoculated using spray method with pure culture of *X. axonopodis* pv. *punicae* under glass house conditions at of Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri. The pathogenic bacterium was multiplied by inoculating the loopful of culture in 200 ml of nutrient broth and incubated at 28^0C for 48 hours in shaker. The broth was diluted 5 times before spraying. The plants were provided with water spray and covered with polythene sheet for 24 hours (pre-incubation) before inoculation. The leaves were slightly injured with sterilized needles. Then bacterial suspension (after incubation) was sprayed onto the surface of injured leaves of different plants with hand sprayer. The plants after inoculation were covered with a polythene sheet and observed regularly for the development of symptoms.

After 10 days of inoculation initially small, water-soaked lesions were noticed on the lower surface of the leaves. Correspondingly on the upper surface, small brown to black color spots were seen. Spots were rounded, angular to irregular in shape. As the disease progressed, these spots also grew, increased in size, coalesced and extended up to midrib in a week's time covering the major portion of the leaf lamina. Similar symptoms of bacterial blight have been earlier reported by several authors (Hingorani and Mehta, 1952; Hingorani and Singh, 1959; Sohi *et al*., 1964; Kanwar, 1976; Kishun, 1993; Rani and Verma, 2002).

After development of typical bacterial blight symptoms initial per cent disease intensity (PDI) was recorded and calculated then biologically synthesized CuNPs and silver nanoparticles (AgNPs) at different concentrations were applied to infected pomegranate plants in four spray at 15 days intervals. Per cent disease intensity (PDI) was calculated after each spray for evaluation of CuNPs and AgNPs effect on *X. axonopodis* pv. *punicae*. At the end of 60^{th} days (After fourth spray), the fallen leaves were randomly picked up and disease intensity were calculated.

4.10.1 Per cent disease intensity (PDI)

4.10.1.1 Per cent disease intensity (PDI) 2020-2021

The results obtained from pot study conducted during 2020-21 are presented in Table 6, graphically presented in Figure 4 and photograph shown in Plate 13. Its revealed that the bactericides treatments T_{11} 2-Bromo-2-Nitro-1,3-Propanediol at the concentration of 250 ppm was found most effective to reduce per cent disease intensity up to 7.20 per cent and which was found to be statistically at par with T_{10} of Streptocycline @ 250 ppm with 9.60 per cent disease intensity followed by T_9 of Captan 50 % WP @ 0.24 per cent (12.26 %). In the present study bactericides were found effective to manage disease than the eco-friendly molecules i.e. Copper and Silver nanoparticles. However, to obtained residue free yield of pomegranate with less damage to environment, it is better to use eco-friendly molecules. The alternatives for bactericide treatment T_4 i.e. CuNPs @ 100 ppm was found most superior and which was at par with T_8 of AgNPs @ 100 ppm with 18.93 per cent disease intensity. The next best trearment is T_3 of CuNPs @ 75 ppm with per cent disease intensity 24.26 followed by T_7 (AgNPs @ 75 ppm) (25.33 %), T_2 (CuNPs @ 50 ppm) (28.80 %), T_6 (AgNPs @ 50 ppm) (29.06 %), T_1 (CuNPs @ 25 ppm) (29.60 %) and T_5 (AgNPs @ 25 ppm) (29.93 %) effectively controlled bacterial blight disease intensity after first spray. The maximum per cent disease intensity was observed in control T_{12} (untreated control) with 30.13 per cent disease intensity.

The bacterial blight disease intensity further was reduced effectively after second sprays. The maximum disease intensity was reduced in treatment T_{11} of 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm with 9.86 per cent disease intensity and it was statistically at par with T_{10} (Streptocycline @ 250 ppm) with 12.26 per cent disease intensity, followed by T_9 (Captan 50 % WP @ 0.24 %) (16.26 %). However, next to bactericide treatments, innovative biomolecules were found effective and competant for production of residue free yield. The ecofriendly treatment i.e. T_4 of CuNPs @ 100 ppm showed minimum per cent disease intensity 20.00 which was found to be statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 21.06 followed by T_3 (CuNPs @ 75 ppm) (29.60 %), T_7 (AgNPs @ 75 ppm) (31.73 %), T_2 (CuNPs @ 50 ppm) (34.40 %), T_6 (AgNPs @ 50 ppm) (34.73 %), T_1

(CuNPs @ 25 ppm) (36.00 %) and T_5 (AgNPs @ 25 ppm) (36.50 %) and the highest bacterial blight per cent disease intensity was recorded in T_{12} (untreated control) with 38.13 per cent disease intensity.

Table 6. ***In vivo*** **evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against** ***X. axonopodis*** **pv.** ***punicae*** **(2020-21)**

