STUDIES ON CROWN GALL OF STONE FRUITS

Thesis

by

SANDEEP KUMAR

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Affectionately
dedicated
to my
reverend parents
and
teachers

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CERTIFICATE - I

This is to certify that the thesis entitled **"Studies on crown gall of stone fruits"**, submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE in MYCOLOGY AND PLANT PATHOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.) is a bonafide research work carried out by **Mr. Sandeep Kumar (H-2001-40-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations have been fully acknowledged.

Place: Nauni, Solan
Dated: December 31, 2003

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CERTIFICATE – II

This is to certify that the thesis entitled **"Studies on crown gall of stone fruits"**, submitted by **Mr. Sandeep Kumar (H-2001-40-M)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **MYCOLOGY AND PLANT PATHOLOGY** has been approved by the student's Advisory Committee after an oral examination of the same in collaboration with the External Examiner.

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Sandeep

Place: Nauni (Solan) **(Sandeep Kumar)**

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INTRODUCTION

Stone fruits are widely distributed throughout the temperate zones of world. These include peaches, nectarines, apricots, almonds, plums and cherries occupying an area of 6,9,31, 772 hectares of the world with the total annual production of 2,94,62,777 MT (Anonymous, 2002). The peach (*Prunus persica* (L.) Batsch.) originated in China (near Sian) where it was grown as far back as 2000 BC is found commercially around the world between 24° and 45° latitude above and below the equator. The peach cultivation is now extended close to equator because most cultivars are more tolerant to heat and require less cold to break the dormancy in the rest period. Apricot (*Prunus armeniaca* L.) was grown in Virginia as early as 1720 AD. The commercial culture of this fruit is limited largely to semi-arid irrigated districts of Pacific scope. Almonds (*P. amygdalus* Batch.) probably came from Western Asia, whereas cherries were cultivated in Greece in prehistoric times and now widely grown around the world. The cultivated cherries are divided into two main groups, i.e. sweet cherries (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.). Besides peach, apricot, almond and cherry, plum is also an important stone fruit. The cultivated plums belong to two species viz., *Prunus domestica* L. (European plum) and *P. saliciana* Lindl (Japanese plum) and are widely grown in low hills and sub mountainous tracts. Major peach, apricot, plum and almond producing

countries are USA, Italy, China, Romania and Spain, whereas major producers of cherries are erstwhile USSR followed by USA.

In India, the stone fruit cultivation is however, confined at present to Jammu and Kashmir, Punjab, Himachal Pradesh and Uttaranchal and parts of Uttar Pradesh. They are also grown to a small extent at suitable elevations in South India in the Niligiris. As per FAO estimates total area in India under stone fruits is 36,000 hectares with total annual production of 2,48,000 MT which exclude almond, whereas, almond cultivation is restricted to Jammu and Kashmir, areas of Himachal Pradesh bordering China and some parts of Punjab. In Jammu and Kashmir and Himachal Pradesh, the area under almond cultivation is 28,225 hectare with 14,600 MT production (Ananda, 2002). Asian continent is the largest producer of stone fruits with an area of 36,25,030 hectares and a total annual production of 1,37,93,293 MT followed by Europe with 95,17,012 MT from 20,95,225 hectares of cultivated area (Anonymous, 2002).

In Himachal Pradesh alone, 31,088 hectares area is under stone fruit cultivation with a total production of 25, 116 MT (Anonymous, 1997). Hence these stone fruits contribute a large to the economy of orchardists and as a result the fruit growers are becoming increasingly interested in them.

Because of increasing cultivation the incidence due to diseases has increased considerably. The major diseases affecting stone fruits plants are crown gall, rusts, peach leaf curl, brown rot, shot hole, frosty mildew, cankers, bacterial spot, bacterial canker

(Ved Ram and Gupta, 1999). Among various diseases, crown gall caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. (Syn.: *Rhizobium radiobacter* (Beijerinck and vanDelden) Young *et. al.*) causes huge economic losses in stone fruit nursery plants making seedlings unfit for further plantation in orchards by production of gall on the stem and roots of the plants just below the soil surface. Galls develop on roots, crowns and occasionally aerial parts of the stone fruit trees. Galls vary in size from microscopic to more than 10 cm in diameter. The galls reduce root development and plant vigor; galled plants in nurseries remain stunted and show chlorotic leaf growth. The plants with tumors are unsalable as their crowns or their main roots grow poorly and resulted in reduced yield and ultimate death of plants. Crown gall has been reported from most countries of the world and is world wide in distribution, it affects many woody and herbaceous plants belonging to 140 genera of more than 60 families. Almost every peach nursery in Rajgarh and Sangrah block of district Sirmaur has been affected by crown gall. Diseased plants in the nurseries are culled and sold at a reduced price, resulting in significant economic losses to nursery growers (Sharma *et al.*, 2001).

The pathogen *A. tumefaciens* incorporate its Tumour Inducing Principle (TIP) in the host genome and modify host cells. Incorporation of bacterial genetic material (tumour inducing plasmid) in the host genome makes the bacterium a genetic parasite, which hampers the prospects to develop resistant cultivars against this disease. No effective chemical management strategies are available except the dip treatment of healthy nursery plants in one per cent copper sulphate solution as preventive measures before planting in orchards.

Although effective control of crown gall has been achieved by the use of *A. radiobacter* strain K84 (New and Kerr, 1972) but it is still not yet available to growers in most of the countries including India because legal requirements for registration have either not been explored or not been granted. Moreover, breakdown in biological control activity of strain K84 and use of genetically engineered strain K1026 has further implicated the prospects of using these strains.

Keeping in view of the limited success in chemical management, non-availability of other effective management strategies, resistant root stocks/clones/cultivars against crown gall disease and constraints in use of *A. radiobacter* strain K84 and K1026, there is a need to find other suitable antagonist(s) for its further use under field conditions.

Keeping in view of the recurrent losses in stone fruit nurseries due to crown gall, the present investigations were carried out with the following objectives:

i) To record the prevalence and incidence of crown gall in stone fruit nurseries of Himachal Pradesh and

ii) To find out suitable biological agent(s) for the management of crown gall disease in peach.

REVIEW OF LITERATURE

Chapter-II

REVIEW OF LITERATURE

The year 1907 will be remembered for centuries ahead since a landmark finding was reported in the field of bioscience – the association of a bacterium with Trade Mark plant disease 'Crown gall'. Smith and Townsend quoted it as a 'Plant tumour of bacterial origin', the one and only tumour of its kind ever reported to be caused by bacteria.

Perhaps the pioneering workers had never anticipated that their *Bacterium tumefaciens* now known as *Agrobacterium tumefaciens* (Smith and Townsend) Conn. (Syn.: *Rhizobium radiobacter* (Beijerinck and vanDelden) Young *et al.*) would one day revolutionize the world of science with its unique mode of pathogenesis – genetic transformation.

Could we ever imagine reproduction between two unrelated genera or even species? That we mean genome transfer. No, not at all. Thanks to *A. tumefaciens* which taught us that genetic transformation can take place not only within two species or genera but even within prokaryotes and eukaryotes, the basic line of demarcation in the living world, which *Agrobacterium* learned perhaps million of years ago.

Now, scientists across the globe are working on this great principle, introducing genetic material from diverse living groups into the plants and animals and getting the desired results. Now, we can have a dog of green colour, a banana with antibiotics,

golden rice-rich in vitamin A, genetically modified cotton, potato, corn and what not. These transgenics have provided a hope to we people that we will be able to feed the burgeoning human population on this earth, which will cross a 10 billion mark by AD 2100.

Though, man has made outstanding advances in food generation still competition from different pest remains a great challenge, but not to *Agrobacterium*. We know that opines (the sugar – amino acid conjugates) induced by particular strain of *Agrobacterium* can only be utilized by the same strain. This is surely the biggest example of food security in this world.

Also, none of the biocontrol agents screened against a particular plant pathogen proved its efficacy in different part of the world, but this *Agrobacterium* has undoubtedly shown promising results all over the world.

All these things make *Agrobacterium* a wonderful organism.

2.1 Historical background

In 1907, Erwin Smith and Charles Townsend reported a unique plant disease which has probably piqued the imaginations and spurred the research efforts of more plant scientists than any other plant disease. This disease, crown gall, is one of the several types of tumors, which occur on plants. It is unique in that (i) the causative organism is a gram-negative bacterium, *Agrobacterium tumefaciens* (Smith and Townsend) Conn. and (ii) the disease occurs on an extremely broad range of host plants. The second notable observations were the discoveries of Jensen (1910, 1918) and White and Braun (1941) that, once formed, tumour tissue persists in the absence of bacteria and can be maintained

indefinitely in axenic culture without loss of tumourous characteristics. This observation dramatically highlighted the parallelism between crown gall tumours and many animal neoplasms.

Another recent milestone in the elucidation of the mechanism of crown gall induction was the report of Zaenen *et al.* (1974) of the presence of a large plasmid (Ti plasmid) in all tumorigenic strains of *Agrobacterium* examined. It was subsequently shown that genes controlling virulence of the bacterium (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975) and the catabolism of octopine (Chilton *et al.*, 1976) or nopaline (Watson *et al.*, 1975), as well as induction of synthesis of these compounds in tumours (Montoya *et al.*, 1977), are all plasmid-coded traits. Taken together, these observations support the notion that bacterial plasmid DNA is transferred to and subsequently expressed in the host cell.

Finally, we must consider the physical confirmation of the presence (Chilton *et al.*, 1977) and transcription (Drummond *et al.*, 1977) of plasmid DNA sequences in the nucleic acids extracted from cloned tobacco tumour tissues that had been maintained axenically for several years. Indeed, these reports are especially important for several reasons: (i) they conclusively demonstrate what had previously been conjecture based on circumstantial evidence, namely, that *Agrobacterium* DNA is transferred to and expressed in the transformed plant cell, (ii) they demonstrate that no natural barrier exists that prevents the exchange of genetic information between prokaryotes and eukaryotes, and (iii) they suggest that there may be an underlying mechanism common to biologically induced tumours, since in the case of both crown gall and animal tumour viruses

analogous events (insertion of foreign DNA into the host cell) are associated with malignant transformations.

Since the induction process and resultant neoplasms of crown gall have been the most thoroughly studied of the plant tumours, it behooves us to examine the various stages in crown gall tumour induction, and to speculate on mechanisms which may result in the loss of control over morphology and division of a healthy plant cell. It will divide the process of tumour induction into several steps, and treat each step in lesser or greater details as a current data and available models allow. The following steps of tumour induction will be considered: (i) wounding of host plant tissue followed by specific binding of bacterial cells of plant cells, (ii) transfer of bacterial DNA to the host cell, and its subsequent processing, and (iii) uncontrolled growth of the transformed plant cell.

2.2 Economic importance of crown gall disease

Crown gall is economically very important disease of pome and stone fruits around the world wherever these crops are cultivated. In terms of its host range, *Agrobacterium* species is extremely broad in contrast to other plant bacterial pathogens. It affects many woody and herbaceous plants belonging to 140 genera of more than 60 families (Agriose, 1997). However, the host range of *Agrobacterium* species is much more restricted in nature since the trade mark crown gall disease is limited to grapevine, stone and pome fruit trees, nut trees and a few ornamental (eg. rose) on which most economical damage is done (Kerr, 1992).

Crown gall disease has cosmopolitan occurrence. Numerous reports of its occurrence have come up from across the world on almost all economically important temperate fruits, nuts and ornamentals causing huge losses to the fruit growers. It is the most serious disease from Albania (Isufi and Myrta, 1996), Hungary (Sule, 1994), Oregon (Moore, 1976), Italy (Bazzi and Mazzucchi, 1978), South –West Ontario, Canada (Dhanvantri, 1976), South Africa (Matthee *et al.*, 1977), and Australia (Htay and Kerr, 1974).

The disease was observed in early 1940's by Singh (Jindal *et al.*, 1990) on pome fruits in Kumaon Hills of Uttaranchal, Punjab and Kashmir. Durgapal (1971) was the first to isolate and prove the pathogenicity of *A. tumefaciens* from cherry, peach, pear and plum plants from Shimla Hills. Jindal and Sharma (1988) observed the occurrence of biovar 1 of *A. tumefaciens* infecting almond in Himachal Pradesh.

2.3 Crown gall: symptomatology

The formation of tumours or galls of varying size and form characterize crown gall. The disease first appears as small overgrowths or excrescencies on the stem and roots particularly near the soil line (Hedgcock, 1910). It is common on roots and shoots of various nursery plants, which are thus unsalable because the crown gall is likely to continue on plants when they are planted in orchards and gardens. In early stages of gall development the galls are more or less spherical, white and soft (Cook, 1923). Plants with tumours at their crowns or on their main roots grow poorly and provide reduced yield. Severely infected plants and vines may eventually die (Burr and Reid, 1995).

Since galls originate in a wound, at first they cannot be distinguished from callus, however, development of these galls is more rapid than callus. As the tumours enlarge, their surfaces become more or less convoluted. Later on the tissues become dark brown or black, due to death and decay of the peripheral cells. Sometimes there is no line of demarcation between the tumours and the plant proper, the tumours appearing as an irregular swelling of tissues and surrounding stem or root. Almost as often, however, the tumours lie outside but close to the outer surface of the host being connected only by a narrow neck of tissues (Agriose, 1997).

