PLANT GROWTH PROMOTING RHIZOBACTERIA-INDUCED SYSTEMIC RESISTANCE AGAINST ToLCV DISEASE IN TOMATO (*Lycopersicon esculentum* Mill.)

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SHEFALI MISHRA

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD – 580 005

JUNE, 2012

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1. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a herbaceous fruiting plant. It was originated in Latin America and become one of the most widely grown vegetables with ability to survive in diverse environmental conditions (Rice *et al*., 1987). Tomato fruit is considered to be rich in vitamins A and C, it has high cash value with potential for value-added processing. Recently, there has been more emphasis on tomato production not only as a source of vitamins, but also as a source of income and food security. Apart from this, lycopene present in this fruit is valued for its anti-cancer property, since it acts as an antioxidant and scavenger of free radicals. Tomato cultivation has become increasingly popular since the mid-nineteenth century because of its varied climatic tolerance and high nutritive value.

India is the third largest tomato producer in the world after China and USA, accounting for about 8% of the World tomato production. Tomato is one of the most popular vegetables at both the national and state level. In terms of area, it occupies third largest place after potato and onion at the national level (Anon., 2010). During 2008–09, the area of tomato, in India, was about 5,99,000 ha with a production of 11,149,000 MT, respectively (Anon, 2010). In Karnataka, it occupies an area of 46,000 hectares with a production of 17.65 lakh tonnes (Anon., 2001). Bangalore and Kolar districts produce nearly 35 per cent of total tomato production in Karnataka (Anon., 1998). Though, the area under tomato cultivation is high, the productivity (15 t/ha) is rather low. This is attributed to the potential loss in yield due to a number of diseases.

Besides fungal, bacterial and phytoplasmal infections, it is also affected by a large number of viral diseases (Anon., 1983). Of all the viral diseases reported on tomato, tomato leaf curl virus (ToLCV), a geminivirus (Geminiviridae: subgroup – III) is the most important and destructive viral pathogen in many parts of India (Vasudeva and Samraj, 1948: Sastry and Singh, 1973; Saikia and Muniyappa, 1989; Harrison *et al*., 1991). The incidence of ToLCV in tomato growing areas of Karnataka ranged from 17-100 per cent in different seasons and 50 to 70 per cent yield loss was observed in tomato Cv. Pusa Ruby grown in February – May (Saikia and Muniyappa, 1989). Devaraja *et al.,* (2005) reported cent per cent infection in summer month which caused yield losses ranging from 27 to 90 per cent in Karnataka. The growth of the plants due to leaf curl virus infection was retarded very much and produced comparatively very few leaves and fruits when the plants were infected within four weeks after transplanting, resulting in losses up to 92.3 per cent (Sastry and Singh, 1973).

Tomato leaf curl virus disease is characterized by the curling and twisting of leaves followed by marked reduction in leaf size. The diseased plants look pale and stunted due to shortening of internodal length with more lateral branches resulting in a bushy appearance (Vasudeva and Samraj, 1948). Whitefly *Bemisia tabaci* (Gennadius) (Homoptera : Aleyrodidae) is the vector of the virus (Vasudeva and Samraj, 1948 ; Butter and Rataul, 1973; Muniyappa and Veeresh, 1984). The vector life period is very short during May-September being 11-14 days, as against 43-83 days during December to February (Mohanty and Basu, 1987) due to variation in temperature.

The incidence of ToLCV has become a major limiting factor and challenge to farmers as well as scientists. Tomato plants of all ages are vulnerable to ToLCV and generally exhibit symptoms after two to three weeks of infection. Development of strategies for integrated disease management would significantly reduce yield loss. Many approaches like checking the vector population by using trap crops, barriers and application of systemic insecticides might reduce the ToLCV incidence to certain extent (Sastry and Singh., 1974; Saikia and Muniyappa, 1989). Use of genetically resistant varieties, integration of selected cultural practices, application of insecticides to control insects that might serve as vectors and their combinations (Hull, 1994) have been attempted. Two additional approaches for managing viruses include cross protection and development of genetically engineered plants that express a viral structural or non-structural protein (Fitchen and Beachy, 1993). The use of genetically resistant varieties is clearly the most economically and environmentally sound choice. However, commercially acceptable varieties which resist virus infection are not always available.

Cross protection has been used successfully with several virus host systems. But this approach is not feasible with some crops and there are obvious risks associated with inoculation of a crop with an infectious agent (Lecoq, 1998). The efficacy of reducing virus infection via control of its vector through application of insecticides is dependent on the mode of transmission. To be effective, this approach requires timely insecticidal application, based on the knowledge of vector ecology within a given area. Since there are no viricides to control viruses, vector control through spray of chemicals in many instances does not bring about the desired result. The application of insecticides, however, also has associated environmental concerns. Insecticides are non-biodegradable and toxic complexes. When the doses exceed, they become phytotoxic and pollute the environment. Further, they are also toxic to beneficial microorganisms in the soil (Newell *et al.*, 1981).

Under the above circumstances, it becomes inevitable to develop a bio-based, ecofriendly, biodegradable, plant derived or microbial derived method in order to control plant pathogens. Hence, in this context, management of tomato leaf curl virus through biocontrol agents is an alternative strategy, which is also ecologically sound and environmentally safe. Recent investigation on the mechanism of biological control by plant growth promoting fluorescent *Pseudomonas* revealed that these strains protect plants from various pathogens in several crops, by activating defense genes encoding chitinase, beta-1,3 glucanase, peroxidase, phenylalanine ammonia lyase and other enzymes (Maurhofer *et al.*, 1994). Induced systemic resistance once expressed, activates multiple potential defense mechanisms that enhance the increased activity of chitinase and peroxidase, which showed resistance to various pathogens (Xue *et al.*, 1998).

The investigation of plant response to elicitiors is one of the most rapidly developing lines of inquiry in plant physiology. The elicitiors stimulate the contacts of plant-phyto-pathogens and thereby trigger defensive mechanisms that constrain the invasion of pathogenic fungi, bacteria and viruses. Chitosan is one of the most studied elicitiors. It regulates the expression of resistance genes and induces jasmonate synthesis (Doares *et al.*, 1995). Fragments from chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infection, inducing the accumulation of phytoalexins, pathogen-related (PR) proteins and proteinase inhibitors, lignin synthesis and callose formation. Chitin and chitosan are naturally-occurring compounds that have potential in agriculture with regard to controlling plant diseases. These molecules were shown to display toxicity and inhibit fungal growth and development. They were reported to be active against viruses, bacteria and other pests (Abdelbasset *et al.*, 2010). Based on these and other proprieties that help strengthen host plant defenses, interest has been growing in using them in agricultural systems to reduce the negative impact of diseases on yield and quality of crops.

The Departments of Agricultural Microbiology and Biotechnology, University of Agricultural Sciences, Dharwad has a large collection of rhizobacterial strains. These strains have been earlier screened for biocontrol of early blight of tomato, Tobacco Mosaic Virus in tomato and Yellow Vein Mosaic of okra (Bhendi). These strains have been found effective in controlling these diseases and promoting plant growth under green house conditions. Keeping these points in view, the following objectives were addressed during the present investigation.

1. Preliminary screening of the rhizobacterial collection for their ability to control ToLCV in tomato and selection of the potential strains.
2. Elucidation of the mechanisms of biocontrol by the selected strains.
3. To assess the effect of combination of PGPR strains and chitosan on the efficacy of biocontrol of ToLCV.
4. Field evaluation of the selected efficient combination of PGPR and chitosan.