Tr. No.	Treatment details	PDI before spray (%)	Mean PDI 15 days after spray (%)				PDC (%)
			I	II	III	IV	
T_1	CuNPs @ 25 ppm	3.73 (11.13)	29.60^{e} (32.96)	36.00^{ef} (36.86)	40.53^{ef} (39.54)	40.53^{de} (39.54)	8.42
T_2	CuNPs @ 50 ppm	3.46 (10.72)	28.80^{e} (32.45)	34.40^{de} (35.91)	38.13^{de} (38.13)	38.13^{cd} (38.13)	13.84
T_3	CuNPs @ 75 ppm	3.73 (11.13)	24.26^{d} (29.50)	29.60^{d} (32.96)	33.06^{d} (35.09)	33.06^{c} (33.06)	25.30
T_4	CuNPs @ 100 ppm	4.80 (12.65)	16.53^{c} (23.98)	20.00^{c} (26.56)	22.13^{ab} (28.06)	21.06^{b} (27.31)	50.00
T_5	AgNPs @ 25 ppm	4.00 (11.53)	29.93^{e} (33.16)	36.50^{ef} (36.10)	41.03^{ef} (39.83)	41.03^{de} (39.83)	7.29
T_6	AgNPs @ 50 ppm	3.20 (10.30)	29.06^{e} (32.62)	34.73^{de} (36.10)	38.86^{de} (38.56)	38.86^{cd} (38.56)	12.20
T_7	AgNPs @ 75 ppm	3.46 (10.72)	25.33^{d} (30.21)	31.73^{d} (34.28)	35.73^{d} (36.70)	35.73^{c} (36.70)	19.27
T_8	AgNPs @ 100 ppm	3.46 (10.72)	18.93^{c} (25.79)	21.06^{c} (27.31)	25.33^{bc} (30.21)	22.40^{b} (28.24)	42.76
T_9	Captan 50 % WP @ 0.24 %	3.73 (11.13)	12.26^{b} (20.49)	16.26^{b} (23.78)	18.40^{a} (25.40)	16.96^{a} (24.31)	58.42
T_{10}	Streptocycline 250 ppm	2.93 (9.85)	9.60^{a} (18.04)	12.26^{a} (20.49)	16.40^{a} (23.88)	14.80^{a} (22.62)	62.94
T_{11}	2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm	3.46 (10.72)	7.20^{a} (15.56)	9.86^{a} (18.30)	15.46^{a} (23.15)	13.76^{a} (21.77)	65.07
T_{12}	Control	3.20 (10.30)	30.13 (33.29)	38.13 (38.13)	44.26 (41.70)	48.30 (44.02)	00.00
	SE±	0.6533	1.4131	1.7396	2.3240	1.8981	-
	CD at 5 %	NS	2.9165	3.5904	4.7965	3.9175	-

*Figures in parentheses are arc sine transformed values
*Per cent disease control (PDC) were calculated based on the per cent disease intensity (PDI) were recorded after third spray, because highest per cent disease intensity (PDI) were recorded after third spray

From the results interpreted after third spray, it was noticed that among the all treatments of bactericides and eco-friendly biomolecules the treatments T_{11} of 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was found with least per cent disease

intensity 15.46 and which was found to be statistically at par with T_{10} of Streptocycline @ 250 ppm and T_9 of Captan 50 % WP @ 0.24 per cent with per cent disease intensity of 16.40 and 18.40 respectively. However, in respect of the eco-friendly biomolecules after third spray treatments T_4 (CuNPs @ 100 ppm) was found to be best with minimum 22.13 per cent disease intensity and which was found to be statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 25.33 per cent. The next best treatment is T_3 (CuNPs @ 75 ppm) with per cent disease intensity 33.06 per cent followed by T_7 (AgNPs @ 75 ppm) (35.73 %), T_2 (CuNPs @ 50 ppm) (38.13 %), T_6 (AgNPs @ 50 ppm) (38.86 %), T_1 (CuNPs @ 25 ppm) (40.53 %) and T_5 (AgNPs @ 25 ppm) (41.03 %) and the highest bacterial blight per cent disease intensity was recorded in T_{12} (untreated control) with 44.26 per cent disease intensity.

After last spray i.e. fourth spray, range of per cent disease intensity on leaves in different treatment was found varied from 13.76 to 41.03 per cent as compared to control 48.30 per cent. Least disease intensity was observed in plant sprayed with T_{11} (2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm) with 13.76 per cent disease intensity and which was at par with T_{10} (Streptocycline @ 250 ppm) and T_9 (Captan 50 % WP @ 0.24 %) with 14.80 and 16.96 per cent disease intensity, respectively.

Among the ecofriendly green nanoparticles treatments T_4 of CuNPs @ 100 ppm was found with minimum per cent disease intensity 21.06 per cent and which was found statistically at par with T_8 of AgNPs @ 100 ppm with per cent disease intensity 22.40 per cent. The next best treatment was T_3 of CuNPs @ 75 ppm with per cent disease intensity 33.06 per cent followed by T_7 (AgNPs @ 75 ppm) (35.73 %), T_2 (CuNPs @ 50 ppm) (38.13 %), T_6 (AgNPs @ 50 ppm) (38.86 %), T_1 (CuNPs @ 25 ppm) (40.53 %) and T_5 (AgNPs @ 25 ppm) (41.03 %). The maximum per cent disease intensity was observed in T_{12} (untreated control) 48.30 per cent.

4.10.1.2 Per cent disease intensity (PDI) 2021-2022

During 2021-22 the bacterial blight disease intensity was reduced effectively after first spray were presented in Table 7 and graphically presented in Figure 5, Photograph shown in Plate 13 B, it is revealed that, treatments of bactericide T_{11} i.e., 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was again found effective with least

per cent disease intensity (9.60 %) and which was found to be statistically at par with T_{10} (Streptocycline @ 250 ppm) with per cent disease intensity of 12.26, followed by T_9 (Captan 50% WP @ 0.24 %) (15.20 %). The similar trends of bactericidal effect were observed in succeeding year 2021-22 in which bactericides were found effective than the nanoparticle for the management of bacterial blight disease.