Several galls may occur at the same root, or stem continuous or in branches, along the same or different lines parallel to length of plant growth (Riker, 1923). The galls are usually ¼ to 4 inch in diameter, spherical to irregular with hard rough surface, generally present on the stem more often at the graft union of the fruit tree and also on root or area just below soil line (Jindal *et al.,* 1990). However, the gall size may reach upto 30 cm in diameter and can appear as big as 150 cm in diameter on the stems, branches, petioles and leaf (Riker and Keitt, 1926).

2.4 Isolation, characterization and morphology

Earlier methods directed towards isolation of *A. tumefaciens* involved the use of simple media. Patel's medium (1926) contained dextrose, agar; sodium taurocholate- a substance found inhibitory to other contaminants that time. Carrot slices also remained a preferred base for *A. tumefaciens* isolations (Ark and Schroth, 1958). But these media had certain limitations. Many soil-harbouring fungi, which grew rapidly on Patel's medium, obscured and hindered the development of *Agrobacterium* colonies. The limitation of the

carrot technique, on the other hand was that it did not quantitatively reflect the populations of *A. tumefaciens* in the soil. A major breakthrough was achieved when a medium was sought by Schroth *et al.* (1969), which effectively limited the growth of many fungi and bacteria without reducing the growth of *A. tumefaciens*. This medium contains mannitol and sodium nitrate ($NaNO_3$) as the basic carbon and nitrogen source, respectively, as *A. tumefaciens* readily utilizes both these substances whereas many other microbes do not. Also the medium contains many toxic substances such as sodium selenite, calcium propionate and an alkaloid berbarine, which hindered the other microbes without adversely affecting Agrobacteria.

Later half of 20^{th} century saw a sea change in the isolation media for *Agrobacterium* sp. A number of growth media were devised and tested viz., Clarks' medium, Kado and Heskett medium and New and Kerr medium. However, the ecological distribution of different biotypes has been studied by Schroth *et al.* (1971) who detected the pathogenic form of biotype 1 in 18 out of total 28 Californian soil isolates. The biotype 2 pathogen is known to occur in Greece, Israel, New South-Whales, New Zealand (Kerr, 1971) and USA (Kerr and Panagopoulos, 1977) as well as South Australia where it is much more prevalent than biotype 1 pathogen (New and Kerr, 1971). Panagopoulos *et al.* (1978) devised the key for differentiation of different biovars of *A. tumefaciens*. Zhang *et al.* (1991) used Kado and Heskett 's selective medium for isolating *A. tumefaciens* from tobacco galls and characterized the isolates on the basis of morphological characteristics. They also reported that 34 strains induced hyper-virulent reactions on carrot disc and were identified as biotype 1 and 2 that formed large galls as compared to rest isolates studied. In India, Jindal and Sharma (1988) reported occurrence

of biovar 1 of *A. tumefaciens* on all the commercial cvs. of almond viz., Non –Peril, Ribba selection and Thin shelled. Al-Karablieh and Khlaif (2002) isolated two hundred isolates of *A. tumefaciens* from stone fruit rootstocks in various parts of Jordan and found that 67 per cent belonged to biovar 1.

2.4.1 Morphology and cultural characteristics

The crown gall pathogen is a rod shaped bacterium about 1-3 µm long by 0.4-0.8µm in diameter. It occurs singly or in short chains and has 2-4 flagella situated peritrichously. The bacterium also forms capsules. On certain media *A. tumefaciens* forms star shaped arrangements which have been considered as stages in sexual processes of these bacteria by which the recombinations and segregation of nuclear material takes place (Agriose, 1997).

Jindal and Sharma (1988) while studying the cultural characteristics of *A. tumefaciens* on nutrient agar observed the bacterium forming characteristically small, circular, and smooth, glistening opaque to translucent convex white colonies with smooth margins.

2.5 Pathogenicity

Smith and Townsend made the first confirmation of association of *A. tumefaciens* with crown gall disease in 1907. Since then, a number of dicotyledonous plants have been shown to respond positively to *A. tumefaciens* gall development upon artificial inoculation. Tomato seedlings (*Lycopersicon esculentum* Mill.) remained the most popular material among several workers for proving pathogenicity (Kerr, 1969; New and

Kerr, 1972; Cooksey and Moore, 1980). However, apart from tomato, sunflower (*Helianthus annuus*), *Kalanchoe diagremontiana* and grape (*Vitis vinifera* L.) have been extensively used for pathogenicity tests upon artificial inoculation (Burr and Reid, 1995). Moore (1976) used radish plants for pathogenicity tests.

2.6 Effect of disease on plant growth

Few attempts have been made to study the effect of disease on the plant growth as well as its extent. There is no conclusive evidence that the disease significantly impairs subsequent performance of rootstocks. A few workers have attempted to study the effect of crown gall disease on the growth of susceptible temperate fruit trees. Apple trees with tumours planted in rich soils grew normally and did not differ significantly from normal (Popova, 1972). Garrett *et al.* (1984) concluded that crown gall infection had only a slight effect on the girth of trees of cherry cv Napoleon during a 6 year experiment. Garrett (1987) observed no clear, constant difference between healthy and galled trees when over 1000 cherry trees, as rootstocks, nursery sweet cherry or ornamental cultivars of orchard trees were studied upto 7 years. However, Jindal *et al.* (1990) reported that crown gall infected temperate fruit trees eventually died in advanced stages. Strobel and Nachmias (1985) while working on almond hairy root found that almond trees treated with *A. rhizogenes* 232 had a larger root numbers and root mass after 90 days than those treated with autoclaved or filtered sterilized bacterial suspension. This also led to significant increase in leaf number, stem diameter and shoot elongation during the first growing season. Hence, they concluded that reaction of almond to *A. rhizogenes* is not pathological one even though this bacterium is classified as plant pathogen.

2.7 Disease management

Crown gall management in the economically important fruit plants still remains a great challenge despite of the fact that many attempts have been made by several workers around the globe.

Numerous reports of efficacy of several antibacterial compounds (Klemmer *et al.*, 1955; Deep, 1958b; Deep and Young, 1965), fumigants (Kapshuk, 1933; Dickey, 1962; Deep *et al.*, 1968) and biological control (Kerr, 1972) against crown gall have come up from several places. Strain K-84 of *A. radiobacter* gave promising results in reducing crown gall development in several countries (Kerr, 1972; Moore, 1979a). Yet occurrence of *A. tumefaciens* strains insensitive to bacteriocin 84 (Dhanvantari, 1978) opens up new vistas in bio-control research.

2.7.1 Soil amendments

Organic amendments are believed to play a major role in alteration of physical, chemical and biological conditions of soil. These amendments act both on host as well as pathogen and enrich the soil with the micro flora potentially competitive or antagonistic to pathogen by producing inhibitory substances during decomposition thereby reducing the incidence of several soil-borne plant diseases. In addition to controlling the soil-borne pathogens certain soil amendments improve the soil structure, which contribute to the growth of plants.

Various oil cakes have been used as soil amendments for controlling soil-borne plant pathogens. The oil cakes alter the soil environment thereby affecting the soil micro flora. Amendment of soil with oil cakes has resulted in providing stimulatory as well as inhibitory effect on micro flora (Singh and Pandey, 1967).

Huber and Watson (1970) have indicated frequent incorporation of fresh leaves, green manure, crop residue and other organic materials. These, they believe prevent the spread of pathogens to nearby fields and produce less favourable conditions to the pathogen in established cropland. Khanna and Singh (1975) observed that with the addition of oil cakes like margose, castor and linseed, there is stimulation in the number of bacteria and actinomycetes but saw dust was inhibitory to these microbes. Khalis and Manoharachary (1985) studied the quantitative and qualitative change in mycoflora in soil amended with oil cakes and observed increase in fungal population in soil amended with neem, groundnut and safflower oil cakes. An increase in the population of *Trichoderma viride, Penicillium* sp., *Aspergillus flavus, A. niger, Bacillus subtilis* and *Streptomyces* sp. after 15-45 days of application of groundnut and safflower cake has been reported by Palakshappa *et al.* (1989). A marked suppression of soil-borne disease have been observed by Nitta (1991) due to enhanced microbiostatis as a result of increased diversity of root micro- flora.

Supplementing soils with deodar needles has been reported to provide protection against white root rot of apple (Gupta and Jindal, 1990). Hence, organic amendments forms integral part of disease management strategies especially against soil-borne disease.

2.7.2 Nutritional aspects

The effect of various metabolites on growth of crown gall tissues in culture has been studied extensively, but relatively little had been done on the interaction among microbial nutrients supply to the host, growth of the host and development of crown gall on the host. Link *et al.* (1953) while studying nitrate-nitrogen nutrition, found that gall tissue developed almost as rapidly on – N (No nitrogen supplied) plants as on + N (Nitrogen supplied) plants although the – N plants were severely stunted. Rives (1932) and Verona and Colestri (1938) found that plants with low potassium developed larger galls, but Sempio (1935) observed stimulation of growth of galls by adding potassium, lead and zinc.

Although crown gall is primarily an economic problem of woody hosts, most research on the correlation among mineral nutrient supply, host growth and gall development had been done with herbaceous hosts. Hussin and Deep (1965) performed experiments to compare the interaction of these factors in a woody host, Cherry (*Prunus avium* L. 'Mazzard'), with a herbaceous host, tomato (*Lycopersicon esculentum* Mill. 'Browny Best'). They found that the relative size of crown galls decreased with an increase in the level of nitrogen (N) and relative size of galls on Browny Best tomato decreased more markedly when amounts of both nitrogen and potassium were increased than when either element was increased. Their investigation results further indicated that optimum growth rate of tumour tissue is reached when nutritional supply is relatively low. Godara *et al.* (1995) observed that dual inoculation of *A. chrococcum* and *G. fasciculatum* completely inhibited infection of crown gall in potted peach plants.

2.7.3 Chemical control

A number of fungicides, antibiotics and other chemicals such as fumigants, soil sterilizing agents, and herbicides have been used against the emerging crown gall problem. Balanchard (1951) reported aureomycin effective against crown gall. Deep and Young (1965) applied fungicides to the soil prior to sowing nursery plants and observed higher incidence of crown gall. They concluded that this was due to reduction in soil fungal micro flora leading to less competition to *A. tumefaciens* and hence increased incidence. Several authors used antibiotics and reported the efficacy of terramycin (Deep, 1958a) and penicillin and streptomycin (Hampton, 1948). However, Peter and David (1958) observed that neither streptomycin nor tetracyclin gave a successful control of crown gall in either pear or cherry rootstocks having the disease in incipient form, when applied as 60 minutes dip. Deep *et al.* (1968) tested 7 fumigants for their effectiveness in controlling crown gall on Mazzard cherry. They reported that methyle-bromide chloropicrin-propurgyl bromide appeared to give some control of crown gall. Dickey (1962) observed more than 90 per cent reduction in *A. tumefaciens* in artificially infected soil at all depths of 3, 9 and 15 inches by methyl bromide (2 and 3 pounds/ 100 sq. feet soil). Kapshuk (1933) also found promising result with chloropicrin (@ 10 cc to 1 cm of the enclosed space. He reported that chloropicrin penetrated to the depth of 20 cm when applied to soil killing the *Agrobacterium* effectively. However, he experimentally showed that mercuric chloride, copper sulphate and chloride of lime were useless for the disinfections of the soil naturally or artificially infested with *A. tumefaciens*. Ogg and Graf (1974) tested 8 herbicides as pre-plant treatment both in green house and field conditions on apples, pears, cherries and plums. They reported increase in the incidence

of crown gall and hairy root on sweet cherry by the use of trifluraince application @ 1.7 kg/ha. Grimm (1987) obtained good control of crown gall in M-9 rootstocks with ammonical cupric sulphate and copper oxychloride. In unsterilized soil, metam-sodium or formaldehyde failed to control crown gall (Utkhede and Smith, 1990)

2.7.4 Biological control

The credit for pioneering work in the field of biological control of crown gall goes to New and Kerr (1972), who for the first time obtained 100 per cent control of this disease on almond seedlings by the use of non-pathogenic strain (*A. radiobacter* strain K-84). Since then this strain of *A. radiobacter* has been effectively and widely used for the control of crown gall across the globe. It was found effective in Australia on peach and other stone fruits as root dip and seed treatment (Kerr, 1972), in Canada on peach, cherry, almond and grape as soil drench, seed treatment, tomato assay and bare root dip (Dhanvantari, 1976), in Greece on almond (Panagopoulos and Psallidas, 1973), in Swiss and Hungarian nurseries of peach, cherry and apple (Grim *et al.*, 1982), in Italy on cherry, Myrobalan and peach rootstocks (Bazzi and Mazzucchi, 1978), on *Prunus* and tomato in Oregon (Moore, 1976), in UK on cherry at East Malling (Crosse *et al.*, 1976), in California on almond, apricot, peach and plum (Moller and Schroth, 1976), in Spain in stone fruits (Lopez *et al.*, 1989), China (Zhang *et al.*, 1991) and several other countries.