2. REVIEW OF LITERATURE

Biological control of plant diseases using microorganisms offers an excellent alternative to chemical control. A vast number of microorganisms present in the rhizosphere have been considered as important in sustainable agriculture because of their biocontrol potentials and plant growth promotional activities. This chapter will provide an overview of the current status of the research on the biocontrol of viral diseases by rhizobacteria.

2.1 Viral diseases of tomato

About 146 viruses infect tomato worldwide (Green, 1991). They are grouped in 33 genera, but 15 genera are most economic important, i.e. *Alfalfamovirus, Begomovirus, Carlavirus, Crinivirus, Cucumovirus, Ilarvirus, Luteovirus, Nepovirus, Potexvirus, Potyvirus, Tobamovirus, Tombusvirus, Topocuvirus, Tospovirus,* and *Tymovirus*. These fifteen genera belong to families *Bromoviridae, Bunyaviridae, Closteroviridae, Flexiviridae, Geminiviridae, Luteoviridae* and *Potyviridae* (Pringle, 1999). Family *Bunyaviridae* has only one assigned plant-infecting genus (*Tospovirus*) to which *Tomato spotted wilt virus* (TSWV) belongs. Other genera of this family consist of virus species that infect animals only. Family *Flexiviridae* has been recently approved by ICTV (Mayo and Brunt, 2005); its major tomato virus is *Potato virus X*, which belongs to the genus *Potexvirus*. Amongst the viruses discussed above, tomato leaf curl virus disease of family *Geminiviridae* is the most important and causes huge economical losses in India (Vasudeva and Samraj, 1948, Banerjee and Kalloo, 1987, Saikia and Muniyappa, 1989).

2.1.1 Symptomology

Viruses that cause tomato leaf curl, yellowing and chlorosis, and that are transmitted by whiteflies, belong to families *Geminiviridae* and *Closteroviridae*. Viruses of the family *Geminiviridae* cause mostly leaf curl, small round leaflets and marginal yellowing (Czosnek and Laterrot, 1997, Cohen and Nitzany, 1966), whereas those of the *Closteroviridae* induce infectious chlorosis .The symptoms of tomato leaf curl virus (ToLCV) include vein clearing, reduction in leaf size, stunted growth, deformation of leaflets, inward and outward curling and puckering of leaflets. The infected plants produce few fruits in case of late infection and no fruits, if infected at very early stage (Plate1).

The diseased plants usually develop purple patches especially on older leaves (Sastry and Singh, 1973, Saklani and Mathai, 1977; Seetharama Reddy, 1978; Capoor, 1981 ; Saikia and Muniyappa, 1989).Yassin and Nour (1965) described tomato leaf curl symptoms *viz.,* leaf curling, stunting of plants, thickening, greening of the veins of leaves. Similar symptoms were described by Vasudeva and Samraj (1948). Gevorkyan *et al.* (1976) reported that the growth and development of tomato plants infected by leaf curl virus were considerably delayed. The disease was accompanied by a decreased content of green and yellow pigments and increased total nitrogen and accumulation of hexose and saccharose.

2.1.2 Family *Geminiviridae*

According to ICTV (International Committee on Taxonomy of Viruses), this family is divided into four genera: *Mastrevirus, Curtovirus*, *Begomovirus* and *Topocuvirus* (Pringle, 1999). The first two genera include viruses infecting maize (*Maize streak virus*) and beet (*Beet curly top virus*), respectively. Members of the genera *Begomovirus* and *Topocuvirus* infect tomato. Genus *Begomovirus,* to which *Bean golden mosaic bigeminivirus* belongs as the type species, is the only genus of the family *Geminiviridae* that has viruses infecting tomato in both the New and Old Worlds. Genus *Topocuvirus* has only one member, *Tomato pseudo curly top virus*, which is the type species. It has a monopartite genome and also infects dicotyledonous plants. Geminiviruses have either a monopartite genome or a bipartite genome. According to Brown (1997), monopartite genomes have sizes of 2.7 to 2.8 kb and contain at least six genes. On the other hand, bipartite genomes are 5.2 to 5.4 kb in size, and have two genomic components, named A and B or DNA 1 and DNA 2. Geminiviruses with the bipartite genome were especially reported in the New World (Davies *et al*., 1989, Rochester *et al*., 1994).

Stnted plant growth with prominent Yellow margins

Upward curling of leaves

Withhering of flowers

Reduced Leaflets size erect shoots

Reduced Leaflets size with Puckered leaves

Distored leaves with yellow margins

Plate 1. Typical symptoms of ToLCV

2.1.3 Tomato leaf curl viruses

Tomato leaf curl disease is caused by a number of different Gemini viruses vectored by the whitefly, *Bemisia tabaci* Gennadius (McGrath and Harrison, 1995, Ramappa *et al.,* 1998). Begomovirus genomes described to date can be seen to fall into a number of genome categories, the main division being into monopartite or bipartite depending on whether they have one or two circular ssDNA components. Most of the described begomoviruses are bipartite containing DNA-A and DNA-B molecules, each of these being approximately 2600–2800 nucleotide in size, which are prevalent under north Indian conditions. However, some old World tomato viruses, Tomato yellow leaf curl virus (TYLCV) and Tomato leaf curl virus-[AU] lack a DNA-B component, requiring only their DNA-A component to systemically and symptomatically infect plants (Dry *et al.,* 1993, Navot *et al.,* 1991).

The symptoms caused by this virus are leaf curling, blistering, reduced leaf size, shortened internodes, leathery leaves, chlorosis of leaf margins, rounding of leaflets, flower abscission and poor bearing (Cohen and Nitzany, 1966, Saikia and Muniyappa, 1989).

According to Kumar *et al* (2008), there are two distinct TLCVs under north Indian conditions based on nucleotide sequence comparisons. It is also considered that viruses of the genus *Begomovirus,* which have nucleotide sequence similarity levels below 90 per cent are distinct from each other (Padidam *et al*, 1995), although later on, ICTV reported that this could only be concluded when complete genome sequences have been compared, and not on the basis of the intergenic region (IR) or coat protein gene alone. Similarity comparisons have previously been done on the basis of the intergenic region and partial sequences for other TYLCVs including isolates from Egypt and Israel, which are similar but different from isolates from Spain (GenBank No. L 277081) and Sicily (GenBank No. Z28390) (Noris *et* al, 1993, Cohen and Antignus, 1994). So there is a need to characterize and compare ToLCV isolates, with other isolates.

2.1.4 Distribution of Tomato Leaf Curl Viruses

ToLCV is quite general in the tropics. It is widely spread in the Old World and in the New World, e.g. in South East Asia and East Asia, the Americas and the Mediterranean (Green and Kallo, 1994, Chiang *et al.,* 1996, Czosnek and Laterrot, 1997). In Africa, it has been reported from South Africa, Senegal, Tanzania, Malawi, Zambia, Zimbabwe, Nigeria, Ivory Coast, Egypt and Sudan (Yassin and Nour, 1965, AVRDC 1987, Czosnek *et al.*, 1990, Nakhla *et al.*, 1993, AVRDC 1994, Nono-Womdim, 1994 and Chiang *et al.*, 1996).

2.1.5 Transmission of Tomato Leaf Curl Viruses

ToLCV is transmitted by whitefly (*Bemisia tabaci* Gennadius) of the Family *Aleyrodidae*, (Gerling and Mayer 1995). *Bemisia tabaci* occurs in biotypes A and B. Biotype B is more common than A and is regarded by some as a separate species designated as *B. argentifolii* (Bellows *et al.,* 1994). Others continue to regard it as a biotype of *B. tabaci*, even though there are many biotypes, which include biotype Q (Demichelis *et al.,* 2000). In some circumstances, the incidence and rate of spread of TYLCV are directly proportional to the whitefly population present in the environment (Mansour and Al-Mousa 1992 and Mehta *et al* 1994). Both adults and nymphs can acquire the virus by feeding on infected plants with a minimum access and acquisition period (AAP) of 15 minutes. The virus has a latent period of 21-24 hours and persists for 10 to 20 days in viruliferous *B. tabaci* adults (Cohen and Nitanzy, 1966). For the whitefly to transmit the virus persistently, it must have adequate inoculation access periods (IAP) following acquisition access periods (AAP) (Cohen and Harpez, 1964).