Among the innovative biomolecules treatments, the minimum per cent disease intensity 18.93 was noticed with treatment T_4 of CuNPs @ 100 ppm, followed by T_8 of AgNPs @ 100 ppm (21.86 %), T_3 (CuNPs @ 75 ppm) (26.93%), T_7 (AgNPs @ 75 ppm) (27.46 %), T_2 (CuNPs @ 50 ppm) (30.93%), T_6 (AgNPs @ 50 ppm) (31.60 %), T_1 (CuNPs @ 25 ppm) (31.73 %) and T_5 (AgNPs @ 25 ppm) (32.00 %) effectively controlled bacterial blight disease intensity. The maximum per cent disease intensity was recorded in T_{12} (untreated control) 32.80 per cent.

The maximum bacterial blight per cent disease intensity was recorded in T_{12} untreated control (41.33 %). The bacterial blight disease intensity was further reduced effectively after second spray treatment T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm) effectively reduced bacterial blight disease intensity with 12.80 per cent disease intensity and it was at par with T_{10} (Streptocycline @ 250 ppm) with 15.20 per cent disease intensity, followed by T_9 (Captan 50 % WP @ 0.24 %) (19.46 %). Among the eco-friendly treatments T_4 i.e. CuNPs @ 100 ppm showed minimum per cent disease intensity 23.20 and which was found statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 23.73. The next best treatment is T_3 (CuNPs @ 75 ppm) (32.00 %) followed by T_7 (AgNPs @ 75 ppm) (34.40 %), T_2 (CuNPs @ 50 ppm) (36.80 %), T_6 (AgNPs @ 50 ppm) (37.46%), T_1 (CuNPs @ 25 ppm) (39.20 %), T_5 (AgNPs @ 25 ppm) (39.86 %).

After third spray, it was noticed that among the all treatments of chemical bactericides and eco-friendly biomolecules, the treatments T_{11} i.e., 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was found effective to reduced bacterial blight disease intensity with 18.13% and which was found statistically at par with T_{10} of Streptocycline @ 250 ppm with per cent disease intensity of 18.93, followed by T_9 (Captan 50 % WP @ 0.24 %) (21.60%).

Table 7. ***In vivo*** **evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against *X. axonopodis* pv. *punicae* (2021-22)**

Tr. No.	Treatment details	PDI before spray (%)	Mean PDI 15 days after spray (%)				PDC (%)
			I	II	III	IV	
T_1	CuNPs @ 25 ppm	4.26 (11.91)	31.73^{f} (34.28)	39.20^{ef} (38.76)	42.80^{e} (40.86)	42.80^{e} (40.86)	9.81
T_2	CuNPs @ 50 ppm	4.80 (12.65)	30.93^{f} (33.78)	36.80^{de} (37.34)	41.60^{e} (40.16)	41.60^{e} (40.16)	12.34
T_3	CuNPs @ 75 ppm	3.73 (11.13)	26.93^{e} (31.26)	32.00^{d} (34.44)	36.00^{d} (36.86)	36.00^{d} (36.86)	24.14
T_4	CuNPs @ 100 ppm	5.60 (13.68)	18.93^{c} (25.79)	23.20^{b} (28.79)	25.60^{c} (30.39)	24.60^{c} (29.73)	46.05
T_5	AgNPs @ 25 ppm	4.00 (11.53)	32.00^{f} (34.44)	39.86^{ef} (39.14)	44.13^{e} (41.62)	44.13^{f} (41.62)	7.01
T_6	AgNPs @ 50 ppm	5.60 (13.68)	31.60^{f} (34.20)	37.46^{de} (37.73)	42.26^{e} (40.54)	42.26^{e} (40.54)	10.95
T_7	AgNPs @ 75 ppm	4.53 (12.28)	27.46^{e} (31.60)	34.40^{d} (35.91)	38.13^{d} (38.13)	38.13^{d} (38.13)	19.65
T_8	AgNPs @ 100 ppm	4.26 (11.91)	21.86^{d} (27.87)	23.73^{bc} (29.15)	28.00^{c} (31.94)	25.83^{c} (30.54)	41.17
T_9	Captan 50 % WP @ 0.24 %	4.53 (12.28)	15.20^{b} (22.94)	19.46^{b} (26.17)	21.60^{ab} (27.69)	18.80^{b} (25.69)	54.48
T_{10}	Streptocycline 250 ppm	4.00 (11.53)	12.26^{a} (20.49)	15.20^{a} (22.94)	18.93^{a} (25.79)	17.66^{a} (24.84)	60.11
T_{11}	2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm	5.06 (12.99)	9.60^{a} (18.04)	12.80^{a} (20.96)	18.13^{a} (25.20)	16.50^{a} (23.96)	61.79
T_{12}	Control	5.86 (14.00)	32.80 (34.93)	41.33 (40.00)	47.46 (43.54)	50.13 (45.07)	00.00
	SE±	0.8757	1.3608	1.9152	1.5232	1.0667	-
	CD at 5 %	NS	2.8086	3.9529	3.1436	2.2015	-

*Figures in parentheses are arc sine transformed values

*Per cent disease control (PDC) were calculated based on the per cent disease intensity (PDI) were recorded after third spray, because highest per cent disease intensity (PDI) were recorded after third spray

Among the eco-friendly biomolecule's treatments T_4 (CuNPs @ 100 ppm) was found effective with minimum 25.60 per cent disease intensity and which was found to be statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 28.00. The next best treatment is T_3 (CuNPs @ 75 ppm) (36.00 %) followed by T_7 (AgNPs @ 75 ppm) (38.13 %), T_2 (CuNPs @ 50 ppm) (41.60 %), T_6 (AgNPs @ 50 ppm) (42.26 %), T_1 (CuNPs @ 25 ppm) (42.80 %) and T_5 (AgNPs @ 25 ppm)

(44.13 %) are found to be least effective. The maximum per cent disease intensity was recorded in T_{12} untreated control (47.46 %).