Dhanvantari (1976) reported that Australian isolate of *A. radiobacter* when applied as soil drench one week before soil inoculation with *A. tumefaciens* gave excellent protection against crown gall development for five months on peach seedlings in green house conditions. In the same year, Kerr found that galling was reduced from 79

to 31 per cent when peach seeds were treated with K-84. Successful biological control of crown gall has also been reported from several other countries (Kerr, 1980; Moore, 1979b). However, several strains of *A. tumefaciens* were insensitive to bacteriocin (Agrocin 84) produced by strain K-84 *in vitro* (Kerr and Htay, 1974; Kerr and Panagopoulos, 1977; Moore, 1979; Schroth and Moller, 1976) and in some instances strain k-84 did not prevent tumour production on susceptible hosts (Kerr and Panagopoulos, 1977; Moore, 1979). Even though the success of *A. radiobacter* undoubtedly has encouraged several other workers to look for new antagonists for the strain K-84 insensitive pathogens but other *A. radiobacter* strains that inhibit pathogenic *Agrobacterium* species *in vitro* have been ineffective as control agent on plants (Garrett, 1979; Kerr and Panagopoulos, 1977). Johnson and Dileone (1999) used antibiotic agrocin 84 a derivative of *A. radiobacter* strain 84 and found that control of crown gall was nearly complete with the combination of *A. radiobacter* strain 84 and an agrocin sensitive strain of *A. tumefaciens* in tomato. Soil fungi are potential source of antagonists that have been largely ignored in crown gall research. Deep and Young, (1965) showed that pre-planting fungicide treatment increase the incidence of crown gall of cherry seedlings suggesting the presence of natural fungal competition. They later used a suspension of unidentified fungi as a pre-planting treatment and reduced galling by about 25 per cent. Cooksey and Moore (1980) tested several fungal and bacterial soil micro flora and achieved the same level of control with species of *Penicillium* and *Bacillus* comparable to that achieved by Kerr (1972). The results from their study showed that the incidence of crown gall of cherry could be reduced from 61.9 to 4.1 per cent by using a *Penicillium* antagonist against group II pathogens. They also reported that the biological control was

enhanced when some of the antagonists were inoculated into wounded plant tissues 24 hours before the pathogen.

Among the other bacterial antagonists strain 1-1-4 *Pseudomonas fluorescens* has been reported to provide more than 95 per cent control of crown gall on peaches (Zhang *et al.*, 1991).

MATERIALS AND METHODS

Chapter-III

MATERIALS AND METHODS

The studies were carried out at Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (Himachal Pradesh) during 2001-2003 situated at an elevation of 1250 meters at 30°50'45" latitude and 70°08'50" longitude above mean sea level. The laboratory experiments were carried out in the Bacteriology Laboratory of Department of Mycology and Plant Pathology, Laboratories of Department of Soil Science and water management and Pomology, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan while field experiments were carried out in experimental fields of Department of Mycology and Plant Pathology at Naganji Farm , Nauni ,Solan (H.P.).

Glassware used

Petri plates (100X17mm), Test tubes (10 and 20 ml), microscopic glass slides (2.5x 7.0 x 2.0 mm), Erlenmeyer flasks (100, 250, 500 and 1000 ml), pipettes (0.1, 1.1, 2.5, 10.0 ml) were used during the course of investigations. The glassware used was of Borosil make.

Equipments, apparatus and other materials used

Digital electronic balance was used to take the weight, BOD incubators were used to incubate the test materials and magnetic stirrer was used for mixing soil homogenously in sterilized distilled water for isolation of microorganisms from the soil. Vertical laminar airflow chamber was used for aseptic culturing of test bacterium, rhizosphere bacteria and other microbes. Autoclave was used for sterilization of media, glassware and muslin cloth. Orbital shaker cum incubator was used for preparation of broth cultures of different test bio-control agents. Portable leaf area meter (AM-200) was used to measure the area of leaves. Earthen pots of 30 cm diameter were used to grow tomato seedlings for pathogenicity test.

Sterilization

The sterilization of various culture media and glassware (wrapped in butter paper) was done by autoclaving at 1.05 Kg/cm^2 pressure for 20 minutes. The autoclaved glassware was dried in hot air oven at 80^0C for half an hour. Polyethylene bags and butter papers used for carrying soil samples and shade drying of soil were sterilized with the rectified spirit. These were packed in a cardboard box for further use.

The methodologies and techniques adopted during the course of these investigations have been categorized under the following broad headings:

3.1 Occurrence, incidence and severity of crown gall disease in stone fruit nurseries

3.2 Effect of crown gall disease on plant growth parameters in peach

3.3 Isolation and identification of the causal organism

3.4 Establishment of pathogenicity of different isolates of *Agrobacterium tumefaciens*

3.5 Isolation of rhizobacteria and evaluation of their antagonistic activity against *Agrobacterium tumefaciens*

3.6 Effect of different biocontrol agents; organic amendments; soil sterilizing agents; manures, fertilizers & biofertilizers and antibacterial compounds on the incidence and severity of crown gall on peach

3.1 Occurrence, incidence and severity of crown gall disease in stone fruit nurseries

In order to record the prevalence of crown gall, extensive surveys were conducted during 2001 and 2002 in Solan, Sirmaur, Shimla, Kullu and Mandi districts of Himachal Pradesh in the registered stone fruit nurseries and Progeny cum Demonstration Orchards (PCDO's), Department of Horticulture, Himachal Pradesh. Disease incidence and disease severity was recorded in nurseries of peach, plum, apricot, almond, cherry and Colt suckers.

The disease incidence was calculated by the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{No. of diseased plants}}{\text{Total number of plants examined}} \times 100$$

For calculating the disease severity; a disease severity scale was devised as following:

S. No.	Description	Category
1.	No galls	0
2.	Less than 5 galls of small size (10 % crown area covered)	1

5-10 galls of medium size covering (25 % of crown area of plant covered)	2
10-15 galls of medium size (50 % crown area covered)	3
> 25 galls of large size (100% crown area covered)	5

Gall description	**Size (cm)**
Small	< 1
Medium	1-5
Large	> 5

The per cent infection index (designated as disease severity) was calculated by the following formula described by Mc Kinney (1923).

$$\text{Disease severity (\%)} = \frac{\text{Sum of all disease ratings}}{\text{Total number of ratings X Maximum Disease grade}} \text{ X } 100$$

3.2 Effect of crown gall disease on plant growth parameters in peach

Trials were laid during the year 2002 and 2003 to study the effect of crown gall disease on different growth parameters of peach plants (cv. Redhaven) in an experiment laid out in Randomized Block Design with four replications. Diseased peach plants having gall size of 5-8 cm diameter were planted in the fields having no history of pome and stone fruit cultivation. Nine diseased peach plants were planted in each plot of size 1 m^2 during the last week of February with plant-to-plant and row-to-row distance of 30 cm. A control was also maintained where healthy peach plants were planted in the same

size plots for comparison. The recommended package and cultural practices were followed in the growing season and plants were uprooted in last week of November. The data pertaining to the growth parameters namely shoot length, stem girth, root length and plant biomass was recorded after uprooting, whereas number of leaves per plant and leaf area were recorded during August, at the time when plants had attained maximum growth. Ten leaves from each portion - top, bottom and middle of the plant canopy were collected at random and bulked together. A sample of 15 leaves from each plot was taken and leaf area was measured with the help of portable leaf area meter (AM-200). The plant biomass was calculated by weighing all the plants of each treatment after uprooting and value obtained was divided by the total number of plants in each treatment.

3.3 Isolation and identification of the causal organism

3.3.1 Isolation from gall

The peach plants showing tender galls were uprooted with the help of crow bar with intact crown portion and roots showing gall development. The plants from different locations were bundled separately and brought to laboratory within 24-48 hours. The young tender and milky galls separated from diseased peach plants were washed with non-chlorinated water for 24 hours and shade dried. The necrotic tissues were removed with the help of a sharp sterilized razor and the galls were diced to 2 mm cubes (approx.). These cubes were surface sterilized in 0.1 per cent $HgCl_2$ for 30 seconds and rinsed in sterile distilled water for three times to remove mercuric chloride from the cubes. These cubes were then crushed in a sterilized Petri plate containing 1 ml of sterilized distilled water with the help of sterilized glass rod. The suspension was kept undisturbed for 10

minutes and 2 drops of it were added with the help of sterilized pipette of 1 ml capacity to each sterilized Petri plate. Twenty-five ml of sterilized respective isolation medium in each Petri plate was poured. The plates were gently rotated to disperse the suspension evenly in the medium. The medium was allowed to solidify in the plates. The bacterial suspension (0.1ml) was also streaked (streak plate method) over the solidified surface (24 hours prior poured) of twenty-five ml sterilized agar media contained in sterilized Petri plates. For each sample, three Petri plates were maintained representing three replications. Different media employed for isolation of the causal agent were nutrient agar (NA), soil extract agar, yeast extract mannitol agar (YEMA) supplemented with Congo Red (500 ppm), Schroth *et. al.* and Kado and Heskett (Appendix I). The Petri plates were incubated in the BOD incubator at 25 $\pm 1^0$C and were examined after every 24 hours for development of colonies. A single colony was further streaked to obtain pure progeny of single cell colony.

3.3.2 Isolation from soil

The soil adhering to the roots of the disease peach plants was collected and carried to the laboratory in sterilized polyethylene bags (sterilized with the help of 95 % ethyl alcohol). The soil samples were later dried in shade for 24 hours on sterilized butter paper. Ten grams of this soil from each sample was filtered through the sterilized muslin cloth and was added into 90 ml of sterilized distilled water. The suspension was homogenously mixed with the help of a magnetic stirrer (Johnson, 1957). The suspension was diluted to 10^{-3} by serial dilution method. The suspension (0.1 ml) was spread over the solidified surface of 24 hours prior poured sterilized nutrient agar (NA), soil extract agar,

yeast extract mannitol agar supplemented with Congo red (500 ppm), Schroth *et. al.* and Kado and Heskett media (Appendix I) contained in sterilized Petri plates. The suspension was spread over the solidified surface of the medium with the help of sterilized L-shaped glass rod. Three replications were maintained for each soil sample. Petri plates were later incubated in BOD incubator at 25 ± 1^0C and were examined after every 24 hours to observe any growth. A single colony was further sub streaked to obtain progeny of a single cell colony. The cultures so obtained were maintained in slants of yeast extract mannitol agar medium and were kept in refrigerator. Periodic sub- culturing of these isolates was done after every two months to maintain different isolates.

3.3.3 Identification

The growth obtained on the selective media was critically studied for its cultural and morphological characteristics. The following cultural and morphological characteristics were studied:

Cultural characteristics

1. Colony shape (round, circular, oblong, ovate etc.)
2. Colony colour (white, transparent, opaque, or coloured)
3. Colony size (cm)
4. Colony margins (smooth, rough, zigzag etc.)
5. Other specific characters, if any

Morphological characters

1. Cell shape (rod, coccus, vibrio, helical etc.)
2. Cell size (under PC based fluorescent microscope: X1000)

3. Cell colour (hyaline or coloured)
4. Other specific characters, if any

Gram staining (Appendix II) was employed for establishing the identity of the bacterium under compound microscope (X 1000). Also the pure obtained cultures of the causal agent were subjected to different biochemical tests namely; 3-ketoglycoside test, acid production from erythritol, growth on Schroth *et al* medium, growth at 35 ^{0} C and litmus milk test (Appendix III) to ascertain the identity of the test bacterium as descried by Panagopoulos *et al.*, 1978.

3.4 Establishment of pathogenicity of different isolates of *Agrobacterium tumefaciens*

Isolates thus obtained and preliminary identified as *A. tumefaciens* were further tested for pathogenicity on tomato seedlings (Kerr, 1969b). *A. tumefaciens* culture suspension (containing 10^{8} c.f.u./ml) were wound inoculated on tomato stem.. It gave two wrong results out of 60, an acceptable level of error, and was employed for routine testing because of considerable saving in time and labour.

3.4.1 Standard method of wounding and inoculation

The method used by New and Kerr (1972) was employed for wound inoculation on tomato seedlings under pot culture conditions. Stem of 4 weeks old tomato plants were wounded at crown, stem and tip portions to a depth of 3 mm with the help of a sterilized blunt cylindrical sterilized steel rod of 2 mm (diameter) and 0.004 ml suspension of pathogen containing 10^{8} c.f.u. /ml was then deposited in each wound by an

autoclaved micropipette. A control was kept where only 0.004 ml of sterilized distilled water was deposited with the help of autoclaved micropipette in the wounds at crown, stem and tip portion of tomato plants. The wounds were wrapped with sterilized cotton to avoid secondary invasion by saprophytes. The pots were kept at room temperature for development of galls at the wounded and inoculated portions. The observations for any gall development were made everyday after third day of inoculation.