Cohen and Antignus (1994) demonstrated that TYLCV could be found as double-stranded DNAs in viruliferous whiteflies, which implies replication of viral DNA in the vector. Once the whitefly has acquired the virus, it can continue transmission through out its life (Brown, 1997). These observations are opposed to earlier findings (Cohen and Nitzany, 1966), which stated that TYLCV triggers an antiviral mechanism in *B. tabaci,* preventing multiplication of the virus in the vector and necessitating the need for repeated acquisition (Cohen and Harpaz 1964). In another study, Briddon *et al.* (1990) and Hiebert *et al.* (1995) reported that, factors involved in virus transmission included presence of viral coat protein and genes on the complementary DNA strand with open reading frames ACI, AC2, AC3, and AC4.

The presence of viral DNA in the vector is a proof of vector transmission of that particular virus (Navot *et al* 1992). It can be determined by using modern techniques like Polymerase Chain Reaction (PCR). Navot *et al* (1992) used specific primers to achieve high level DNA amplification in PCR experiments. However, the best approach for use in developing countries with limited research facilities would be the use of DNA hybridisation to check for viral DNA in plants and whiteflies (Navot *et al.*, 1991). Even though serological techniques are the easiest, they have limited sensitivity (Credi *et al*, 1989, Chiemsombat *et al*. 1991). While assessing other methods of TYLCV transmission, Makkouk *et al.* (1979) established that direct contact between plants, natural root grafting through adjacent roots, seed infection, and soil contamination are not effective in transmitting TYLCV, and that the only efficient method of transmission is by *B. tabaci* or *B. argentifolii*.

2.1.6 Epidemiology of Tomato Leaf Curl Viruses

Tomato cultivation, especially in autumn in north India and in summer season in south India is adversely affected due to the high incidence of tomato leaf curl virus disease and losses often exceeds 90 per cent (Butter and Rataul, 1981, Saikia and Muniyappa, 1989). Sastry *et al*, (1978) demonstrated that tomato crop planted during December to May, resulted in lower yield due to tomato leaf curl virus incidence. Shaheen (1983) suggested that *Bemisia tabaci* attacked tomato in April-November with infestation peak in August-October. Saikia and Muniyappa (1989) reported that tomato plants were susceptible for infection to ToLCV at all stages of their growth. Epidemiological studies by Moustafa (1991), in the semi-tropical climatic zone of Egypt, indicated that at the beginning of spring and early summer (February - April), when tomato plants have just established, TYLCV incidence was very low. The latter became high towards the end of summer (September– mid-October), and then coincided with peak whitefly population density (Riley *et al*, 1995). This was followed by high TYLCV incidence and severe damage in the fall (autumn) when production losses rise to 80 per cent and almost all plants were infected. Similarly, Cohen and Antignus (1994) observed that in the Jordan Valley, the spread of TYLCV was significantly correlated with *B. tabaci* population size. In Egypt also, peak whitefly population occurred between the first week of September and Mid-October. In Tanzania, TYLCV symptoms and whitefly vector presence reported to be most common during November to February (Nono-Womdim *et al*, 1996).

Another factor contributing to high incidence of ToLCV is proximity to old host crop fields. Mazyad *et al*., (1994) found that adjacent old fields of vegetables and other field crops present at tomato planting played a big role in harboring whiteflies, which eventually infested tomatoes. Similar observations were made in Egypt, Cyprus, Lebanon, Jordan, Saudi Arabia and Israel (Ioannou and Hadjinicolis, 1991, Mazyad *et al*., 1986).

2.2 Importance of ToLCV disease

Tomato leaf curl virus was reported to be a serious disease on tomato throughout India. The disease may cause up to 75 per cent or more reduction in fruit yield and due to its devastating nature, it has become a national problem (Sastry and Singh, 1973; Saikia and Muniyappa, 1989). Sastry and Singh (1973) reported that ToLCV infected plants produced very few fruits when infected within 20 days after planting, resulting up to 92.3 per cent yield loss. Plants infected 35 and 50 days after transplanting, resulted in 74 and 22.9 per cent yield loss respectively. An average of 39 per cent losses in tomato yield due to ToLCV was reported annually from Somalia (Castellani *et al.,* 1981). Banerjee and Kalloo (1987) reported that major constraint in the cultivation of tomato was the outbreak of ToLCV during summer in South India and autumn in North-India. Saikia and Muniyappa (1989) studied in detail the incidence of ToLCV in some tomato growing areas of Karnataka and reported that the incidence ranged from 17-53 per cent in July-November to 100 per cent during February – May grown crop resulting in heavy yield losses. ToLCV resulted up to 75 per cent or more reduction in fruit yield (Yasin and Nour, 1965).

2.3 Detection of tomato leaf curl viruses

Serological tests have played a big role in identifying tomato leaf curl viruses. They are widely used, but have limitations because of the need to obtain sufficient purified coat protein for the production of antisera (Czosnek *et al* 1988, Credi *et al* 1989, Chiemsombat *et al* 1991). Initially, polyclonal antibodies were used until Muniyappa and Veeresh (1984) and others began using monoclonal antibodies.

They found that the advantages of using monoclonal antibodies are (1) though different viruses have the same epitopes, there are antibiotics that are specific to one particular virus; and (2) geminiviruses from the same geographic areas tend to share more epitopes than viruses from different regions. This, therefore, served as a basis for use of monoclonal antibodies to study relationships among geminiviruses using monoclonal antibodies raised against *African cassava mosaic virus* (ACMV) (Macintosh *et al* 1992).

However, Polymerase chain reaction (PCR) is now more widely adopted because of easy application, sensitivity and specificity for detection and identification of geminiviruses in epidemiological and disease management studies. Navot *et al* (1992) were able to amplify the genomic DNA molecule of an Israel isolate of *Tomato yellow leaf curl virus* (TYLCV-Is) from total DNA extracts of TYLCV-infected plants. Rojas *et al* (1993) took advantage of geminiviruses replicating via a double-stranded, circular DNA form to characterize bipartite geminiviruses from the Americas. Deng *et al* (1994) and Wyatt and Brown (1996) designed universal primers for the identification of the begomoviruses. Nakhla *et al* (1994), Aref and Doug dong (1996), McGlasham *et al* (1994) and Martino *et al.* (1993) used PCR for confirmation of virus presence in the sample with help of different primers. Through the PCR process, a specific DNA fragment that lies between two primer-annealing points is amplified. Degenerate (general) primers are used for general amplification of part of the viral genome sequence. Oligonucleotide (specific) primers, which anneal to either C1 or V1, are used for specific amplification of desired fragments of TYLCV DNA sequence (Nakhla *et al* 1994). Khan *et al*. (2002) could detect ToLCV both in its host *Lycopersicon esculentum* and vector *B. tabaci* by employing geminivirus specific degenerated primers using PCR. Muniyappa *et al.* (2000) used polymerase chain reaction for detection of ToLCV using degeneated primers both in infected tomato plants and viruliferous *B. tabaci*.