In last spray i.e. after fourth spray, range of per cent disease intensity on leaves in different treatment was found varied from 16.50 to 44.13 per cent as compared to control 50.13 per cent. Least per cent disease intensity observed in plant sprayed with T_{11} of 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm and T_{10} (Streptocycline @ 250 ppm) were found effective and which were statistically at par with each other with minimum 16.50 and 17.66 per cent disease intensity, followed by T_9 (Captan 50 % WP @ 0.24 %) (18.80 %). However, it was observed that after fourth spray the per cent disease intensity of effective chemicals i.e., T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm), T_{10} (Streptocycline @ 250 ppm) and T_9 (Captan 50 % WP @ 0.24 %) were decreases as compared to per cent disease intensity were noticed after third spray.

Among the green nanoparticles treatments T_4 i.e., CuNPs @ 100 ppm was found to be effective to reduced bacterial blight disease intensity with 24.60 per cent which was found statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 25.83. The next best treatment is T_3 (CuNPs @ 75 ppm) (36.00 %) followed by T_7 (AgNPs @ 75 ppm) (38.13 %), T_2 (CuNPs @ 50 ppm) (41.60 %), T_6 (AgNPs @ 50 ppm) (42.26 %), T_1 (CuNPs @ 25 ppm) (42.80 %) and T_5 (AgNPs @ 25 ppm) (44.13 %). The maximum per cent disease intensity was recorded in T_{12} untreated control (50.13 %).

4.10.1.3 Per cent disease intensity (PDI) 2020-21 and 2021-22 Pooled results

The pooled data of both 2020-21 and 2021-22 years interpreted in Table 8. Pooled data of per cent disease intensity were calculated by using per cent disease intensity recorded after third spray. The maximum per cent disease intensity were recorded after third spray. It is revealed that during both years the treatment of bactericides i.e. T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm), T_{10} (Streptocycline @ 250 ppm) and T_9 (Captan 50% WP @ 0.24 %) were found effective to reduce per cent disease intensity of bacterial blight and which were statistically at par with each other with minimum 16.79, 17.66 and 20.00 per cent disease intensity.

Table 8. *In vivo* evaluation ecofriendly antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against *X. axonopodis* pv. *punicae* (2020-21 and 2021-22) (Pooled data)

Tr. No.	Treatment details	2021		2022		Mean	
		Final PDI (%)	PDC (%)	Final PDI (%)	PDC (%)	PDI (%)	PDC (%)
T_1	CuNPs @ 25 ppm	40.53^{ef} (39.54)	8.42	42.80^{e} (40.86)	9.81	41.66 (40.19)	9.15
T_2	CuNPs @ 50 ppm	38.13^{de} (38.13)	13.84	41.60^{e} (40.16)	12.34	36.93 (36.62)	13.09
T_3	CuNPs @ 75 ppm	33.06^{d} (35.09)	25.30	36.00^{d} (36.86)	24.14	34.53 (35.98)	24.70
T_4	CuNPs @ 100 ppm	22.13^{ab} (28.06)	50.00	25.60^{c} (30.39)	46.05	23.86 (29.23)	47.97
T_5	AgNPs @ 25 ppm	41.03^{ef} (39.83)	7.29	44.13^{e} (41.62)	7.01	42.58 (40.73)	7.15
T_6	AgNPs @ 50 ppm	38.86^{de} (38.56)	12.20	42.26^{e} (40.54)	10.95	40.56 (39.55)	11.55
T_7	AgNPs @ 75 ppm	35.73^{d} (36.70)	19.27	38.13^{d} (38.13)	19.65	35.59 (37.42)	19.47
T_8	AgNPs @ 100 ppm	25.33^{bc} (30.21)	42.76	28.00^{c} (31.94)	41.17	26.66 (31.08)	41.86
T_9	Captan 50 % WP @ 0.24 %	18.40^{a} (25.40)	58.42	21.60^{ab} (27.69)	54.48	20.00 (26.56)	56.38
T_{10}	Streptocycline 250 ppm	16.40^{a} (23.88)	62.94	18.93^{a} (25.79)	60.11	17.66 (24.84)	61.49
T_{11}	2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm	15.46^{a} (23.15)	65.07	18.13^{a} (25.20)	61.79	16.79 (24.18)	63.38
T_{12}	Control	44.26 (41.70)	00.00	47.46 (43.54)	00.00	45.86 (42.62)	00.00
	SE±	2.3240		1.5232		1.9836	
	CD at 5 %	4.7965		3.1436		3.8560	

*Figures in parentheses are arc sine transformed value

*Per cent disease intensity (PDI) after third spray considered as final per cent disease intensity (PDI) in both (2020-21 and 2021-22) year, because highest per cent disease intensity (PDI) were recorded after third spray

Among the green nanoparticle's treatments T_4 i.e. CuNPs @ 100 ppm was found to be effective to reduced bacterial blight disease intensity with 23.86% which was found statistically at par with T_8 (AgNPs @ 100 ppm) with per cent disease intensity 26.66. The next best treatment is T_3 (CuNPs @ 75 ppm) (34.53 %) followed by T_7 (AgNPs @ 75 ppm) (35.59 %), T_2 (CuNPs @ 50 ppm) (36.93 %), T_6 (AgNPs

@ 50 ppm) (40.56 %), T_1 (CuNPs @ 25 ppm) (41.66 %) and T_5 (AgNPs @ 25 ppm) (42.58 %). The maximum per cent disease intensity was recorded in T_{12} untreated control (45.86 %).