3.5 Isolation of rhizobacteria and evaluation of their antagonistic activity against *Agrobacterium tumefaciens*

3.5.1 Isolation of rhizobacteria

Different bacteria were isolated from the rhizosphere soil of healthy and infected peach plants for their antagonistic activity. Samples were collected from March to August, 2001 from stone fruit nurseries located at PCDO Jadari (district Solan), PCDO Rajgarh, Fruit Nursery Sanahari (district Sirmaur) and nurseries of Department of Pomology and Fruit Breeding Dr, Y. S. Parmar University of Horticulture and Forestry, Nauni that had recurrent high incidence of crown gall for the past several years on fruit especially on Peach. The peach seedlings uprooted with the help of a crow bar with rhizosphere soil intact were brought to the laboratory. Rhizosphere soil was collected on a sterilized butter paper and shade dried for 24 hours. The soil was diluted to 10^{-1}, 10^{-2} and 10^{-3} with a sterile distilled water (serial dilution method) and 0.1 ml of each dilution was spread over the solidified surface of 25 ml nutrient agar (NA) medium contained in each Petri plate with the help of sterilized L-shaped glass rod. The Petri plates were incubated in BOD incubators at 25 ± 1^0C and observed for development for any growth

at 24 hours interval. After 72 hours of growth, different bacterial colonies were picked with the help of sterilized bacterial loop and were sub-streaked to obtain pure progeny of rhizobacteria. The cultures so obtained were maintained in slants of NA medium and were kept in refrigerator. Periodic sub- culturing of these isolates was done after every two months to maintain different isolates.

3.5.2 *In vitro* evaluation of antagonistic activity of rhizobacteria against *Agrobacterium tumefaciens*

The purified rhizobacterial culture identified as *Bacillus* sp., *Pseudomonas fluorescens* and a soil isolate of *A. tumefaciens* were evaluated for their antagonistic activity against the crown gall pathogen by employing dual culture technique (Boosalis, 1956). Cork borer cut bits (5 mm dia.) of both the rhizobacteria and the causal agent (pathogen) were put at 2 cm apart from center on the other side of the plate, so the distance between discs were 4 cm in Petri plate containing NA medium. Cultures of both test antagonist and pathogen were also streaked at two cm apart from each other over the solidified surface of sterilized NA contained in sterilized Petri plate. The plates were incubated for 7 days at 25± 1^{0}C in a BOD. The observations on growth inhibition of test pathogen in Petri plates were made by comparing the standard where only *A. tumefaciens* was grown on NA in sterilized Petri plate after similar conditions of incubation in BOD. Development of zone of inhibition, if any was also observed after 7 days of incubation at 25± 1^{0}C.

3.5.3 Evaluation of antagonistic activity of rhizobacteria against *Agrobacterium tumefaciens* on tomato plants

Evaluation of possible antagonistic activity of different isolated rhizobacteria was also done on four weeks old tomato seedlings under pot culture conditions by cross inoculation methods by causing 3 mm deep injuries with a sterilized blunt cylindrical steel rod. With the help of sterilized micropipette, 0.004 ml of rhizobacterial suspension (having 10^8 c.f.u. /ml) was deposited in each wound. After 24 hours 0.004 ml pathogen suspension (having 10^8 c.f.u. /ml) was applied to the same wound. Observations on gall development over inoculated wounds on tomato seedlings were made after third day and on each successive day.

3.6 Effect of different biocontrol agents, organic amendments, soil sterilizing agents, manure, fertilizers and bio-fertilizers, and antibacterial compounds on the incidence and severity of crown gall on peach

All the trials were laid out in Randomized Block Design. In all treatments, four replications were taken and in each replication, nine one year- old peach seedlings (cv. Redhaven) were planted in nursery bed of 1 m^2 size with plant-to-plant and row-to-row distance of 30 cm. The recommended package and practices (Appendix IV) were followed in entire crop season of the year 2003.

3.6.1 Effect of field application of potent antagonists on the incidence and severity of crown gall on peach

The rhizosphere bacteria and an *A. tumefaciens* isolate isolated from rhizosphere soil were further employed for their antagonistic activity against crown gall pathogen under field conditions in the year 2003. A broad-spectrum fungal antagonist *Trichoderma viride* as talc-based formulation having 10^8 c.f.u./g was also tested against crown gall.

3.6.1.1 Mass culture preparation of rhizobacteria and *Trichoderma viride* for field application

Mass culture preparation of Rhizobacteria

Broth cultures of *Bacillus* species, *Pseudomonas fluorescens* and *A tumefaciens* (soil isolate) obtained from the rhizosphere soil were prepared. Three bits (5 mm diameter) of 5 days old cultures of above-mentioned bacteria were added to the 100 ml sterilized nutrient broth in individual 250 ml conical flasks under aseptic conditions. These flasks were incubated at 25±1^{0}C in BOD incubators for four days. After four days of incubation the flasks were kept in orbital shaker-cum incubator at 25±1^{0}C for 36 hours. Spore load of each bacterium (10^8 spores /ml) in broth culture was estimated by dilution plate method.

***Trichoderma* formulation**

Talc based formulation of *Trichoderma viride* earlier tested against seedling blight of apple (Gupta *et. al.*, 2003) was used to test its efficacy under field conditions against crown gall disease. Broth culture of *T. viride* was prepared in yeast extract

molasses broth (yeast extract 5 g+ molasses 30 g in one liter of water) by growing 3 bits (5 mm diameter) of 10 days old culture of *T. viride* in 100 ml broth medium at 25± 1^0 C for 10 days in BOD incubator. Afterwards the broth culture was mixed in talc powder in 1:2 ratio (one part broth+ two parts talc). After 48 hours of shade drying of this mixture, carbooxymethyl cellulose @5g/ Kg was mixed. Spore load of *T. viride* (10^8 c.f.u. /g) was estimated by dilution plate method. The formulation was tightly packed in sterilized polythene bags. The bags were stored at room temperature in dark till further use within 3 months.

3.6.1.2 Field application of biocontrol agents

In a Randomized Block Design experiment with four replicates, each bio control agent was tested as root dip, soil drench and root dip + soil drench treatments. Fifty ml of broth culture of each rhizobacteria (containing 10^8 c.f.u. /ml) was added to ten liters of water and was used for drenching each plot of 1 m^2 size. While for root dip treatment, the roots of peach seedlings were dipped upto collar portion for one and half hour in 10 liters of water containing 50 ml of bacterial suspension prepared in the way mentioned above. One hundred and fifty gram of talc based formulation of *T. viride* having 10^8 c.f.u. /g was added in 10 liters of water for soil drenching of each bed. Also same quantity of *Trichoderma* formulation in 10 liters of water was used for root dip treatment of peach seedlings for one and half hour. Nine peach seedlings were planted in each plot immediately after treatment during first week of February with plant-to-plant distance and row-to-row distance of 30 cm. The recommended package and practices were

followed throughout the growing season and the trials were uprooted in the last week of November 2003 to record the data on incidence and severity of crown gall.

3.6.2 Effect of different soil amendments on incidence and severity of crown gall

Various soil amendments such as neem cake, mustard cake, pine + deodar needles (1:1) and eucalyptus leaves were tested for their efficacy in reducing the incidence and severity of crown gall at 0.5, 1.0 and 1.5 per cent of the soil in $1m^2$ plots upto 30 cm soil depth. The total soil content in per $1m^2$ plot upto 30 cm of soil depth was calculated by the following formula:

Soil up to 30 cm of depth in one ha. = 2.24×10^6 kg

An experiment was laid out in Randomized Block Design with four replicates at Research Farm of Department of Mycology and Plant Pathology, main university campus, Nauni in 2003 crop season. One year old, nine peach seedlings were planted in last week of February in $1m^2$ plots with a row to row and plant to plant distance of 30 cm and neem cake, mustard cake, pine+deodar needles (1:1) and eucalyptus leaves at above mentioned concentrations were added in mid March when plants had already established. The pine, deodar and eucalyptus leaves were chopped whereas neem cake and mustard cake were crushed to powder before field application (Singh and Pandey, 1967). These treatments were thoroughly mixed in the soil and beds were irrigated thoroughly every third day for proper and homogenous mixing of different amendments. The recommended package and cultural practices were followed throughout the growing

season and plants were uprooted in last week of November in each cropping season to record data on incidence and severity of crown gall.

3.6.3 Effect of pre-plant application of different soil sterilizing agents on incidence and severity of crown gall

Research trial was laid out in February, 2003 at Nauni in Randomized Block Design having four replications in each treatment alongwith control to evaluate the efficacy of different soil sterilizing agents namely formaldehyde (40%) @ 3, 4 and 5 per cent, bleaching powder (33% available chlorine) @ 0.15, 0.25 and 0.35 per cent and sodium hypochlorite (5% available chlorine) @ 0.10, 0.15 and 0.20 per cent were used as soil drench. Ten liters of water containing above-mentioned concentrations of soil sterilizing agents was used for drenching each plot of size $1m^2$ during first week of February. In case of formaldehyde the plots were covered with a polyethylene sheet for 21 days; afterwards removed followed by everyday raking of soil for 7 days. Nine peach seedlings (one year old) were planted in each treated plot in the first week of March. The recommended package and cultural practices were followed in the entire crop season and plants were uprooted in the last week of November to record the disease incidence and severity of crown gall.

3.6.4 Effect of manure, fertilizers and bio-fertilizers and their combinations on the incidence and severity of crown gall

Effect of recommended doses of FYM (60 T/ha), nitrogen (90 kg/ha), phosphorus (30 kg/ha) and potash (50 kg/ha) were studied on crown gall incidence and severity. A

Randomized Block Design experiment was laid out with four replicates at Nauni with suitable control. The half, three- fourth and double the amount of the recommended doses alongwith their combinations were also tested. Also vesicular mycorrhizal fungi (VM) containing 250 spores/ 100 g in air dried soil culture was tested alongwith commercial formulation of *Azotobactor chrococcum* procured from Division of Microbiology, IARI, New Delhi. Half Kg of *Azotobacter* culture was mixed with 10 Kg of farmyard manure and was mixed in the soil of 1 m^2 plot followed by irrigation. One Kg soil culture of VM was mixed with 15 Kg FYM and four Kg of this were applied per plot of 1 m^2 size. All the treatments including half dose of nitrogen were applied as pre plant application during last week of January. One-year-old nine peach seedlings were planted in each bed of 1 m^2 size during first week of February. Remaining half dose of nitrogen was applied to each bed during first week of August. The uprooting of peach plants was done in the last week of November to record the effect of bio-fertilizers, fertilizers and manures.

3.6.5 Effect of pre-plant application of antibacterial compounds on the incidence and severity of crown gall

Different antibacterial compounds namely Bordeaux mixture (0.8%), copper-sulphate (1.0 %), copper oxy-chloride (0.3 %), cupric-sulphate (0.3 %), cupric chloride (0.3 %), streptocycline (0.01 %) and bleaching powder (0.3 %) were used as root dip, soil drench and root dip + soil drench alongwith suitable controls during 2003 crop season (Kapshuk, 1933). An experiment was laid out in Randomized Block Design. Each plot served as one replication and in each treatment four replications were taken. Ten liters of water containing above-mentioned concentrations of test compounds was used to drench

each plot of 1 m^2 size in mid February and after 7 days nine one- year old peach plants were planted in each plot. For root dip treatment, the roots of peach plants were dipped for one and half hour in above-mentioned concentrations of each chemical. The dipped peach seedlings were immediately planted in the experimental plots. The recommended package and cultural practices for peach plants were followed during the growing season. Plants were uprooted in the last week of November and data on disease incidence and severity was recorded.

EXPERIMENTAL RESULTS

Chapter-IV

EXPERIMENTAL RESULTS

The results of the present investigations on studies on crown gall of stone fruits have been described under the following headings:

4.1 Incidence and severity of crown gall in stone fruit nurseries

4.2 Effect of crown gall disease on plant growth parameters in peach

4.3 Isolation and identification of causal organism of crown gall

4.4 Pathogenicity of different isolates of *Agrobacterium tumefaciens*

4.5 Evaluation of test antagonist(s) against *Agrobacterium tumefaciens*

4.6 Effect of different antagonists, organic amendments, soil sterilizing agents, manure, fertilizers and bio-fertilizers and antibacterial compounds on the incidence and severity of crown gall on peach

4.1 Incidence and severity of crown gall in stone fruit nurseries

In order to determine the incidence and severity of crown gall in nurseries of stone fruit plants, a systematic survey of stone fruit growing areas of Solan, Sirmour, Shimla, Kullu and Mandi districts of Himachal Pradesh were carried out in the month of December to February during 2001 and 2002 when the uprooting of nursery plants was being carried out. The data recorded on the incidence and severity of crown gall is presented in Table 1.