Therefore, both degenerate and specific primers could be used to identify and characterize ToLCV occurring in Karnatka and India. A more recent and improved PCR now exists. It employs more than one primer pair, mostly specific ones, to target specific parts of the replication gene and intergenic region. This method is called multiplex PCR (Potter *et al.,* 2003) and it is reported to be faster and even cheaper than the PCR technique described above.

2.4 Histopathological changes due to ToLCV infection

Histopathological and biochemical changes take place in tomato leaves after infection by ToLCV resulting in external manifestation in the form of different symptoms Cook (1925) in a study on histology and cytology of sugarcane affected by sugarcane mosaic virus reported that the host nuclei were usually very much enlarged in diseased tissues especially in leaves. He further reported that nuclei were very irregular in form and it was very difficult to find intracellular bodies associated with them. The chloroplasts were smaller and fewer in mosaic virus affected leaf cells.

Sorokin (1927) reported that mosaic affected cells of tomato leaves were almost isodimetrical and there was no differentiation of tissues into palisade and spongy parenchuma. The chloroplasts were destroyed due to dissolution of proteins in the stroma.

Easu (1933) stated that the curly top disease induced pronounced changes in affected leaves of sugarbeet involving hypertrophy, hyperplastic, hypoplasia and necrosis. He further reported that vein clearing in the leaves of sugar beet affected by curly top was de to cell enlargement near the veins. Lack of intercellular spaces and chloroplast degeneration. Stone (1942) found little effect of the mild mosaic virus on the leaf thickness in potato and chloroplasts were reduced in size and number.

Bawden (1943) suggested that the common absence of the symptoms in matured leaves when plants were infected with virus could be probably due to low virus content in such leaves rather than by the inability of the virus to affect mature plastids. He further reported that under development and actual destruction of plastids play a role in the differentiation of chlorotic patterns in virus diseased plants.

Abbot and Sass (1945) reported that yellow streaks in sugarcane affected with chlorotic streak showed reduction in size and number of chloroplasts, often thinner than the adjacent areas which eventually became necrotic.

Jeyarajan and Ramakrishnan (1961) have reported the reduction in polyaccharides in chilli plants affected with a sap transmissible virus.

Mishra and Singh (1973) showed that leaves of *Capsicum* plants attacked by chilli mosaic virus were smaller with little differentiation between palisade and spongy parenchyma.

Joshi and Dubey (1974) stated that RNA content of CMV infected leaves in chilli plants was first higher than in the healthy plants, but declined later on compared to that of healthy plants. He observed the length and breadth of palisade cells of diseased chilli leaves infected with cucumber mosaic virus which showed a clear distinction between palisade and spongy parenchyma and were quite reduced in size in comparison to healthy ones.

Russo and Martell (1975) reported that the nuclei of infected tobacco cells were deformed and reacted to cytochemical stains for DNA and histones differently from nuclei of healthy cells. There was also variation in chromatin distribution. They further reported that these changes were due to nuclear involvement in virus multiplication.

Ehara and Misawa (1975) reported the occurrence of abnormal chloroplasts in tobacco leaves infected systemically with ordinary strain of cucumber mosaic virus. They further reported that chloroplasts appeared to be only partially involved in virus synthesis and accumulation of chloroplast changes may be caused mostly by changes in cell metabolism after infection.

Alok *et al.* (1986) studied the histopathological changes in tomato induced by TMV and or *Alternaria solani*. The plants inoculated with virus alone exhibited the disintegration of palisade tissue with wider space in spongy parenchyma. The *A. solani* alone inoculated plats showed leaves with broken epidermal cell wall and necrosis with collapsed tissue, while there was broken and thinner palisade tissue in plants inoculated with both the pathogens.

Giri and Mishra (1990) reported that the loss in number and weight of tomato fruits was depended on viruses involved in mosaic. The reduction in fruit number and weight due to TMV was 34.02 and 59.77 per cent, respectively, while it was 40.87 and 40.23 per cent, respectively for CMV infection. The sterility due to CMV and TMV was 12.20 per cent and 5.59 per cent respectively, while it was 7.23 per cent in healthy plants.

Channarayappa *et al.* (1992) reported that there was hypertrophy of the nucleolus, segregation of nucleolar components into discrete granular and fibrillar regions with electron dense regions in the nucleus and the virus particles was loosely or hexagonally packed in symmetrical arrays. Further, the chloroplasts of ToLCV infected cells showed considerable disturbance in internal and disorganized with excessive accumulation of osmiophilic bodies in degenerating chloroplasts.

Thind *et al.* (1996) also recorded reduction in the amount of reducing sugars, non-reducing sugars, total sugars, starch and total chlorophyll in the plants infected with yellow mosaic virus as compared to healthy plants. While, there was increase in total phenols, ribonucleic acid (RNA), Fe, Mn and Cu due to virus infection.

Banerjee and Kalloo (1998) recorded high phenol and high crude protein content in highly resistant *Lycopersicon hirsutum* f. sp. Glavbratum B6013 and resistant *L. pimpinellifolium* A1921 lines to tomato leaf curl virus as compared to susceptible varieties.

Singh (1971) observed that, in papaya leaves infected with the PRSV, the palisade layers and spongy cells became smaller. Chloroplast size and number got reduced. The leaves had broad inter cellular spaces. Three types of symptoms were also observed *viz.,* hypoplasia, hyperplasia and hypertrophy, which often occurred in combination. The virus infection reduced the latex flow from the infected fruits of papaya and also the sugar content by 42 to 55 per cent as well as reduced the yield (Singh, 1969, Singh and Mishra, 1969 and Khurana, 1970).

The PRSV destroyed the chlorophyll A and B, the latter being in greater extent. The chlorophyll and protein contents reduced by 24.3 and 41.0 per cent respectively (Singh *et al.,* 1977 and Sun, 1985). Diseased plants had higher amount of total nitrogen. The virus infection resulted in a significant decrease in the dry weight (25%), total chlorophyll (76%), total sugars (43%), reducing sugars (33%) and non reducing sugars (51%) as compared to healthy leaves (Khurana, 1970, Singh, 1973 and Johri, 1975a).

Sun (1985) studied effects of leaf mosaic infection in photosynthetic electron transport chain of chloroplast from papaya eaves. The results indicated that the photo reduction cytochrome F is partially blocked when the leaves were infected, but there was no effect on the cyclic electron transport chain.

2.5 Management of tomato leaf curl virus

The most effective way of managing *Tomato leaf curl virus* is by use of an integrated management package, which combines cultural practices, insecticide application, UV-absorbing plastic films, insect vector proof nets, and variation of weather conditions like light intensity, photoperiod and temperature (Cohen and Antignus, 1994, Palumbo *et al,* 2001, Hilje *et al.,* 2001, Muniyappa *et al.,* 2000, Maruthi *et al* 2003, Mutwiwa *et al* 2005, Kumar and Poheling 2006). Mazyad *et al* (1986), and Chan and Jeger (1994) controlled ToLCV by eliminating or reducing the sources of initial inoculum through uprooting diseased plants. Chan and Jeger (1994) reported that uprooting was the most effective method where plants were sparsely distributed and with minimal contact. This may also relate to young plants whose canopies are still smaller. Ioannou *et al*, (1987) recommended the use of healthy transplants, and later in 1991 reported successful control of ToLCV by timely planting, as the whitefly population is lower during the rainy season and higher in the dry season. Careful tomato seedling protection against whiteflies in the dry season, and transplanting at the beginning of the rainy season helped to avoid ToLCV infection. Prabhaker *et al*. (1988), Mason *et al*. (2000) and Palumbo *et al*. (2001) managed vector populations by using chemical pest control options, such as Imidacloprid (a nicotinoid), buprofezin (a chitin synthesis inhibitor), and pyriproxyfen (a juvenile hormone analog). However, Palumbo *et al*. (2001) recommend use of cultural and biological options for controlling *B. tabaci* because of resistance of insects to insecticides. Cohen and Antignus (1994), Mazyad *et al.* (1994), as well as Greer and Dole (2003) used cultural practices, such as yellow traps and mulches, intercropping tomato with other crop species, and physical barriers to reduce whitefly movement. Kasrawi *et al* (1988), Lapidot and Friedmann (2002), Yang *et al.* (2004), and De Castro (2005), aimed at controlling ToLCV by breeding or engineering for resistance, while Berlinger and Dahan (1987), Lapidot *et al*, (1997) and Pico *et al*., (1998) researched into host plant resistance to whitefly vector. All these approaches depend on accurate virus identification to avoid targeting a wrong pathogen (Hamilton *et al.,* 1981 and Bock, 1982).