Per cent disease intensity of bacterial blight disease of pomegranate caused by *X. axonopodis* pv. *punicae* was revealed that the trends of effectiveness of all chemicals and innovative biomolecules were found to be same up to the third spray in the pot study of both the year (2020-21 and 2021-22) and after fourth spray were found some different results, whereas per cent disease intensity in untreated control was increased (Table 6 and 7 and graphically presented in Figure 4 and 5). After fourth spray during the pot study of 2020-21 year shown in photograph of Plate 13, it were observed that the treatments i.e., T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm), T_{10} (Streptocycline @ 250 ppm), T_9 (Captan 50% WP @ 0.24%), T_4 (CuNPs @ 100 ppm) and T_8 (AgNPs @ 100 ppm) were noticed best with minimum per cent disease intensity of 13.76, 14.80, 16.96, 21.06 and 22.40 respectively it was less than the per cent disease intensity were noticed after third spray. The treatments i.e. T_3 (CuNPs @ 75 ppm), T_7 (AgNPs @ 75 ppm), T_2 (CuNPs @ 50 ppm), T_6 (AgNPs @ 50 ppm), T_1 (CuNPs @ 25 ppm) and T_5 (AgNPs @ 25 ppm) were found with same pattern of per cent disease intensity after third and fourth spray i.e., 33.06, 35.73, 38.13, 38.86, 40.53 and 41.03 respectively.

During the pot study of 2021-22 year (Plate 13), it were observed that the treatments i.e., T_{11} of 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm, T_{10} (Streptocycline @ 250 ppm), T_9 (Captan 50 % WP @ 0.24 %), T_4 (CuNPs @ 100 ppm) and T_8 (AgNPs @ 100 ppm) were noticed best with minimum per cent disease intensity of 16.50, 17.66, 18.80, 24.60 and 25.83 respectively it was less than the per cent disease intensity were noticed after third spray, Whereas rest of the treatments i.e., T_3 (CuNPs @ 75 ppm), T_7 (AgNPs @ 75 ppm), T_2 (CuNPs @ 50 ppm), T_6 (AgNPs @ 50 ppm), T_1 (CuNPs @ 25 ppm) and T_5 (AgNPs @ 25 ppm) were found with same per cent disease intensity after third and fourth spray i.e., 36.00, 38.13,41.60, 42.26, 42.80 and 44.13 respectively, From the above results it was observed that in both year pot study, after fourth spray of chemicals and eco-friendly biomolecules per cent disease intensity were decreases and cease, therefore it was

proved that there is no need of next spray i.e., fifth spray for the management of *X. axonopodis* pv. *punicae,* causing bacterial blight disease of pomegranate.

The results of above study revealed that the chemical treatments were found to be effective than the eco-friendly nanoparticles for the management of bacterial blight disease of pomegranate caused by *X. axonopodis* pv. *punicae.* After fourth spray the per cent disease intensity of effective chemical treatment i.e., T_{11} of 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm in the year 2020-21 and 2021-22 pot study was 13.76 and 16.50, respectively. Thus, average per cent disease intensity from both year pot study was 15.13, whereas after fourth spray per cent disease intensity in effective eco-friendly nanoparticle treatment i.e., T_4 of CuNPs @ 100 ppm in the year 2020-21 and 2021-22 pot study was 21.06 and 24.60, respectively. Thus, the mean average per cent disease intensity from both year pot studies was 22.83. The difference between average per cent disease intensity of effective chemical treatment i.e., T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm) and effective eco-friendly nanoparticle treatment i.e., T_4 (CuNPs @ 100 ppm) in the year 2020-21 and 2021-22 pot study is 7.70, these difference between effectiveness of chemical treatment and eco-friendly nanoparticle treatment is not much more. Hence, it is proved that the use of eco-friendly nanoparticle best alternative to chemicals for the management of *X. axonopodis* pv. *punicae,* causing bacterial blight disease of pomegranate, because chemical has harmful effect on the environment, residual effect in fruit and hampering export- import business activity and such disadvantages haven't seen with eco-friendly nanoparticles.

4.10.2 Per cent Disease Control (PDC)

During the both (2020-21 and 2021-22) years study of *in vivo* evaluation of antibacterial potential of biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles under pot culture condition against *X. axonopodis* pv. *punicae,* per cent disease control was calibrated after third spray, because highest per cent disease intensity was recorded after third spray and it was found to be decreased after last spray i.e., fourth spray.

4.10.2.1 Per cent Disease Control (PDC) 2020-21

During the *in vivo* study of year 2020-21 (Table 6, Plate 13A and Figure 6), it was observed that highest per cent disease control was recorded in treatment T_{11} i.e. of 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm (65.07 %). The next superior treatment T_{10} i.e. Streptocycline @ 250 ppm was found with maximum per cent disease control of 62.94 per cent, followed by T_9 (Captan 50 % WP @ 0.24 %) (58.42 %). Among the ecofriendly innovative biomolecules treatments T_4 of CuNPs @ 100 ppm was found best and recorded maximum per cent disease control of 50.00 per cent, followed by T_8 (AgNPs @ 100 ppm) (42.76 %), T_3 (CuNPs @ 75 ppm) (25.30 %), T_7 (AgNPs @ 75 ppm) (19.27 %), T_2 (CuNPs @ 50 ppm) (13.84 %), T_6 (AgNPs @ 50 ppm) (12.20 %), T_1 (CuNPs @ 25 ppm) (8.42 %) and T_5 (AgNPs @ 25 ppm) (7.29 %).