Table 1: Incidence and severity of crown gall disease in stone fruit nurseries in different districts of Himachal Pradesh during 2001 and 2002

Location	Crop	2001		2002	
		Disease incidence(%)	Disease severity (%)	Disease incidence(%)	Disease severity (%)
Solan					
Nauni	Peach	20.00	46.00	26.36	79.28
	Cherry	28.0	15.20	46.76	52.72
	Apricot	3.00	1.60	12.61	9.00
Jadari	Colt	13.20	49.00	17.40	65.21
Gaura	Apricot	10.00	1.20	1.71	53.00
	Peach	5.70	24.80	22.33	12.00
Sirmaur					
Daro Deria	Apricot	3.00	2.40	25.62	13.22
	Peach	5.00	29.20	17.77	42.22
Nohradhar	Peach	11.40	32.40	13.60	72.00
Rajgarh	Almond	33.60	39.60	13.27	12.22
	Peach	24.30	39.60	4.33	80.00
Sanahari	Peach	70.00	39.60	58.00	72.00
Shimla					
Gaggar	Colt	5.00	2.20	10.42	12.32
Chaithla	Colt	10.00	5.80	25.00	28.23
Jauni	Colt	10.00	5.80	25.22	31.20
Kullu					
Bajaura	Plum	13.30	16.00	12.50	16.00
	Peach	50.00	14.00	28.50	13.00
	Apricot	2.30	5.00	38.89	16.00
	Almond	25.00	37.00	28.26	57.00
	Cherry	36.26	42.42	38.17	23.00
Banjar	Plum	10.20	7.00	14.60	12.00
	Apricot	8.60	9.00	21.74	19.00
PCDO Bajaura	Plum	10.00	12.00	8.00	13.00
	Peach	5.00	7.00	28.00	31.00
	Apricot	20.00	17.00	62.00	9.00
	Almond	28.00	30.00	34.64	27.00
Bhattgran	Plum	5.00	7.00	6.00	4.00
Mandi					
Palsehar	Cherry	100.00	50.00	24.23	13.17
Harabagh	Apricot	6.00	20.00	13.75	18.22
	Plum	14.00	18.00	11.72	13.34
Jhamar	Plum	16.00	21.00	4.00	2.00
	Apricot	8.00	13.00	6.00	21.00
Nalhas	Peach	6.00	10.00	14.00	7.00
	Almond	14.00	22.00	17.46	54.00

The crown gall disease was widely prevalent in all the stone fruit nurseries in the district Solan. Minimum disease incidence (3.00%) was observed at Nauni on apricot with 1.60 per cent disease severity during the year 2001. In the year 2002, the minimum disease incidence (1.71%) was again observed on apricot having 53.00 per cent disease severity at Gaura. In district Sirmaur, the disease incidence ranged from 3.00 to 70.00 per cent and 4.33 to 58.00 per cent, respectively during the year 2001 and 2002. The disease severity ranged from 2.40 to 39.60 and 12.22 to 80.00 per cent, respectively during the year 2001 and 2002. In district Kullu, the disease incidence ranged from 2.30 to 50.00 per cent with disease severity ranging from 5.00 to 42.42 per cent during the year 2001. In the year 2002, the disease incidence ranged from 8.00 to 62.00 per cent. The disease severity also ranged from 4.00 to 57.00 per cent. In district Mandi, hundred per cent cherry plants were found infected with crown gall disease during 2001 having 50.00 per cent disease severity. Whereas on peach, maximum incidence (70.00%) was observed at Sanahari in district Sirmaur having 39.60 per cent disease severity. At all these locations, cherry and peach plants were found highly affected with crown gall disease followed by almond, apricot and plum (Plate –I).

4.2 Effect of crown gall disease on plant growth parameters in peach

From the data in Table 2, it is evident that in 2002 the diseased plants had a significant reduction in all the growth parameters. The maximum per cent reduction (in diseased over healthy) was observed in shoot length (26.60%) followed by leaf area (15.46%). The diseased peach plants also had reduction in number of leaves per plant (13.59%), root length (10.91%) and stem girth (8.87%). Similar trend was observed in the

Table 2: Effect of crown gall disease caused by *Agrobacterium tumefaciens* on growth parameters of peach during the years 2002 and 2003

Growth parameter	2002		Per cent reduction in diseased	2003		Per cent reduction in diseased	Pooled
	Healthy	Diseased		Healthy	Diseased		
Shoot length (cm)	102.82	76.11	26.60 (29.75)	165.55	158.25	22.48 (28.26)	24.54 (29.01)[a]
Root length (cm)	20.07	17.89	10.91 (18.29)	24.77	19.90	19.46 (24.32)	15.19 (21.31)[ab]
Stem girth (cm)	1.06	0.95	8.87 (15.60)	1.59	1.46	9.69 (17.44)	9.28 (16.52)[b]
No. of leaves/plant	76.46	65.85	13.59 (16.75)	79.01	66.31	15.83 (21.81)	14.71 (19.29)[ab]
Leaf area (cm^2)	24.75	20.85	15.46 (22.64)	27.19	24.32	18.97 (24.19)	18.22 (24.23)[ab]
Crown area covered (%)	0.00	68.50	-	0.00	33.50	-	-
Over all mean			15.09 (20.61)[A]			17.69 (23.53)[A]	
SE±			(8.15)			(6.72)	
$CD_{0.05}$			(17.77)			(14.64)	

Figures in the parentheses are aresine transformed values

	SE±	$CD_{0.05}$
Parameters (P)	(4.96)	(10.18)
Year(Y)	NS	NS
P x Y	(7.01)	(14.38)

year 2003, where maximum per cent reduction (22.48%) was observed in shoot length followed by root length (19.46%) and leaf area (18.97%). Overall mean values also showed the maximum reduction (24.54%) in shoot length followed by leaf area (18.22%) and root length (15.19%)in diseased peach plants. Significant per cent reduction was also observed in stem girth (9.28%) and number of leaves per plants (14.71%).

4.3 Isolation and identification of causal organism of crown gall

4.3.1 Isolation

The attempts were made to isolate the crown gall pathogen from gall and from soil on different isolation media viz., nutrient agar, soil extract agar, yeast extract mannitol agar, Kado and Heskett and Schroth *et al.* The best growth of bacteria from gall was obtained on yeast extract mannitol agar (Plate -I) followed by Kado and Heskett medium (Table 3). Schroth *et al.* medium was best in case of soil isolation though the causal agent also appeared on nutrient agar yet it supported the growth of other contaminants as well.

Table 3: Isolation of *Agrobacterium tumefaciens* from crown gall infected stone fruit plants and soil on different media

Medium	Growth on medium	
	Gall	Soil
Nutrient agar	-	+
Soil extract agar	-	-
Yeast extract mannitol agar	+	+
Kado and Heskett	+	-
Schroth *et al.*	-	+

+ Growth obtained - No growth

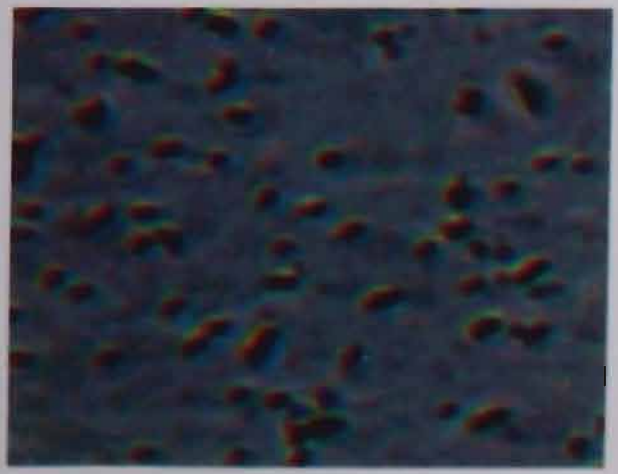
Plate-I

Crown gall infected stone fruit plants showing large galls

Artificially inoculated tomato plant showing galls induced by isolate of *Agrobacterium tumefaciens*

***Agrobacterium tumefaciens* colonies on yeast extract mannitol agar medium**

Development of young creamy galls induced by *Agrobacetrium tumefaciens* on stem of artificially inoculated tomato plant



Microphotograph of cells of *Agrobacterium tumefaciens* (X 1000)

4.3.2 Identification

The identification of causal organism of crown gall disease of stone fruits was done on the basis of cultural and morphological characters.

4.3.2.1 Cultural and morphological characters

Gram staining of all the five isolates revealed that all the isolates were Gram negative in their reaction. The colonies on yeast extract mannitol agar and Kado and Heskett medium of the three isolates from peach, one from cherry and one from soil isolate were off white with a circular to ovate or elongated in shape with smooth margin and the colony diameter ranged from 0.7 to 1.5 cm on yeast extract mannitol agar and 1.20 to 2.20 cm on Kado and Heskett medium except in the soil isolate where the colony diameter ranged from 0.8 to 1.3 cm after five days of growth on yeast extract mannitol agar and 1.1 to 1.33 cm on Schroth *et al.* medium. All the isolates were having rod shaped hyaline cells which were either born singly or in chains of 2 to 6 cells having cell size ranging 1–4 x 0.2-0.45 μm (Plate –I). The colonies were transparent to translucent and squishy –squashy in texture (Table 4).

On the basis of above mentioned cultural and morphological characteristics, the pathogen was tentatively identified as *Agrobacterium tumefaciens* and identification of different isolates was further confirmed by different biochemical tests and pathogenicity on tomato plants.

Table 4: Cultural and morphological characteristics of *Agrobacterium tumefaciens* isolated from different stone fruit plants and soil

Cultural characters	Isolates from different locations				
	Rajgarh (Peach)	**Nauni (Peach)**	**Nauni (Cherry)**	**Sanahari (Peach)**	**Soil isolate**
Gram reaction	Negative	Negative	Negative	Negative	Negative
Colony shape	Circular to ovate	Circular to elongated	Circular to ovate	Circular	Circular to ovate
Colony colour	Off white	Off white	Off white	Off white	Off white
Colony size (cm)	0.8-1.3 after 5 days on YEMA and 1.2 to 2.2 on Kado and Heskett	0.7-1.3 after 5 days on YEMA and 1.2 to 2.2 on Kado and Heskett	0.8-1.4 after 5 days on YEMA and 1.2 to 2.2 on Kado and Heskett	0.8-1.5 after 5 days on YEMA and 1.2 to 2.2 on Kado and Heskett	0.8- 1.3 after 5 days on YEMA and 1.1-1.33 on Schroth *et al.* medium
Colony margin	Smooth	Smooth	Smooth	Smooth	Smooth
Other characters	Colonies translucent, protruding towards outside squishy-squashy (spread on medium surface on slight touch)	Colonies translucent, convex, squishy-squashy	Colonies transparent to translucent, convex and squishy-squashy	Colonies translucent, protruding towards outside, convex and squishy-squashy	Colonies translucent, protruding towards outside, convex and squishy-squashy
Morphological characters					
Cell shape	Rod shaped occurring generally singly, sometimes in chains of 2-5 cells	Rod shaped occurring generally singly, occasionally in chains of 2-3 cells	Rod shaped occurring generally singly, occasionally in chains of 2-6 cells	Rod shaped occurring generally singly but occasionally in chains of 3-5 cells	Rod shaped occurring generally singly, occasionally in chains of 3-4 cells
Cell size	Measuring 1-4 µm x 0.2 to 0.4 µm	Measuring 1-4 µm x 0.25-0.3 µm	Measuring 1-3 µm x 0.2 to 0.35 µm	Measuring 1-4 µm x 0.2 to 0.4 µm	Measuring 1-3 µm x 0.3 to 0.45 µm
Cell colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline

4.3.2.2 Reaction of different isolates of *Agrobacterium tumefaciens* to different biochemical test for biovar differentiation

To establish the identity of different biovars, different biochemical tests were conducted (Table 5). All the five isolates, three from peach, one from cherry and one from soil showed positive reaction towards 3-Ketoglycoside test, growth on Schroth *et al.* medium and growth at 35°C whereas no acid production from erythritol was observed in any of the isolate. All the isolate showed alkaline reaction in litmus milk test. Thus by comparing the description of different biovars (Panagopoulos *et al.*, 1978), it could be concluded that all the five isolates belonged to biovar-I group of *A. tumefaciens* (Plate – II).

4.4 Pathogenicity of different isolates of *Agrobacterium tumefaciens*

The pathogenicity test of crown gall causing bacterium, *A. tumefaciens* was conducted on four-week-old tomato plant by inoculating the bacterial suspension in the wounds made with the help of a blunt cylindrical rod. All the four isolates, three from peach and one from cherry produced typical galls on tomato plants, which were initially soft and creamy, smaller in size appeared after 12 days of inoculation at all the inoculated portions of the plants. These galls grew progressively. After 25 days, galls of 2-3 cm diameter were observed on all the inoculated plant parts (Plate I). Reisolation of the bacterium from these galls (produced on tomato plants) further resulted in obtaining small, smooth, glistening, translucent, circular to ovate or elongated colonies typically resembling to those obtained from cherry and peach. The soil isolate although produced galls on tomato plants but the gall were tiny and it did not attain the size as observed in pathogenicity test of other isolates. Reinoculation of these isolates on tomato plants

Table 5: Reaction of different isolates of *Agrobacterium tumefaciens* obtained from different stone fruit plants and soil to different biochemical tests for biovar differentiation

Diagnostic test	Comparison of reaction of isolates of *Agrobacterium tumefaciens* to biovar tests described by Panagopoulos *et al.* (1978)							
	Characteristics of biovar III of *Agrobacterium tumefaciens* compared to biovars I and II			Reaction of different isolates to biovar tests				
	Biovar I	Biovar II	Biovar III	I_1	I_2	I_3	I_4	I_5.
3-keto glycoside test	+	-	-	+	+	+	+	+
Growth on Schroth *et al.*	+	-	-	+	+	+	+	+
Growth at 35^{0}C	+	V	+	+	+	+	+	+
Acid from erythritol	-	+	-	-	-	-	-	-
Litmus milk test	alk.	acid.	alk.	alk.	alk.	alk.	alk	alk.