2.5.1 Rhizobacteria in the management of plant diseases

Of late, rhizobacterial strains have emerged as potential biocontrol agents for the control of root and foliar diseases of many crops (Anuratha and Gnanamanickam, 1990; Ramamoorthy *et al.,* 2002 and Earnapalli, 2005). PGPR have the ability to protect plants against bacterial, fungal and even viruses by induced systemic resistance (ISR) (Kloepper *et al*., 1992). In the past three decades, numerous strains of fluorescent *Pseudomonas* have been isolated from soil and plant roots by several workers and their biocontrol activity against soil borne and foliar pathogens assessed (Rosales *et al.,* 1993, Vidhyasekaran and Muthamilan, 1995, Nandakumar *et al*., 2001a, Vishwanathan and Samiyappan, 2002, Ramamoorthy *et al.* 2002, Jagadish, 2006, KiranKumar, 2007 and Patil, 2010).

2.6 Screening and selection of biocontrol agents

Microorganisms isolated from roots or rhizosphere of a specific crop are adapted better to that crop and provide effective control of diseases than organisms isolated from other plant species. Such plant associated microorganisms serve as effective biocontrol agents since they are co-evolved. Screening of such locally adapted strains has yielded improved biocontrol in a number of cases (Cook and Baker, 1983 and Jagadeesh, 2000). The first step towards development of effective biocontrol agents is to identify the potent strains through preliminary screening of a large collection of the isolates. For effective implementation on a practical level, the isolates must be ecologically fit to survive, become established and function within the particular condition of an ecosystem. Understanding the mechanisms of biocontrol and interactions with the environment will enable realizing the full potential of biocontrol agents and to develop strategies for management and implementations.

2.7 Induction of systemic resistance by rhizobacteria

Induced systemic resistance, broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack has been hypothesized to be desirable mechanism of biocontrol in several rhizobacteria. Van Loon (1998) defined induced systemic resistance as a state of increased defensive capacity developed by plants when appropriately stimulated, through activation of latent resistance induced by diverse agents including rhizobacteria. ISR activates multiple potential defense mechanisms that induce increased activity of chitinases, peroxidases (PO) and polyphenol oxidase (PPO), resulting in resistance against various plant pathogens (Kandan *et al.*, 2003). ISR is associated with increased synthesis of certain enzymes such as peroxidase (Rajinimala *et al*., 2003) increased level of certain acid soluble proteins (Zdor and Anderson, 1992) and the accumulation of phytoalexins in the induced plant tissue (Vanpeer *et al*., 1991).

Peroxidase shows affinity to substances involved in cellular lignifications and the products of its activity also have direct antimicrobial activity in the presence of hydrogen peroxidase (Ride, 1975). Phenylalanine ammonia lyase activity generates precursors of lignin biosynthesis and other phenolic compounds that accumulate in response to infection (Klessig and Malamy, 1994). Peroxidase, lipooxygenase and phenylalanine ammonia lyase (PALase) are linked to the ISR pathway regulated by Jasmonate and ethylene which are activated by saprophytic microorganisms including rhizobacteria (Van Loon, 1998).

Chitinase is the major cell component which hydrolyses chitin. It cleaves the bond between C1and C4 of two consecutive N-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanisms. The production of chitinases in plants has been suggested to be a part of their defence mechanisms against fungal pathogens (Schlumbaum *et al.,* 1986). The pathogenesis related protein (PR) such as chitinase and ß-1, 3-glucanases showed synergistic, antifungal activity and were related to the systemic acquired resistance (SAR) pathway that induced salicylic acid that was activated by necrotizing pathogens and chemical inducers (Mauch and Stachelin, 1989). In response to application of *Pseudomonas* sp. to roots, enhanced accumulation of chitinases in tobacco and bean leaves were observed (Zdor and Anderson, 1992). Induced systemic resistance in plants is attractive from an environmental point of view, as the organism does not inhibit or kill the pathogen directly by a toxic metabolite, but restricts penetration of pathogens into plants by optimizing plant's defense system. A variety of defense molecules are synthesized by rhizobacteria which are involved in the control of viral diseases in different crops. These are furnished in Table 1.

2.8 Biocontrol of viral diseases by plant growth promoting rhizobacteria

2.8.1 Seed treatment

Seed treatment with *P. fluorescens* strain 89B-27 and *Serratia marcescens* strain 90-166 has consistently reduced the number of CMV-infected plants and delayed the development of symptoms in cucumber and tomato (Raupach *et al*., 1996).

Murphy *et al.* (2000) demonstrated that treatment of tomato seeds with powder formulation of PGPR, *Bacillus amyloliquefaciens, B. subtilis and B. pumilus* reduced symptom severity of tomato mottle virus and increased fruit yield. Kandan *et al.* (2003) reported that the tomato spotted wilt virus disease intensity in *Pseudomonas fluorescens* COT-I +CHAO and *Pseudomonas fluorescens* CHAO treated (seed treatment) cowpea plants was reduced by 63 and 53 per cent respectively. *Pseudomonas fluorescens* treated cowpea plants significantly enhanced oxidative enzymes like phenylalanine ammonia lyase and peroxidase. The phenolic content was also enhanced in the treated plants compared to the untreated plants. The bitter-gourd plants treated (seed treatment) with *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* reduced the bitter gourd yellow vein mosaic virus (BGYMV) disease incidence and increased the plant growth. It showed higher plant height and increased activity of peroxidase, polyphenol oxidase and phenol content (Rajinimala *et al.,* 2003). Kandan *et al.*, (2005) demonstrated that *Pseudomonas fluorescens* strains applied to seed significantly reduced Tomato spotted wilt virus, with a concomitant increase in growth promotion in both the glasshouse and field. In *Pseudomonas fluorescens* treated tomato plants, increased activity of polyphenol oxidase, ß-1, 3 glucanase and chitinase were observed.