4.10.2.2 Per cent Disease Control (PDC) 2021-22

The results obtained during *in vivo* study of year 2021-22 (Table 7, Plate 13 B and Figure 6), it is revealed that among all treatments the per cent disease control was highest in T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm) (61.79 %). The next superior treatment was T_{10} i.e., Streptocycline @ 250 ppm was found with maximum per cent disease control of 60.11 per cent, followed by T_9 (Captan 50 % WP @ 0.24 %) (54.48 %). From the eco-friendly biomolecule's treatments i.e., T_4 (CuNPs @ 100 ppm) was recorded with maximum per cent disease control of 46.05 per cent. followed by T_8 (AgNPs @ 100 ppm) (41.17 %), T_3 (CuNPs @ 75 ppm) (24.14 %), T_7 (AgNPs @ 75 ppm) (19.65 %), T_2 (CuNPs @ 50 ppm) (12.34 %), T_6 (AgNPs @ 50 ppm) (10.95 %), T_1 (CuNPs @ 25 ppm) (9.81 %) and T_5 (AgNPs @ 25 ppm) (7.01 %).

4.10.2.3 Per cent Disease Control (PDC) 2020-21 and 2021-22 Pooled results

Pooled results of both (2021 and 2022) year *in vivo* study presented in Table 8. Its noticed that among all treatments, more per cent disease control was found in T_{11} (2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm) (63.38 %). The next superior treatment T_{10} (Streptocycline @ 250 ppm) (61.49 %), followed by T_9 (Captan 50 % WP @ 0.24 %) (56.38 %). Among the ecofriendly innovative biomolecule's treatments T_4 of CuNPs @ 100 ppm was noticed with maximum per cent disease

control of 47.97 per cent followed by T_8 (AgNPs @ 100 ppm) (41.86 %), T_3 (CuNPs @ 75 ppm) (24.70 %), T_7 (AgNPs @ 75 ppm) (19.47 %), T_2 (CuNPs @ 50 ppm) (13.07 %), T_6 (AgNPs @ 50 ppm) (11.55 %), T_1 (CuNPs @ 25 ppm) (9.15 %) and T_5 (AgNPs @ 25 ppm) (7.15 %).

The results of two-year data of *in vivo* study after four spray of different chemicals and innovative green nanoparticles revealed that the minimum per cent disease intensity and maximum per cent disease control were found with chemical treatments i.e., 2-Bromo-2-Nitro-1,3-Propanediol, Streptocycline and Captan 50 % WP, followed by eco-friendly innovative green nanoparticles i.e., copper (CuNPs) and silver (AgNPs) nanoparticles (Table 6, Table 7 and Figure 6). But from the Table 8 it was observed that among different concentrations of green nanoparticles i.e., 100, 75, 50 and 25 ppm were used in *in vivo* study. The maximum mean per cent disease control was noticed with 100 ppm concentration i.e., 47.97 and 41.86 for copper and silver nanoparticles respectively, whereas the rest of the concentrations i.e., 75, 50 and 25 ppm noticed with least mean per cent disease control i.e., 24.70, 13.09 and 9.15, respectively for copper nanoparticles and 19.47, 11.55 and 7.15, respectively for silver nanoparticles. Therefore, it is proved to use both nanoparticles (copper and silver) at 100 ppm concentration for effective results than the rest of concentrations i.e., 75, 50 and 25 ppm for the management of *X. axonopodis* pv. *punicae,* causing bacterial blight disease of pomegranate.

The references in respect of evaluation of nano formulation under pot culture condition for controlling bacterial blight disease in pomegranate is not traceable in literature. However, various chemicals *viz*. Copper oxy chloride (COC), Captan, Bromopal, Karate, Tafgor, Monocrotophos, Nuvan, Blue Copper etc. and antibiotics such as Streptocyclin, Kasu-B, Bactrasan, Bactrinashak etc. are commercially available for management of this disease. None of these, individually and in combination are fully effective in controlling total bacterial growth, but have been reported to reduces the disease severity of the disease and minimize the losses to some extent.

Chemical treatments showed complete control under *in vitro* conditions are Bordeaux mixture, captan, Copper oxychloride, Copper hydroxide, 2-Bromo-2-Nitro-

1, 3-Propanediol and streptocycline (Raghuwanshi *et al*., 2013), Streptocycline, copper oxychloride, K-cycline and Ampiclox (Lokesh *et al*., 2014), Tetracycline and Bacterinol (Ambadkar *et al*., 2014), for controlling bacterial blight disease in pomegranate.

Arora *et al*. (2016) reported Blitox (0.3 %) + Streptocycline (250 ppm) as significantly superior to other agro-chemicals in reducing percent disease index. Percent disease index on the plants sprayed by Blitox (0.3 %) + Streptocycline (250 ppm) was 5.10 and 6.50 on leaves and fruits, respectively, which was considerably lower as compared to control where it was 60.0 and 63.50 percent on leaves and fruits, respectively. Blitox + Streptocycline treatment provided maximum disease control (91.50 % on leaves and 89.76 % on fruits) as compared to other treatments. Kocide (0.25 %) + Streptocycline (250 ppm) was the next best treatment in minimizing per cent disease index which gave 89.73 and 88.72 per cent disease control on leaves and fruits, respectively. Similarly, various scientists have used different chemicals combination such chemicals as Streptocycline, copper oxychloride and Bromopal (Ravikumar *et al*., 2011).