V = Variable
\+ = Positive reaction
\- = Negative reaction
I_1 = Rajgarh (Peach)
I_2 = Nauni (Peach)
I_3 = Nauni (Cherry)
I_4 = Sanahari (Peach)
I_5 = *Agrobacterium tumefaciens* soil isolate (Nauni)

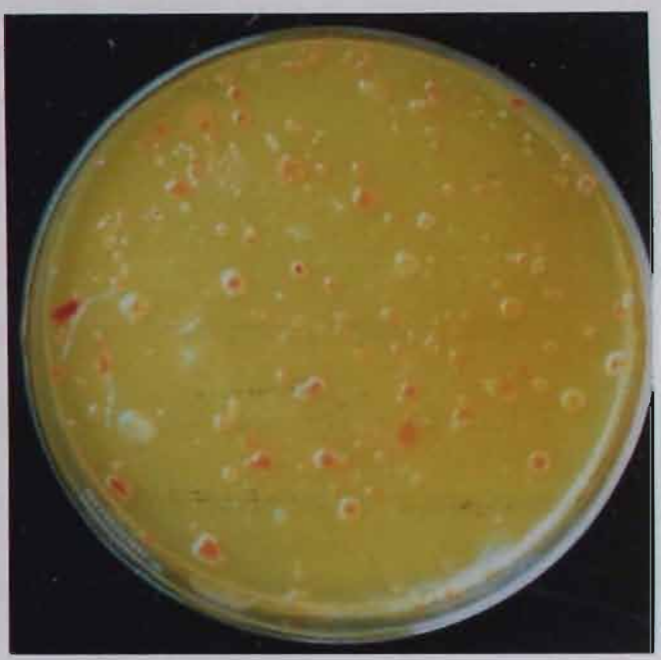
further produced typical galls confirming the Koch's postulates. Based on the above-mentioned observations, it could be concluded that all the isolates were *A. tumefaciens*. The soil isolate, which produced tiny galls on tomato plants, also belongs to *A. tumefaciens* though differing in virulence.

4.5 Evaluation of test antagonist(s) against *Agrobacterium tumefaciens*

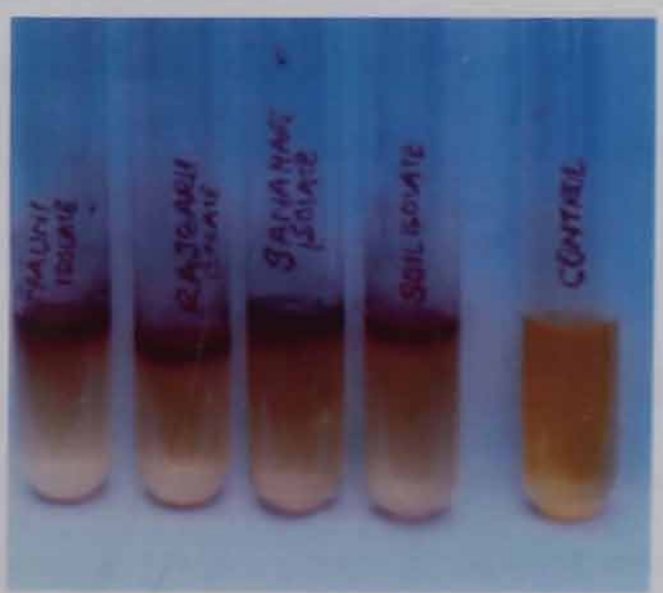
In order to find out the effective biocontrol agent, different test antagonist(s) isolated from soil were tested against *A. tumefaciens* for their antagonistic activity by dual culture technique. In dual culture technique, it was observed that the media, which supported the growth of *A. tumefaciens*, did not support the growth of test antagonist(s). Due to unsynchronized growth pattern of both test antagonist(s) and *A. tumefaciens*, the evaluation of test antagonist(s) against *A. tumefaciens* was done on four-week-old tomato plants. *Bacillus* sp. and *Trichoderma viride* (Plate- II) were found most effective as out of nine inoculated wounds, only three wounds showed gall development followed by *Pseudomonas fluorescens* where six out of nine wounds showed gall development. The soil isolate of *A. tumefaciens* produced galls on six wounds out of nine inoculated wounds as compared to control where hundred per cent gall development was observed on inoculated wounds. The gall produced after the antagonism of different isolate (rhizobacteria and *Trichoderma viride*) also varied in size. *T.viride* resulted in small gall development whereas galls produced as result of antagonism of soil isolate of *A. tumefaciens* were also tiny in size as compared to control, where galls produced were medium to large in size (Table 6).

Plate-II

Development of yellow colour in 3-keto glycoside test (positive reaction) by an isolate of *Agrobacterium tumefaciens*



Growth of *Agrobacterium tumefaciens* at 35°C on Schroth *et al.* medium



Alkaline reaction shown by different isolates of *Agrobacterium tumefaciens* in litmus milk test

***In vitro* inhibition of gall development by *Bacillus* sp. on stem of artificially inoculated tomato plant**

Table 6: Evaluation of test antagonists against *Agrobacterium tumefaciens* on tomato plants under pot culture conditions

Antagonist	No. of wounds inoculated	No. of wounds showing galls	No. of galls/wound	Size of galls
Bacillus sp.	9.00	3.00	2.00	Medium
Pseudomonas fluorescens	9.00	6.00	1.00	Medium
Trichoderma viride	9.00	3.00	1.00	Small
*Agrobacterium tumefaciens**	9.00	6.00	1.00	Tiny
Control	9.00	9.00	3.00	Medium to large
SE±	NS	1.00	0.36	
$CD_{0.05}$	NS	2.13	0.78	

* Soil isolate

Gall size

Tiny = < 0.1 cm
Small = 1 to 3 cm
Medium = 3 to 5 cm
Large = >5 cm

4.6 Effect of different antagonists, organic amendments, soil sterilizing agents, manures, fertilizers and bio-fertilizers and antibacterial compounds on the incidence and severity of crown gall on peach

Field trials were laid out to evaluate the efficacy of different antagonists, organic amendments, soil sterilizing agents, manure, fertilizers and bio-fertilizers and antibacterial compounds against crown gall in peach during 2003 (Plate- III).

4.6.1 Effect of different antagonists on the incidence and severity of crown gall on peach

Agrobacterium tumefaciens, *Pseudomonas fluorescens*, *Bacillus* sp. and *Trichoderma viride* were applied as root dip, soil drench and root dip + soil drench. Application of *P. fluorescens* as soil drench resulted in 100 per cent control of crown gall incidence followed by 2.22 and 6.89 per cent disease incidence in *Bacillus* sp. and *T. viride*, respectively applied as soil drench. The root dip treatment of *Bacillus* sp. and *P. fluorescens* having 3.14 and 3.66 per cent disease incidence, respectively were superior to *T. viride* and *A. tumefaciens* (soil isolate) as compared to control having 16.75 per cent disease incidence. The overall mean values indicated that *P. fluorescens* and *Bacillus* sp. were most effective and statistically at par with each other. There was no significant difference in mode of application. Similar trend was also observed in disease severity as no disease severity was observed in *P. fluorescens* applied as soil drench followed by 5.00, 6.84, 8.33 and 10.14 per cent disease severity, respectively observed in *Bacillus* sp. applied as soil drench, root dip + soil drench, root dip and *T. viride* applied as root dip (Table 7).

Table 7: Effect of different antagonists on the incidence and severity of crown gall on peach under field conditions during 2003

Antagonist	Disease incidence (%)			Mean	Disease severity (%)			Mean
	RD	SD	RD+SD		RD	SD	RD+SD	
Agrobacterium tumefaciens*(50 ml/10 l)	6.06 (11.89)	16.25 (23.36)	10.87 (13.46)	10.06 (16..24)^{ab}	12.50 (17.89)	33.75 (34.69)	16.25 (17.31)	20.83 (23.30)^{ab}
Pseudomonas fluorescens (50 ml/10 l)	3.66 (7.69)	0.00 (0.00)	5.05 (9.14)	2.90 (5.61)^{c}	12.62 (14.49)	0.00 (0.00)	5.00 (9.22)	5.87 (7.90)^{c}
Bacillus sp. (50 ml/10 l)	3.14 (5.18)	2.22 (6.04)	4.78 (8.99)	3.30 (6.74)^{c}	8.33 (8.82)	5.00 9.22)	6.84 (10.84)	6.72 (9.62)^{c}
Trichoderma viride (150g/ 10 l)	5.27 (9.21)	6.89 (12.08)	11.25 (16.95)	7.80 (12.75)^{bc}	10.14 (13.29)	23.75 (24.80)	15.00 (16.45)	16.30 (18.18)^{bc}
Control	16.75 (23.14)	16.75 (23.14)	16.75 (23.14)	16.75 (23.14)^{a}	27.08 (30.73)	27.08 (30.73)	27.08 (30.73)	27.08 (30.73)^{a}
Over all mean	6.97 (11.42)^{A}	8.42 (12.92)^{A}	9.74 (14.34)^{A}		14.13 (17.04)^{A}	17.92 (19.89)^{A}	14.03 (16.91)^{A}	

Figures in the parentheses are square root transformed values

*Soil isolate

	SE±	$CD_{0.05}$	SE±	$CD_{0.05}$
Antagonist (A)	(4.04)	(8.15)	(5.76)	(11.64)
Application (I)	NS	NS	NS	NS
AxI	(6.99)	(14.10)	(9.99)	(20.16)

RD= Root dip

SD= Soil drench

4.6.2 Effect of different soil amendments on the incidence and severity of crown gall on peach

Mustard cake applied @ 1.5 per cent resulted in 10.23 and 18.67 per cent disease incidence and severity, respectively. Eucalyptus leaves when applied @ 1.5 per cent also resulted in 12.35 per cent incidence as compared to control having 31.80 per cent disease incidence. Treatments of pine + deodar needles, neem cake and saw dust were not effective. Mustard cake applied @ 0.5, 1.0 and 1.5 per cent and eucalyptus leaves applied @ 0.5, 1.0 and 1.5 per cent were statistically at par with each other in reducing the disease incidence. Similar trend was also observed in disease severity where neem cake, mustard cake, pine + deodar needles and eucalyptus leaves when applied @ 0.5, 1.0 and 1.5 per cent were equally effective in reducing the disease severity as compared to control where 51.54 per cent disease severity was observed. The overall mean value further indicate that amendment of soil with mustard cake resulted in 12.04 per cent incidence and 22.87 per cent severity of crown gall as compared to control having 31.80 and 51.54 per cent incidence and severity, respectively (Table 8).

4.6.3 Effect of different soil sterilizing agents on the incidence and severity of crown gall on peach

Preplant application of formaldehyde (5%) was highly effective as it resulted in 5.39 and 14.95 per cent disease incidence and severity of crown gall, respectively as compared to control having 30.07 and 58.45 per cent disease incidence and severity of crown gall. However, application of formaldehyde when applied at 3.0, 4.0 and 5.0 per cent were statistically at par with each other in reducing the incidence and severity of crown gall. Application of sodium hypochlorite @ 0.1, 0.15 and 0.20 per cent resulted in

Table 8: Effect of different soil amendments on the incidence and severity of crown gall on peach during 2003

Organic amendment	Disease incidence (%)			Mean	Disease severity (%)			Mean
	Rate of application (%)				Rate of application (%)			
	0.5	1.0	1.5		0.5	1.0	1.5	
Neem cake	22.22 (28.10)	19.91 (26.41)	20.64 (26.93)	20.92 (27.15)[b]	24.16 (29.36)	20.71 (26.99)	23.04 (28.54)	22.64 (28.29)[c]
Mustard cake	13.75 (21.56)	12.13 (20.30)	10.23 (18.62)	12.04 (20.16)[c]	24.89 (29.40)	25.06 (29.80)	18.65 (25.30)	22.87 (28.17)[c]
Pine+Deodar needles	19.06 (25.80)	19.16 (25.860	21.53 (27.09)	19.92 (26.25)[b]	32.01 (34.44)	31.53 (33.87)	24.87 (29.72)	29.33 (22.68)[c]
Eucalyptus leaves	15.35 (22.95)	14.16 (21.94)	12.35 (20.52)	13.95 (21.80)[c]	32.47 (34.54)	32.50 (33.89)	22.50 (27.61)	29.16 (32.01)[c]
Saw dust	28.33 (31.89)	28.54 (32.04)	27.99 (31.77)	28.29 (31.90)[a]	36.50 (37.13)	41.32 (39.85)	44.06 (41.57)	40.63 (39.52)[b]
Control	31.80 (34.49)	31.80 (34.19)	31.80 (34.19)	31.80 (34.19)[a]	51.54 (45.89)	51.54 (45.89)	51.54 (45.89)	51.54 (45.89)[a]
Mean	21.75 (27.41)[A]	20.95 (26.79)[A]	20.76 (26.52)[A]		33.59 (35.13)[A]	363.71 (35.05)[A]	30.78 (33.10)[A]	

Figures within the parentheses are arcsine-transformed values

Figures represented by the same letter do not differ significantly

	SE±	$CD_{0.05}$	SE±	$CD_{0.05}$
Organic amendment (T)	(1.79)	(3.61)	(2.31)	(4.62)
Rate of application(C)	NS	NS	NS	NS
TxC	(3.11)	(6.25)	(3.99)	(8.01)

Plate-III

Field trial on the effect of different combinations of manure, fertilizers and biofertilizers on the incidence and severity of crown gall on peach

Field trial on the effect of different antibacterial compounds on the incidence and severity of crown gall on peach

Field trial on the effect of different soil amendments on the incidence and severity of crown gall on peach

Field trial on the effect of different soil sterilizing agents on the incidence and severity of crown gall on peach

15.08, 14.50 and 14.15 per cent disease incidence. Minimum disease severity (14.95 %) was observed in formaldehyde (5%) whereas application of bleaching powder @ 0.30 per cent resulted in 53.95 per cent disease severity (Table 9).