Table 1: Defense molecules triggered by rhizobacteria involved in biocontrol of viral diseases

Defense molecule	Rhizobacteria	Viral disease controlled	Reference
Phenylalanine ammonia lyase (PALase)	*Bacillus amyloliquefaciens*	Pepper mild mottle virus(PMMoV)	Ahn *et al* . (2002)
Phenol, Peroxidase, Polyphenol oxidase.	*Pseudomonas chlororaphis* *Pseudomonas fluorescens*	Bitter gourd yellow vein mosaic virus	Rajinimala *et al.* (2003)
Phenylalanine ammonia lyase(PALase)	*Pseudomonas fluorescens* CHAO	Tomato spotted wilt virus in cowpea(*Vigna unguiculata)*	Kandan *et al.* (2003)
Phenylalanine ammonia lyase(PALase)	*Pseudomonas fluorescens* CHAO	Banana Bunchy TOP Virus (BBTV)	Kavino *et al.* (2003)
Phenol	*Pseudomonas fluorescens*	Watermelon yellow mosaic	Nallathambi *et al.* (2003)
Phenylalanine ammonia lyase (PALase)	*Bacillus pumilus* strain SE34, *Serratia marcescens* strain 90	Cucumber mosaic virus in *Arabidopsis thaliana*	Ryu *et al* . (2004)
Phenol, Peroxidase, Polyphenol oxidase, Phenylalanine ammonia lyase(PALase	*Pseudomonas* sp. *Sterptomyces* sp.	Sunflower necrosis virus (SNV)	Srinivasan *et al.* (2005)
Polyphenol oxidase, chitinase, β-1,3-gucanase	*Pseudomonas fluorescens* CHAO,COP-I,COT-I	Tomato spotted wilt virus in tomato	Kandan *et al.* (2005)
Phenol, Peroxidase, Phenylalanine ammonia lyase(PALase),Chitinase	*Pseudomonas fluorescens* B-25	Tobacco mosaic virus in tomato	Kirankumar (2007)
Phenol, Peroxidase, Polyphenol oxidase, Phenylalanine ammonia lyase(PALase), chitinase, β-1,3-gucanase	*Pseudomonas fluorescens* pf1 and CHAO	Banana Bunchy TOP Virus (BBTV)	Harish *et al.* (2005)
Phenol, Peroxidase, Phenylalanine ammonia lyase(PALase)	*Pseudomonas* sp.208(1)	Yellow vein mosaic virus in bhendi	Patil (2010)

Tomato plants treated with PGPR, applied as seed treatment, a spore preparation mixed with potting medium (referred to as “powder”), or a combined seed and powder treatment showed significantly lesser disease severity rating in all PGPR powder based treatments than in either of seeds or control treatments. The use of PGPR could become a component of an integrated programme for management of this virus in tomato (Murphy *et al*., 2000) Kirankumar (2007) reported that seed treatment with *Pseudomonas fluorescens* strains reduced TMV in tomato. He also showed that plants inoculated with *Pseudomonas* B-25 exhibited maximum peroxidase, PALase activity and chitinase activity. Harish *et al*., (2005) demonstrated that seed treatment with rhizobacterial and endophytic bacterial formulations was significantly effective in reducing banana bunchy top virus under field conditions, recording 33.33 per cent infection with 60 per cent reduction over control. They also showed that, the expression of defense related enzymes and pathogenesis related proteins were higher in plants treated with rhizosphere and endophytic bacterial formulations than the control plants.

2.8.2 Soil application

The soil application of *P. fluorescens* CHAO induced protection against Tobacco necrosis virus in tobacco (Maurhofer *et al*., 1994, Maurhofer *et al*., 1998) Murphy *et al.* (2000) demonstrated that soil application with powder formulation of PGPRs, *Bacillus amyloliquefaciens, B.subtilis and B.pumilus* reduced symptom severity of tomato mottle virus and increased fruit yield. *P. fluorescens* strains Pf1 and CHAO with and without chitin, when applied to soil, were found to be effective against banana bunchy top virus in banana. Application of *P. fluorescens* significantly increased the peroxidase and PALase activities in banana against BBTV. The mixture of chitin with CHAO and individual strain CHAO showed multifold increase of PALase activities when compared to other PGPR treatments. The phenolic content was also higher in all the PGPR treatments (Kavino *et al.,* 2003) Kandan *et al.* (2003) reported that the plants treated (soil application) with *P. fluorescens* CoP-1 and CoT-1 and CHAO reduced Tomato spotted wilt virus and showed increased activities of polyphenol oxidase , ß-1,3 –glucanase and chitinase. All the *P. fluorescens* treated tomato plants also showed enhanced growth and yields when compared to control plants. Plant growth-promoting rhizobacterial strains *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937, formulated with the carrier chitosan ‘BioYield’ was tested for its capacity to elicit growth promotion and induced systemic resistance against infection by Cucumber Mosaic Virus (CMV) and *P. syringae* pv. Tomato DC3000 in *Arabidopsis thaliana.* The biopreparation promoted plant growth of *Arabidopsis* hormonal mutants, which included auxins, gibberellic acid, ethylene, jasmonate, salicylic acid, and brassinosteroid insensitive lines (Ryu *et al.,* 2004)

2.8.3 Foliar spraying

Nallathambi *et al.* (2003) found that foliar spraying of *P. fiuorescens* (CIAH-III) (@ 1 Kg talc formulation /ha) suppressed the watermelon mosaic virus. It recorded the least viral incidence (21.54 %) compared to the untreated plants (52.24 %). Srinivasan *et al.* (2005) reported that foliar application of two strains of *Bacillus* sp. and one strain each of *Pseudomonas* sp. and *Streptomyces* sp. reduced the sunflower necrosis virus (SNV) incidence and increased polyphenol oxidase and phenylalanine ammonia lyase activities as compared to control plants. Esitken *et al*. (2006) reported that foliar spraying with *Bacillus*-8, *Bacillus* OSU-142 and *Bacillus* 8 + OSU-142 in sweet cherry stimulated plant growth and resulted in significant increase in fruit yield.

2.8.4 Combination of delivery systems

As far as the management of Watermelon mosaic virus is concerned, none of the treatments could completely eliminate the disease. However, seed treatment plus one foliar spray of the bacterial isolate (CIAH-11) recorded the least viral incidence (14.80%) compared to the untreated plants (52.2%) (Nallathambi *et al.,* 2003) Srinivasan *et al.* (2005) reported that two strains of *Bacillus* sp., one each of *Pseudomonas* sp. and S*treptomyces* sp. were effective in reducing the sunflower necrosis virus (SNV) incidence. They were applied as foliar spray, seed treatment and soil application.

Components of induced systemic resistance against sunflower necrosis virus such as the content of total protein, total phenol, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase activity were all significantly higher in all the PGPR treated plants when compared to control plants. Jagadish (2006) reported that combination of all the five methods (Seed treatment + soil application + nursery bed treatment + foliar spray + root dip method) was the best method of inoculation of *Pseudomonas* B-25. This method increased the plant height by 57 per cent, number of leaves by 53 per cent, stem girth by 46 per cent and number of branches by 71 per cent. Combined methods of application of *Pseudomonas* B-25 controlled the early blight disease in tomato caused by *Alternaria solani* 60.2 per cent. Kirankumar (2007) demonstrated that seed treatment plus soil application was the best method of inoculation of *Pseudomonas* B-25. It controlled the TMV disease in tomato plants by 100 per cent and showed increased plant height by 49.66 per cent, number of leaves by 88.64 per cent, biomass by 66.55 per cent and fruit yield by 102.00 per cent when compared to pathogen inoculated control. Patil (2010) found that seed treatment plus soil application plus foliar spray was the best method of inoculation of *Pseudomonas* 218(1*)* which controlled the BYVMV disease in bhendi plants by 86.67 per cent and showed increase in plant height by 46.15 per cent, biomass to an extent of 56.63 per cent and fruit yield by 132.00 per cent when compared to pathogen inoculated control.