5. SUMMARY AND CONCLUSION

Pomegranate (*Punica granatum* L.) is one of the ancient and highly priced favourite table fruits of tropical and sub-tropical regions cultivated all over the world. In India the area under pomegranate cultivation is increasing day by day due to best market prices and pharmaceutical uses. The favourable climatic conditions for pomegranate cultivation in Western Maharashtra zone resulted in increased area, production and productivity too. Among the all stresses, biotic stresses by bacterial blight disease is one of major threat in pomegranate cultivation particularly production and quality of fruits.

The present studies on "Evaluation of innovative nano biomolecules in management of bacterial blight disease of pomegranate" was undertaken during the year 2020-2022 at Department of Plant Pathology and Agricultural Microbiology, PGI, MPKV, Rahuri (M.S) India. Bacterial blight disease of pomegranate caused by *X. axonopodis* pv. *punicae* is one of the most destructive disease of pomegranate (*Punica granatum*) inflicting considerable quantitative and qualitative losses. The disease occurred mostly on leaves, stems and fruits. Considering the economic importance of the fruit crop and attack of bacterial disease, present investigation was undertaken. The studies included isolation, pathogenicity, cultural and morphological characterization of causal agent. The efforts were made for the biosynthesis of Copper ($CuSo_4$) and Silver ($AgNo_3$) nanoparticles and evaluation of their antibacterial potential under *in vitro* and *in vivo* conditions against bacterial blight disease of pomegranate. The results obtained from present investigation are summarized here under.

Different symptoms were observed and recorded during survey and samples collection. The recorded symptoms of the disease were minute, water soaked lesions which later turned brown surrounded by diffused water soaked zone or yellow halo on the both fruits and leaves. The necrotic lesions also increased later leading to L and Y shaped cracking within spots, later on severally affected fruits were found split open. The lesions also appeared on the branches and the twigs leading to die back and death of branches. Affected plant appeared unthrifty weak and died later.

The causal organism *X. axonopodis* pv. *punicae* of bacterial blight of pomegranate was isolated on the basal culture media i.e. Nutrient agar. Then the pure

bacterial colonies were transferred on the fresh nutrient agar plates by streak plate method.

The pathogenicity test was proved in insect proof glass house on healthy pomegranate seedling of cv. Super Bhagwa by pin-prick infiltration method. The test pathogen was inoculated by pin-prick infiltration method on leaves and stem of seedling. After inoculation of pathogen in seedlings, disease symptoms on leaves were developed within 10 to 18 days. First disease symptoms were appeared on lower sides of the leaves as irregular water-soaked spots. Later on, such spots appeared in raised from on the upper side of the leaves and they were noticed rough to touch with yellow haloes. Subsequently these spots became brown in colour and coalesced to form blighting appearance on the leaves. While the uninoculated control plants were found free from infection. The re-isolation attempted from artificially infected plant tissues on nutrient agar consistently yielded the growth of *X. axonopodis* pv. *punicae*, which fulfilled Koch's postulates and association of *X. axonopodis* pv. *punicae* as a causal agent for bacterial blight disease.

The biochemical characterization studies of *X. axonopodis* pv. *punicae* showed their positive reactions for potassium hydroxide (KOH) test, starch hydrolysis test and catalase test whereas, negative response for Gram staining test.

In cultural studies such as colour of colony, growth on the media, growth appearance, elevations, texture and consistency showed variations on different media *viz.* Nutrient Agar, Nutrient Sucrose Agar, Glucose Yeast Chalk Agar and Yeast Extract Dextrose Calcium Carbonate Agar.

Based on typical symptoms of bacterial blight of pomegranate observed on naturally infected and artificially diseased plants as well as cultural, morphological studies, biochemical characterization and pathogenicity test; the test pathogen under study was identified and confirmed as *X. axonopodis* pv. *punicae*, the cause of bacterial blight disease of pomegranate.

Biosynthesis of Copper and Silver nanoparticles was carried out from fresh leaves of *Ocimum sanctum* (Tulsi) and *Azadirachta indica* (Neem), respectively. UV-Visible spectrophotometer characterization revealed that both Copper and Silver nanoparticles showed absorption peak at 397.98 nm and 406.65 nm, respectively. Transmission Electron Microscopy (TEM) revealed that both Copper and Silver nanoparticles are

spherical in shape and their size ranges from 8.97 to 10.89 nm and 10.33 to 12.21 nm, respectively.

In vitro evaluation of antibacterial potential of bactericides and biosynthesized Copper (CuNPs) and Silver (AgNPs) nanoparticles were carried against test pathogen by using paper disc method. The results revealed that the treatment 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was found most effective for controlling *X. axonopodis* pv. *punicae* by forming 20.86 mm mean inhibition zone, followed by Streptocycline @ 250 ppm (18.44 mm), Captan 50 % WP @ 0.24 % (17.68 mm), CuNPs @ 100 ppm (16.58 mm) AgNPs @ 100 ppm (16.18 mm), CuNPs @ 75 ppm (15.43 mm), AgNPs @ 75 ppm (15.01 mm), CuNPs @ 50 ppm (12.36 mm), AgNPs @ 50 ppm (11.66 mm), CuNPs @ 25ppm (8.16 mm) and AgNPs @ 25 ppm (7.96 mm).