Table 9: Effect of different soil sterilizing agents on the incidence and severity of crown gall on peach during 2003

Soil sterilizing agent	Disease incidence (%)	Disease severity (%)
Formaldehyde (3%)	9.33 (17.67)	26.25 (30.80)
Formaldehyde (4%)	7.00 (15.24)	16.03 (23.54)
Formaldehyde (5%)	5.39 (13.37)	14.95 (22.47)
Sodium hypochlorite (0.1%)	15.08 (22.48)	35.04 (36.15)
Sodium hypochlorite (0.15%)	14.50 (22.25)	32.58 (34.22)
Sodium hypochlorite (0.20%)	14.15 (21.93)	42.91 (40.90)
Bleaching powder (0.15%)	25.48 (30.18)	33.20 (34.08)
Bleaching powder (0.25%)	25.08 (29.90)	48.95 (44.38)
Bleaching powder (0.30%)	25.52 (30.29)	53.95 (47.41)
Control	30.07 (33.10)	58.45 (50.02)
SE±	(2.59)	(6.45)
$CD_{0.05}$	(5.32)	(13.24)

Figures within the parentheses are arcsine-transformed values

4.6.4 Effect of manure, fertilizers and bio-fertilizers on the incidence and severity of crown gall on peach

Recommended dose of FYM + N + P + K and their further combination with *Aztotobacter chrococcum* and vesicular mycorrhizal fungi (VM) resulted in increase in disease incidence and disease severity. Minimum disease incidence (24.86%) was observed when phosphorus was applied ¾th of the recommended dose + FYM+N+K. Maximum incidence (44.16%) was observed in treatment where half of the recommended

dose of FYM was applied along with normal dose of N+P+K. In control, 25.37 and 15.44 per cent disease incidence and severity were observed, respectively, where no manure and fertilizer application was given. Minimum disease severity (15.95%) was observed in K (¾) + FYM+N+P and maximum (35.64%) in VM + *A. chrococcum*. Statistical analysis of data further revealed that all the combination of manures, fertilizer and biofertilizers except FYM (1/2) + N+P+K and N (1/2) +FYM+P+K were statistically at par with control. The reduction of FYM and N upto half of the recommended dose resulted in increase of disease incidence (Table 10).

4.6.5 Effect of different antibacterial compounds on the incidence and severity of crown gall on peach

Minimum disease incidence (16.79%) was observed in copper sulphate (1%) applied as soil drench as compared to control having 27.50 per cent disease incidence. Bleaching powder (0.3%) applied as root dip resulted in 28.98 per cent disease incidence. Minimum severity (25.00%) was observed in copper sulphate (1%) applied as root dip + soil drench as compared to control having 46.91 per cent disease severity. Treatments of copper oxychloride (0.3%) applied as root dip + soil drench, cupric chloride (0.3%) applied as soil drench and root dip + soil drench resulted in 44.52, 42.53 and 47.33 per cent disease severity, respectively (Table 11).

Table 10: Effect of manure, fertilizers and bio-fertilizers on the incidence and severity of crown gall on peach during 2003

Treatment	Disease incidence (%)	Disease severity (%)
FYM+N+P+K*	38.00 (37.13)	28.75 (32.03)
FYM(1/2)+N+P+K	44.16 (41.38)	30.00 (32.09)
FYM(3/4)+N+P+K	40.13 (38.96)	30.66 (33.36)
FYM(Double)+N+P+K	35.91 (36.43)	28.99 (31.89)
N(1/2)+ FYM+ P+ K	44.80 (42.01)	32.18 (34.280
N(3/4)+ FYM+ P+ K	37.28 (37.31)	29.58 (32.18)
N(Double)+FYM+ P+K	32.03 (33.87)	29.58 (32.18)
P (1/2)+FYM+ N+ K	37.83 (37.92)	28.37 (38.48)
P (3/4)+FYM+ N+ K	24.86 (29.20)	25.25 (29.49)
P (Double)+FYM+ N+ K	32.62 (34.70)	28.48 (31.56)
K(1/2)+FYM+N+P	39.06 (38.47)	21.56 (27.47)
K(3/4)+FYM+N+P	28.50 (32.03)	15.95 (23.21)
K(Double)+FYM+N+P	34.00 (35.41)	29.62 (32.20)
FYM + N+P+K*+VM	37.41 (37.65)	21.83 (27.47)
FYM +N+P+K*+*Azotobacter chrococcum*	38.75 (38.30)	28.40 (32.12)
FYM +N+P+K*+VM+*A. chrococcum*	37.70 (37.50)	27.45 (30.77)
VM+*A. chrococcum*	37.03 (37.46)	35.64 (36.09)
Control	25.37 (30.03)	15.44 (22.74)
SE±	(4.85)	(6.25)
$CD_{0.05}$	(9.74)	(12.55)

Figures within the parentheses are arcsine-transformed values
*FYM @ 60 MT/ha.+N @ 90 kg/ha.+P @ 30 kg/ha.+K @ 50 kg/ha.(Normal dose)
VM = Vesicular mycorrhizal fungi

Table 11: Effect of different antibacterial compounds on the incidence and severity of crown gall on peach during 2003

Antibacterial compounds	Disease incidence (%)	Disease severity (%)
Bordeaux mixture (0.8 %) RD	26.28 (30.73)	34.60 (35.93)
Bordeaux mixture (0.8 %) SD	26.34 (30.86)	37.52 (37.72)
Bordeaux mixture (0.8 %) RD+SD	27.34 (31.33)	35.76 (36.16)
Copper sulphate (1%) RD	18.70 (25.59)	29.65 (32.96)
Copper sulphate (1%) SD	16.79 (24.15)	25.54 (29.37)
Copper sulphate (1%) RD+SD	17.64 (24.81)	25.00 (29.48)
Copper oxychloride (0.3%) RD	19.19 (25.88)	43.75 (41.34)
Copper oxychloride (0.3%) SD	19.14 (25.87)	31.75 (33.90)
Copper oxychloride (0.3%) RD+SD	17.88 (25.00)	44.52 (41.79)
Cupric sulphate (0.3%) RD	18.89 (25.72)	40.00 (39.07)
Cupric sulphate (0.3%) SD	21.85 (27.76)	39.60 (38.82)
Cupric sulphate (0.3%) RD+SD	18.22 (25.46)	32.54 (34.43)
Cupric chloride (0.3%) RD	25.03 (29.66)	37.29 (37.58)
Cupric chloride (0.3%) SD	23.24 (28.42)	42.53 (40.61)
Cupric chloride (0.3%) RD+SD	22.76 (28.34)	47.33 (43.45)
Streptocycline (0.01%) RD	19.70 (26.26)	25.29 (29.78)
Streptocycline (0.01%) SD	18.50 (25.21)	36.38 (36.88)
Streptocycline (0.01%) RD+SD	20.50 (26.84)	30.50 (33.13)
Bleaching powder (0.3%) RD	28.98 (32.55)	25.63 (30.33)
Bleaching powder (0.3%) SD	22.88 (27.95)	37.65 (37.65)
Bleaching powder (0.3%) RD+SD	24.46 (29.30)	32.62 (33.95)
Control	27.50 (30.78)	46.91 (43.21)
SE±	(3.69)	(5.83)
$CD_{0.05}$	(7.38)	(11.68)

Figures within the parentheses are arcsine-transformed values

RD- Root dip
SD- Soil drench

DISCUSSION

Chapter-V

DISCUSSION

The crown gall disease caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. was widely prevalent in all the stone fruit nurseries in districts Solan, Sirmaur, Shimla, Kullu and Mandi of Himachal Pradesh. Hundred per cent incidence of crown gall and 50.00 per cent disease severity were observed in cherry at Palsehar followed by 70.00 per cent disease incidence in peach at Sanahari having 39.60 per cent disease severity during the year 2001. During the year 2002, the maximum incidence (58.00%) and disease severity (72.00%) were also observed in peach at Sanhari. At all these locations, Colt rootstocks of cherry and peach were badly affected by crown gall. Durgapal (1971) and Jindal and Sharma (1988) also reported the occurrence of crown gall widely prevalent in cherry and peach. Formation of large size tumors or galls on the stem and roots particularly near the soil line were observed by Hedgcock (1910), Jensen (1918), White and Braun (1941), Bazzi and Mazzuchi (1978) and more recently by Al-Karablich and Khlaif (2002) from different countries.

Occurrence of crown gall on stone fruits resulted in reduction of shoot length, root length, stem girth, number of leaves/plant, plant biomass but the crown area increased as a result of production of large galls. Jindal *et al.* (1990) also reported that the infected temperate fruit trees eventually die in advanced stages. The present findings however,

contradict the earlier observation made by Garrett *et al.* (1984) and Garrett (1987) who observed no significant difference in plant growth parameters of crown gall affected trees of cherry cv. Napoleon except slight effect on the girth of trees.

Isolation of *A. tumefaciens* and subsequent characterization of different isolates for biovar differentiation further revealed that all the isolates belonged to biovar I. Similar findings were observed by Jindal and Sharma (1988) who reported the occurrence of biovar I of *A. tumefaciens* on all the commercial cultivar of almond. However, Al-Karabliah and Khlaif (2002) observed the occurrence of biovar I (60.30%), biovar II (23.30%) and biovar III (1%) in addition to an intermediate biovar (15%) which clearly showed the dominance of population of biovar I of *A. tumefaciens* infecting rootstocks of stone and pome fruits in Jordan.

All the isolates showed similar cultural and morphological characteristics as described earlier (Ark and Schroth,1958; Schroth *et al.*, 1969; Zhang *et al.*, 1991; Jindal and Sharma, 1988). The morphological characteristics of different isolates of *A. tumefaciens* also resembled to those described by Agriose (1997). Jindal and Sharma (1998) also observed the small circular and smooth, glistening opaque to translucent convex white colonies on nutrient agar having similar cultural characteristics observed during the course of present investigations. All the isolates of *A. tumefaciens* typically produced small creamy galls on crown and stem portions of tomato plants within 12 days which grew in size as observed earlier (Kerr, 1969; Cooksey and Moore, 1980). On account of establishment of its pathogenicity on tomato plants, all the isolates were further identified as *A. tumefaciens*. However, the soil isolate of *A. tumefaciens* produced

tinny galls on tomato plants, which was presumed due to the less virulence as compared to other tumourgenic isolates of *A. tumefaciens.*

Different rhizobacteria viz., *Pseudomonas fluorescens, Bacillus* sp. and a soil isolate of *A. tumefaciens* and *Trichoderma viride* were further tested for their antagonistic activity against *A. tumefaciens* under *in vitro* conditions as well as cross inoculation method earlier used by Zyl *et al.* (1986), who observed the inhibition of tumour formation when the agrocinogenic strains were applied to wounds 24 hours before pathogen and by concomitant application of agrocin producer and pathogen at cell ratios of 1:10 or 1 :3. In the present studies, *Bacillus* sp., *P. fluorescens* and *T. viride* significantly inhibited gall development on tomato plants. Cross inoculation of soil isolate of *A. tumefaciens* also resulted in development of tiny galls, which showed some inhibition of tumorogenic *A. tumefaciens*. Garrett (1979) and Kerr and Panagopoulos (1977) also observed similar inhibition in gall development by different soil isolates of *A. tumefaciens*. The size of galls was small to medium. However, we were enable to evaluate potential antagonistic activity of different rhizobacteria and *T. viride* against *A. tumefaciens* by dual culture technique because of unsynchronized growth pattern of the antagonists and pathogen.

The success of *A. radiobacter* (non-pathogenic strain of *A. tumefaciens*) in controlling crown gall disease world over (Kerr 1972; Kerr and Htay, 1974; Kerr 1980; Moore, 1979 and Grimm *et al.*, 1982) prompted for the isolation of similar antagonists for its incorporation in the management programme. Isolation of different bacteria from rhizosphere soil of diseased plants of peach resulted the occurrence of hypo virulent strain of *A. tumefaciens, Bacillus* sp and *P. fluorescens*.

Pseudomonas fluorescens when applied as soil drench completely controlled crown gall disease. *Bacillus* sp. also effectively controlled crown gall when applied as soil drench and resulted in 2.22 per cent incidence followed by *P. fluorescens* and *T. viride*. Utkhede and Smith (1993) also observed root dip treatment of young apple trees with *Bacillus subtilis*, highly effective in controlling crown gall under field conditions. *P. fluorescens* provided more than 95 per cent disease control in peach field trials (Zhang *et al.*, 1991). *A. tumefaciens* when applied as root dip treatment also resulted in 6.06 per cent incidence of crown gall which showed that hypo-virulent strains of *A. tumefaciens* also able to control disease to some extent (Garrett, 1979; Kerr and Panagopoulos, 1977). *T. viride* when applied as root dip and soil drench resulted in 5.27 and 6.89 per cent incidence as compared to control having 16.75 per cent disease incidence. Deep and Young (1965) observed the increase in disease incidence of crown gall with pre-planting application of fungicides. They suggested the presence of natural fungal competition and obtained good control by using suspension of unidentified fungi as a pre-planting treatment in reduction of gall development by 25 per cent.