2.9 Induction of systemic resistance by PGPR against diseases and insects

2.9.1 Diseases

PGPR induce resistance in plants against fungal, bacterial and viral diseases (Liu *et al.,* 1995, Maurhofer *et al.,* 1998), insects (Zehnder *et al*., 1997) and nematode pests (Sikora and Murphy, 2005). Induction of systemic resistance by selected strains of PGPR against plant diseases and insect pests has been proved by spatially separating the pathogen and PGPR in plants (Van Peer *et al.,* 1991). Several studies have been carried out to elicit ISR by PGPR in plants. In carnation, application of *Pseudomonas* sp. strain WCS 417r protected plants systemically against Fusarium wilt caused by *Fusarium oxysporum* f.sp. dianthi (Van Peer *et al.,* 1991). PGPR strain applied as a seed -treatment resulted in a significant reduction in anthracnose disease caused by *Colletotrichum orbiculare* in cucumber (Wei *et al.,* 1996). Similarly, induction of systemic resistance by *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 reduced *Fusarium* wilt of cucumber incited by *Fusarium oxysporum* f.sp.*cucumerinum* (Liu *et al.,* 1995). PGPR as a seed treatment alone or as seed treatment plus soil drenching has protected cucumber plants against anthracnose disease (Wei *et al.,* 1996). In rice, seed treatment followed by root-dipping and a foliar spray with *P. fluorescens* strains Pf1 and FP7 showed higher induction of ISR against the sheath blight pathogen, *Rhizoctonia* solani (Vidhyasekaran and Muthamilan, 1995). Similarly, in sugarcane, Viswanathan and Samiyappan (1999) established PGPR-mediated ISR against *Colletotrichum falcatum* causing red rot disease.

PGPR can also induce systemic protection against bacterial diseases. Seeds treated with *P. fluorescens* strain 97 protected beans against halo blight disease caused by *Pseudomonas syringae* pv. *Phaseolicola* (Alstrom, 1991), while treatment of cucumber seed with *P. putida* strain 89B-27 and *S. marcescens* strain 90-166 decreased the incidence of bacterial wilt disease (Kloepper *et al.,*1992). Similarly, seed treatment of cucumber with *P. putida* strain 89B-27*, Flavomonas oryzihabitans* strain INR-5, *S. marcescens* strain 90-166 and *Bacillus pumilus* strain INR-7 provided systemic protection against angular leaf spot caused by *P. syringae* pv*. Lachrymans* by reducing total lesion diameter compared with non treated plants (Liu *et al.,* 1995; Wei *et al.,* 1996). Induction of systemic resistance by PGPR against viral diseases has been reported in cucumber and tobacco plants. Seed – treatment with *P. fluorescens* strain 89B-27 and *S. marcescens* strain 90-166 has consistently reduced the number of cucumber mosaic virus-infected plants (CMV) and delayed the development of symptoms in cucumber and tomato (Raupach *et al.,* 1996). Soil application of *P. fluorescens* strain CHAO induced systemic protection against inoculation with tobacco necrosis virus (TNV) in tobacco (Maurhofer *et al.,* 1994, 1998). These experiments showed that PGPR strains initiated ISR against a wide array of plant pathogens causing fungal, bacterial and viral diseases.

2.9.2 Insect pests

The reports on PGPR–mediated ISR against insects are restricted to very few crops. Generally, fluorescent pseudomonads influence the growth and development of insects at all stages of their growth. *Pseudomonas. maltophila* affected the growth of larval stages of *Helicoverpa zea*, the corn earworm, leading to more than 60 per cent reduction in adult emergence while pupae and adults that emerged from bacteria-infected larvae were smaller (Bong and Sikorowski, 1991). Induction of systemic resistance by PGPR strains, *viz.*, *P. putida* strain 89B-27, *S. marcescens* strain 90-166, *Flavomonas oryzihabitans* strain INR-5 and *Bacillus pumilus* strain INR-7 has significantly reduced populations of the striped cucumber beetles, *Acalyma vittatum* and the spotted cucumber beetle, *Diabrotica undecimpunctata* on cucumber. Among these strains, *S. marcescens* strain 90-166 was more effective in reducing the population of both the beetles and its efficacy was better than application of the insecticide esfenvalerate (Zehnder *et al*., 1999). As certain fluorescent pseudomonads are effective rhizosphere colonizers and are endophytic in nature in the plant system, attempts were made to transfer the insecticidal crystal protein from *B. thuringiensis* to *P. fluorescens. P. fluorescens*, thus genetically engineered, was effective against lepidopteran insect pests and the transgenic *P. cepacia* strain 526 with the crystal protein gene has consistently shown insecticidal activity against tobacco hornworm (Stock *et al*., 1990). Thus, PGPR treatment of crops can be effective for insect pest management and has a greater potential for future use. Zehnder *et al*. (2000) reported that plant growth promoting rhizobacteria reduced the level of aphids transmitting cucumber mosaic virus in tomato plants. The per centage of symptomatic plants in the most effective PGPR treatments ranged from 32 to 58 per cent, compared with 88 to 98 per cent in the non rhizobacterized challenged disease control treatment. Sikora and Murphy (2005) demonstrated that PGPR were significantly effective in controlling cucumber mosaic virus by reducing 80 per cent population of aphids, the vectors of CMV in tomato.

Zehnder *et al*. (2000) reported that delivering of *Serratia marcescens* strain 9 as a seed dip before planting and application of 100 ml of the same @ 10^8 cfu per ml to the sterilized soil-less planting mix controlled cucumber beetles, besides increasing fruit weight. Radjacommare (2002) reported that *P. fluorescens* Pf-1 reduced the incidence of leaf folder by 48 per cent. *Pseudomonas* treated leaves altered the feeding behavior of leaf folder larvae and reduced larval and pupal weight, increased larval mortality and incidence of malformed adults under *in vitro*. An increased population of natural enemies in *Pseudomonas* treated plots under field conditions was also observed which, in turn, yielded 12- 21 per cent more rice production.

2.10 Durability of ISR

Generally, the durability of resistance by PGPR differs from crop to crop and also due to different bacterial strains. Resistance mechanisms attain their maximum effectiveness at four to five days after application of an inducing agent, but the level of persistence of resistance generally decreases over time. These criteria determine the number of applications of PGPR needed to maintain the resistance level in the crop plants (Dalisay and Kuc, 1995). In rice, seed treatment with *P. fluorescens* strain Pf1 induced resistance which was observed upto 45 days after sowing. When a foliar spray was given at 45 days after sowing, the resistance was observed at 4 days after application and persisted for 15 days in rice leaves (Vidhyasekaran *et al*., 1997a).

A foliar spray of *P. fluorescens* formulations has been recommended to be given at every 15 days intervals for managing rice foliar diseases (Vidhyasekaran *et al*., 1997a). A parallel experiment conducted by Nayar (1996) indicated that induction of defense mechanisms using *P. fluorescens* persisted upto 60 days by seed treatment, 30 days by root-dipping and 15 days by foliar spray. In cucumber, PGPR-mediated ISR resulted in protection of cucumber from anthracnose disease for five weeks from the time of sowing. But the consistency of ISR varied with PGPR strains over time (Liu *et al*., 1995). In sugarcane, induction of resistance by PGPR persisted for 90 days of crop growth (Viswanathan and Samiyappan, 1999).

2.11 Growth promotion by rhizobacteria

The prime beneficial traits of PGPR include production of phytohormones, fixation of atmospheric nitrogen, mineral phosphate solubilization and antibiotic resistance. The PGPR play an important role in phytostimulation, phytoremediation and biofertilization and also provide protection to plants against diseases by suppressing the deleterious and pathogenic microorganisms. The Plant growth promoting rhizobacteria have a significant role in plant growth and development in two different ways *viz.*, directly or indirectly.