In vivo evaluation of antibacterial potential of bactericides and biosynthesized Copper and Silver nanoparticles were carried against *X. axonopodis* pv. *punicae,* during 2020-21 and 2021-22 in pot under glasshouse condition. Among the bactericides and biosynthesized Copper and Silver nanoparticles, the per cent disease control ranged between 7.29 to 65.07 per cent during 2020-21 and 7.01 to 61.79 per cent during 2021-2022. During the both years, the maximum per cent disease control was found in 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm up to 65.07 and 61.79 per cent, followed by Streptocycline @ 250 ppm i.e. 62.94 and 60.11 % respectively, Captan 50 % WP @ 0.24 % (58.42 and 54.48 %), CuNPs @ 100 ppm (50.00 and 46.05 %), AgNPs @ 100 ppm (42.76 and 41.17 %), CuNPs @ 75 ppm (25.30 and 24.14 %), AgNPs @ 75 ppm (19.27 and 19.65 %), CuNPs @ 50 ppm (13.84 and 12.34 %), AgNPs @ 50 ppm (12.20 and 10.95 %) whereas, the minimum per cent disease control was recorded in CuNPs @ 25ppm (8.42 and 9.81 %) followed by AgNPs @ 25 ppm (7.29 and 7.01 %) as compared to control treatment.

Conclusions

From the observations recorded during present investigation and results interpreted on various aspects of pomegranate bacterial blight disease, following conclusions are drawn:

1. *X. axonopodis* pv. *punicae* was found as a causal agent of bacterial blight disease of pomegranate.

2. Different types of symptoms like water soaked lesions, water soaked zone or yellow halo on the both fruits and leaves and L and Y shape cracking on fruits were observed in bacterial blight of pomegranate.
3. In biochemical characterization of *X. axonopodis* pv. *punicae* showed their positive reactions for potassium hydroxide (KOH) test, starch hydrolysis test and catalase test whereas, negative response for Gram staining test.
4. *X. axonopodis* pv. *punicae* showed variability in colour of colony, growth, growth appearance, elevations, texture and consistency on different media *viz.* Nutrient Agar, Nutrient Sucrose Agar, Glucose Yeast Chalk Agar and Yeast Extract Dextrose Calcium Carbonate Agar.
5. Copper and Silver nanoparticles biosynthesized from fresh leaves of *Ocimum sanctum* (Tulsi) and *Azadirachta indica* (Neem) showed absorption peak at 397.98 nm and 406.65 nm, respectively under UV-Visible spectrophotometer reading whereas, Transmission Electron Microscopy (TEM) showed that both Copper and Silver nanoparticles are spherical in shape with size range of 8.97 nm to 10.89 and 10.33 nm to 12.21 nm, respectively.
6. *In vitro* evaluation of antibacterial potential of bactericides and nanoparticles carried against *X. axonopodis* pv. *punicae,* by using paper disc method and recorded that the treatment of bactericide 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was significantly superior than all the treatments showed maximum inhibition zone i.e. 20.86 mm. The next best treatment was Streptocycline @ 250 ppm, followed by Captan 50% WP @ 0.24 %, CuNPs @ 100 ppm and AgNPs @ 100 ppm and other treatment except untreated control.
7. *In vivo* evaluation of antibacterial potential of bactericides and eco-friendly nanoparticles against *X. axonopodis* pv. *punicae,* carried during 2020-21 and 2021-22 in pot culture under glasshouse condition. Proved that the treatment of bactericide 2-Bromo-2-Nitro-1,3-Propanediol @ 250 ppm was found most effective than all the treatments during both years with maximum per cent disease control i.e. 65.07 and 61.79 % respectively, followed by Streptocycline @ 250 ppm, Captan 50% WP @ 0.24 %, CuNPs @ 100 ppm, AgNPs @ 100 ppm and other treatment except untreated control.

8. In both *in vitro* and *in vivo* studies all bactericides along with Copper and Silver nanoparticles were found effective with maximum per cent disease control and minimum per cent disease intensity against bacterial blight disease of pomegranate. From the pooled data it was revealed that treatment of chemical bactericide 2-Bromo-2-Nitro-1, 3-Propanediol @ 250 ppm was found most effective with maximum per cent disease control by managing the disease up to 63.38 per cent. As per the objectives of present studies focusing the evaluation and comparison of chemicals and eco-friendly management practices, it is revealed and proved that next to chemical bactericidal treatment the treatment of eco-friendly nanoparticles i.e. CuNPs @ 100 ppm and AgNPs @ 100 ppm was found best treatments with maximum per cent disease control of 47.97 and 41.86 per cent, respectively as an alternative to chemical in eco-friendly management of bacterial blight disease of pomegranate and production of quality and residue free pomegranate fruit yield.

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7. APPENDIX

Composition of media

I. Nutrient Agar medium (NA) : Isolation and cultural characteristics

Peptone	:	5.00 gm
Beef extract	:	3.00 gm
$NaCl_2$	:	5.00 gm
Agar	:	10.00 gm
Distilled water	:	1000 ml

II. Nutrient Sucrose Agar medium (NSA) : Cultural characteristics

Peptone	:	10.00 gm
Sucrose	:	20.00 gm
Beef extract	:	5.00 gm
Agar	:	15.00 gm
Distilled water	:	1000 ml

III. Yeast Extract Dextrose Calcium Carbonate agar (YDCA) : Cultural characteristics

Yeast extract	:	5.00 gm
Dextrose	:	20.00 gm
Calcium carbonate	:	20.00 gm
Agar	:	15.00 gm
Distilled water	:	1000 ml

IV. Glucose Yeast Chalk Agar (GYCA) : Cultural characteristics

Yeast extract	:	5.00 gm
Glucose	:	5.00 gm
Calcium carbonate	:	40.00 gm
Agar	:	15.00 gm
Distilled water	:	1000 ml

8. VITAE

MR. ROTHE ANAND SHIVAJI

DOCTOR OF PHILOSOPHY (AGRICULTURE)

IN

PLANT PATHOLOGY

2022

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