Amendments of soil with mustard cake @ 1.5 per cent reduced the incidence and severity of crown gall followed by eucalyptus leaves which was again statistically at par with mustard cake in providing control of crown gall. Possible reduction in crown gall incidence was attributed to the qualitative and quantitative changes in soil microbial population, which have inhibitory effects on crown gall pathogen (Khalis and Manoharachary, 1985).

Among different soil sterilizing agents, formaldehyde (5%) resulted in minimum incidence (5.39%) as compared to control having 30.07 per cent disease incidence where

as sodium hypochlorite and bleaching powder were less effective. Kapshuk (1933) also reported that chloride of lime and other compounds were lesser effective in controlling crown gall.

Recommended doses of manure, fertilizers and bio-fertilizers and their combinations had no significant effects on crown gall. The enhanced doses of manure, fertilizers and bio-fertilizers also failed to suppress crown gall in peach. *A. chrococcum* and vesicular mycorrhizal fungi failed to control the disease significantly. Godara *et al.* (1995), however observed that fertilizers although had no significant effects on crown gall but dual inoculation of *A. chrococcum* and *G. fasciculatum* completely inhibited crown gall in peach seedlings under pot culture conditions.

Copper sulphate when applied as soil drench or as root dip + soil drench was most effective in controlling crown gall incidence and severity, respectively. Grimm (1987) also obtained similar results with ammonical cupric sulphate. Streptocycline could not effectively control the disease as earlier reported by Peter and David (1958).

SUMMARY

Chapter-VI

SUMMARY

The crown gall disease caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. was widely prevalent in all the nurseries surveyed in Solan, Sirmaur, Shimla, Kullu and Mandi districts of Himachal Pradesh.

The hundred per cent incidence of crown gall was observed on cherry with 50.00 per cent disease severity followed by 70.00 and 39.60 per cent disease incidence and severity, respectively, on peach during 2001. Whereas, maximum disease incidence (58.00%) was observed on peach having 72.00 per cent disease severity during 2002. The maximum incidence of crown gall was observed on peach and Colt rootstock of cherry followed by almond, plum and apricot.

The diseased peach plants had 76.11 cm shoot length, 17.89 cm root length, 0.95 cm stem girth, 65.85 number of leaves per plants, 20.85 cm^2 leaf area and 68.50 per cent crown area covered showing reduction of 26.60, 10.91, 8.87, 13.59 and 15.46 per cent reduction in shoot length, root length, stem girth, number of leaves per plant and leaf area, respectively, over healthy plants.

A.tumefaciens could be isolated on yeast extract mannitol agar and Kado and Heskett media whereas, isolations from soil were obtained on nutrient agar and Schroth *et al.* media.

All the five isolates, three from peach, one from cherry and one from soil were Gram negative having circular to ovate or elongated off-white colonies ranging 0.7 to 1.5 cm in diameter on yeast extract mannitol agar and 1.1 to 2.2 cm in diameter on Kado and Heskett media. All the isolates were having smooth colony margins. The colonies were translucent and squishy – squashy. All the isolates had rod shaped cells generally occurring singly or occasionally in chains of 2 – 6 cells. The cell size ranging from 1-4 x 0.2 – 0.4 µm was observed in all the isolates.

On the basis of different biochemical tests viz., 3-ketoglycoside test, growth on Schroth *et al.* medium, growth at 35°C, acid from erythritol and litmus milk test, it could be concluded that all isolates belonged to biovar I of *A. tumefaciens*.

All the isolates produced typical soft and creamy galls which grew progressively upto 25 days except in soil isolate of *A. tumefaciens* which resulted in production of tinny galls on inoculated tomato plants. The soil isolate of *A. tumefaciens* was therefore considered as a hypo-virulent strain.

Cross inoculation technique was found superior to dual culture technique for screening of test antagonists against *A. tumefaciens*.

Bacillus sp., *Pseudomonas fluorescens* and *Trichoderma viride* inhibited the gall development on tomato plants as compared to *A. tumefaciens* (soil isolate). *P. fluorescens* applied as soil drench resulted in 100 per cent control of crown gall followed by *Bacillus* sp. (applied as root dip) having 3.14 per cent disease incidence as compared to 16.75 per cent disease incidence in control. Minimum disease severity (5.00%) was observed in *Bacillus* sp. applied as soil drench.

Application of mustard cake at the rate of 1.5 per cent resulted in minimum incidence (10.23%) and severity (18.65%) as compared to control having 31.80 and 51.54 per cent disease incidence and severity, respectively.

Formaldehyde (5%) was found most effective against crown gall resulting in 5.39 per cent incidence and 14.95 per cent severity as compared to control having 30.07 and 58.45 per cent disease incidence and severity, respectively.

Application of normal, ¾ , ½ and double of recommended doses of manure, fertilizer and bio-fertilizers and their combinations had no significant effect in reducing the incidence and severity of crown gall.

Among different antibacterial compounds, copper sulphate (1%) applied as soil drench was found most effective resulting in 16.79 per cent incidence and 25.54 per cent

severity, as compared to control having 27.50 and 46.91 per cent disease incidence and severity, respectively.

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*Original not seen

APPENDICES

APPENDIX –I

Culture media used for growing microorganisms

Nutrient agar

Peptone	5.0 g
Beef extract	3.0 g
NaCl	5.0 g
Agar agar	15.0 g
Distilled water	1000 ml

Adjust pH 7.0 at 25°C with 1N NaOH before adding agar

Yeast extract mannitol agar medium

Mannitol	10.0 g
L-glutamate	2.0 g
KH_2PO_4	0.5 g
Yeast extract	0.03 g
$MgSO_4\ 7H_2O$	0.2 g
NaCl	0.2 g
Agar agar	20.0 g
Distilled water	1000 ml

Adjust pH 7.0 at 25°C with 1N NaOH before adding agar

Kado and Heskett medium

Cellobiose	5.0 g
NH_4Cl	1.0 g
$MgSO_4\ 7H_2O$	0.3 g
K_2HPO_4	3.0 g
NaH_2PO_4	1.0 g
Malachite green	0.01 g
Agar agar	15.0 g
Distilled water	1000 ml

Adjust pH 7.0 at 25°C with 1N NaOH before adding agar

Schroth *et al.* medium **Per Litre**

Mannitol	10.0 g
$NaNO_3$	4.0 g
$MagCl_2$	2.0 g
Calcium propionate	1.2 g
$Mg_3(PO_4)_2$	0.2 g
$MgSO_4.7H_2O$	0.1 g
$NaHCO_3$	75.0 mg
$MgCO_3$	75.0 mg
Agar agar	20.0 g
Autoclave, cool to 50-55°C and add (final conc.)	µg/ml
Berberine	275
Sodium selenite	100
Penicillin G (1,625 units/mg)	60
Streptomycine sulphate (78.1% streptomycin base)	30
Cycloheximide (85-100% active ingredient)	250
Tyrothricin (pure)	1
Bacitracin (65 units/mg)	100

Adjusted pH 7.1 at 25°C with 1.0 N HCl before adding agar- agar

Soil extract agar medium

Glucose	1.0 g
K_2HPO_4	0.5 g
Soil extract	100 ml
Peptone	1.0 g
Yeast extract	1.0 g
Cycloheximide	10.0 ml
Agar agar	20.0 g
Distilled water	900 ml

APPENDIX –II

Gram staining

Various steps involving in Gram staining

1. On a clear slide dry a thinly spread bacterial film in air without heat then lightly flame the underside of the slide twice to fix the bacteria to the slide.
2. Flood the smear with a crystal violet solution for one minute.
3. Wash in tap water for a few seconds, drain off excess water and lightly blot dry on a paper towel.
4. Flood the smear with iodine solution for one minute.
5. Wash in tap water for a few seconds, blot dry.
6. Decolorize with solvent e.g. ethyl alcohol until the solvent flows colourlessly from the slide for about 30 seconds, blot dry.
7. Rinse in tap water for few seconds.
8. Counter the stain for about 10 seconds with safrinin solution.
9. Wash briefly in tap water, blot dry and examine.

Here the primary strain is crystal violet, mordant means ionizing agent is iodine. Decolorizer is 95 per cent ethyl alcohol. Secondary stain is safrinin. The bacterial cells, which retain the colour of crystal violet, are Gram positive and the cells, which retain the colour of safrinin, are Gram negative

APPENDIX –III

Biochemical tests to establish the identity of different biovars

3-ketoglycoside test

Smear bacterial inoculum over about a 0.5 cm diameter spot on medium containing 1% lactose, 0.1% yeast extract and 2% agar and incubate the plate at 25-30°C for 1-2 days. Four to six strains can be applied to the same plate. Afterwards, flood the agar surface with a shallow layer of Benedict's reagent and leave at room temperature. If 3-ketolactose is present, a yellow ring of Cu_2O becomes visible around the cell mass, reaching maximal size after about one hour.

Benedict's reagent: Dissolve 173 g of sodium citrate and 100 g of anhydrous sodium carbonate in about 600 ml of distilled water, with heating. Filter the resulting solution if a precipitate forms. Dissolve 17.3 g of cupric sulphate in about 150 ml distilled water. Slowly add the cupric sulphate solution to the sodium citrate-sodium carbonate solution while the latter is contained in a large beaker, with constant stirring. Dilute to one liter.

Growth on Schroth *et al.* medium

Add 0.1 ml bacterial suspension (10^3 c.f.u./ml) to the poured plate of Schroth *et al.* medium and spread the inoculum with the help of sterilized L- shaped glass rod. Incubate the plate at 25 ± 1°C in BOD incubator for 7 days.

Growth at 35°C

Add 0.1 ml bacterial suspension (10^3 c.f.u./ml) to the poured plate of Schroth *et al.* medium and spread the inoculum with the help of sterilized L- shaped glass rod. Incubate the plate at 35 ± 1°C in BOD incubator for 7 days.

Acid production from Erythritol

Agrobacteria strains are inoculated to culture tubes (1x12.5 cm) containing the following medium:

Basal medium	**Per liter**
$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	1.0 g
Bromothymol blue	0.3 ml*

Adjust pH 7.1 with 1 N NaOH before adding agar

Agar agar 1.5 g

*1 per cent (w/v) solution in 50 per cent ethanol

Erythritol solution: Add 1 part of filter- sterilized 10% (w/v) erythritol to 9 parts sterile, cooled basal medium; then dispense aseptically to sterile plugged tubes to a depth of about 4 cm.

Development of a yellow colour in the medium indicates production of acid from the oxidation of erythritol.

Reaction on litmus milk

Take yeast extract mannitol broth medium (10 ml) in test tube. Add a loopful bacteria suspension (10^3 c.f.u./ml) to it. After two days of incubation at 25±1°C add 0.2 ml of litmus milk solution to the test tube. Observe the tube for reaction either alkaline or acidic.

APPENDIX –IV

Recommended Package of Practices for peach plant nurseries

Cultivar: Redhaven

Manure and fertilizer: FYM @60 MT/ha. + N @ 90 kg/ha.+P @ 30 kg/ha+K @ 50kg/ha (Normal dose) applied as CAN (25%), SSP (16%) and MOP (45%).

Remarks

Apply FYM during December-January along with half N,P and K. Apply remaining half N in the month of August.

Cultural practices

Removal of weeds from the beds is must either mechanically after every 15 days of interval. To conserve moisture, mulching of dried grass or hay 10-15 cm in thickness in the bed basin is followed.

Irrigation

The irrigation is very important to nursery beds after every three days of interval upto 50 per cent of field capacity.

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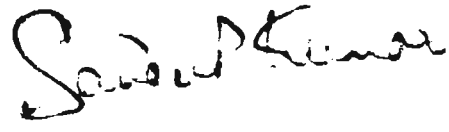

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ABSTRACT

The present investigations on "Studies on crown gall of stone fruits" were undertaken to study the occurrence, incidence and severity of disease, effect on plant growth parameters, cultural and morphological characteristics, differentiation of biovars of *Agrobacterium tumefaciens*, evaluation of test antagonist(s) against *A. tumefaciens* on tomato plants and effect of different antagonists, soil amendments, soil sterilizing agents, manure, fertilizers, biofertilizers and different antibacterial compounds on the management of crown gall disease. Hundred per cent disease incidence along with 50 per cent severity of crown gall were recorded on cherry at Palschar in district Mandi. Crown gall infected peach plants showed reduction in all the growth parameters, maximum in shoot length i.e. 26.60 per cent. The best growth of *A. tumefaciens* was observed on yeast extract mannitol agar and Schroth *et al.* medium from galls and soil, respectively. All the isolates of *A. tumefaciens* were Gram negative having circular to ovate or elongated off-white colonies ranging from 0.7 to 1.5 and 1.1 to 2.2 cm in diameter on yeast extract mannitol agar and Kado and Heskett medium, respectively. The rod shaped cells ranged in size of 1-4 x 0.2 to 0.4µm. All the isolates belonged to biovar I group. *Bacillus* sp., *Pseudomonas fluorescens* and *Trichoderma viride* inhibited the gall development on tomato plants as compared to *A. tumefaciens* (Soil isolate) under pot culture conditions. In field trials, mustard cake (1.5%), formaldehyde (5%), copper sulphate (1%) applied as soil drench were most effective in reducing the disease incidence and severity. Among different antagonists, *Bacillus* sp. and *Pseudomonas fluorescens* were found effective *in vivo*.

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