Glick *et al*. (1999) reported that the direct promotion of plant growth by PGPR generally entails providing the plants with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment. Plant growth benefits due to the addition of PGPR include increase in germination rates, root growth, shoot and root weights (Yeole and Dube, 1997), grain yield (Hoffman *et al*., 1998 and Polyanskaya *et al*., 2000), chlorophyll content (Singh *et al*., 1975) tolerance to drought, salt stress and delayed leaf senescence (Lucy *et al*., 2004). Van Peer and Schippers (1989) demonstrated that potato plants bacterized with plant growth promoting *Pseudomonas* strain showed increased root and shoot fresh weight and observed simultaneous suppression of deleterious pathogenic microflora. Mroz *et al.* (1994) reported that inoculation of wheat plants with *P. fluorescens* in potted soil naturally infested with *Gaeumannomyces graminis* var. *tricici* showed 29 per cent higher grain yield over untreated control. The plant growth promoting strains of *P. fluorescens* ANP15 and *P. aeruginosa* 7 NSK-2 were found to protect maize seeds from cold shock damage and significantly increased germination of maize seeds and enhanced dry matter content of inoculated plants (Hofte *et al.,* 1991) Kloepper *et al*. (1980) reported that two strains of fluorescent *Pseudomonas* isolated from potato epidermis and celery roots significantly increased growth of potato plants upto 50 per cent greater than control in greenhouse assays. Chanway (1995) reported that inoculation of western hemlock (*Tsuga hecterophylla*) with plant growth promoting *B. polymyxa* strain L6-16 R could resulted in significant increase in seedling emergence, height and biomass accumulation. Devananda (2000) observed maximum plant growth yield and nutrient uptake in pigeon pea in the combined inoculation treatment comprising of *Rhizobium*, *Azospirillum* and *P. striata*. Four PGPR strains belonging to fluroscent *Pseudomonas* group increased root length, shoot length and pot yield of groundnut which was attributed to production of siderophores and IAA like substances (Pal *et al*., 2003). Zarger *et al*. (2005) reported that *Pseudomonas* species from rhizosphere of fir and spruce plants grass in forest floor of Kashmir valley have been reported to increase plant height, number of leaves, girth and weight of fir and spruce plants significantly in addition to enhancing the nitrogen, phosphorous and potassium contents of plants. The secretion of succinic and lactic acids was observed by a plant growth promoting rhizobacterial strain *P. putida* which stimulated root growth in *Asparagus* seedlings (Yoshikawa *et al.,* 1993). The seed bacterization with plant growth promoting rhizobacteria and phylloplane bacteria promoted growth and increased yield in groundnut (Krishna *et al*., 2003). Earnapalli (2005) reported that the strain of *P. fluorescens* applied to seed improved growth of tomato. Jayamma (2008) reported that biofertilizer (*Azospirillum* + P. solubilizer + *P. fluorescens*) application enhanced plant height of Jasmine at all stages of growth compared to chemical fertilizer application alone. And, the highest plant height was observed in the plants receiving 100 per cent NPK + biofertilizers. Similar trend was observed even in the experiment on the farmer's field. Kirankumar (2007) demonstrated that out of six PGPR strains, *Pseudomonas* B-25 emerged as the best organism in plant growth promotion of tomato. It increased the plant height by 49.66 per cent, number of leaves by 88.64 per cent, biomass by 66.55 per cent and fruit yield by 102.00 per cent when compared to the pathogen inoculated control. Patil (2010) demonstrated that out of the five promising isolates, *Pseudomonas* 218(1) emerged as the best organism in plant growth promotion. It increased the plant height by 46.15 per cent, biomass by 56.63 per cent and fruit yield by 132 per cent, when compared to the pathogen inoculated control.

2.12 Role of chitosan in plant protection

Chitin and chitosan are naturally-occurring compounds that have potential in agriculture with regard to controlling plant diseases. Chitosan is deacetylated derivative of chitin and one of the most studied elicitiors. It regulates the expression of resistance genes and induces jasmonate synthesis (Doares *et al*., 1995).

Fragments from chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infection. Both chitin and chitosan have demonstrated antiviral, antibacterial, and antifungal properties, and have been explored for many agricultural uses. They have been utilized to control disease or reduce their spread, to chelate nutrient and minerals, preventing pathogens from accessing them, or to enhance plant innate defenses.

When used to enhance plant defenses, chitin and chitosan induced host defense responses in both monocotyledons and dicotyledons. These responses included lignification (Barber *et al*., 1989), ion flux variations, cytoplasmic acidification, membrane depolarization and protein phosphorylation (Felix *et al*., 1998), chitinase and glucanase activation (Roby *et al*., 1987, Kaku *et al*., 1997), phytoalexin biosynthesis (Ren and West,1992), generation of reactive oxygen species (Kuchitsu *et al*.,1995), biosynthesis of jasmonic acid (Nojiri *et al.,*1996)), and the expression of unique early responsive and defense-related genes (Nishizawa *et al*., 1999). In addition, chitosan was reported to induce callose formation (Conrath *et al*., 1989), proteinase inhibitors (Walker-Simmons and Ryan, 1984), and phytoalexin biosynthesis (Hadwiger and Beckman, 1980) in many dicot species. The response to chitin, chitosan, and derived oligosaccharides varies with their acetylation degree.

2.12.1 Antimicrobial properties of chitosan

Chitosan exhibits a variety of antimicrobial activities (Rabea *et al*., 2003., Kulikov *et al.,* 2006), which depend on the type of chitosan (native or modified), its degree of polymerization, the host, the chemical and/or nutrient composition of the substrates, and environmental conditions. In some studies, oligomeric chitosans (pentamers and heptamers) have been reported to exhibit a better antifungal activity than larger units (Rabea *et al*., 2003). In others, the antimicrobial activity increased with the increase in chitosan molecular weight (Kulikov *et al.,* 2006), and seems to be faster on fungi and algae than on bacteria (Savard *et al*., 2002). Chitosan inhibits the growth of a wide range of bacteria (Muzzarelli *et al.,* 1990). The minimal growth-inhibiting concentration varies among species from 10–1,000 ppm. Quaternary ammonium salts of chitosan, such as *N*,*N*,*N*-trimethylchitosan, *N*-propyl-*N*,*N*-dimethylchitosan and *N*-furfuryl-*N,N*-dimethylchitosan were shown to be effective in inhibiting the growth and development of *Escherichia coli* (Jia *et al*., 2007), especially in acidic media. Similarly, several derivatives of chitin and chitosan were shown to inhibit *E. coli, Staphylococcus aureus* (Kim *et al*., 1997), some *Bacillus* species, and several bacteria infecting fish. Rabea *et al*. (2005), reported on the fungicidal activity of 24 new derivatives of chitosan (*i.e.*, *N*-alkyl, *N*-benzylchitosans) and showed, using a radial hyphal growth bioassay of *B. cinerea* and *P. grisea*, that all derivatives have a higher fungicidal action than the native chitosan. Recently, Palma-Guerrero *et al.,* (2009) demonstrated that chitosan was able to permeabilize the plasma membrane of *Neurospora crassa* and killed the cells in an energy-dependent manner. In general, chitosan, applied at a rate of 1 mg/mL, was able to reduce the *in vitro* growth of a number of fungi and oomycetes except Zygomycetes, which have chitosan as a component of their cell walls (Allan and Hadwiger, 1979). Another category of fungi that seems to be resilient to the antifungal effect of chitosan is the nemato-/entomo-pathogenic fungi that possess extracellular chitosanolytic activity (Palma-Guerrero *et al.,* 2008). Helander *et al*. (2001) evidenced that chitosan caused membrane level disrupting and increased cellular leakage in foodborne pathogens.

2.12.2 Against viruses

Chitosan has shown to inhibit the systemic propagation of viruses and viroids throughout the plant and to enhance the host’s hypersensitive response to infection (Pospieszny *et al*., 1991, Chirkov., 2002). The level of suppression of viral infections varied according to chitosan molecular weight (Kulikov *et al*., 2006). Similar observations were reported with the potato virus X, tobacco mosaic and necrosis viruses, alfalfa mosaic virus, peanut stunt virus, and cucumber mosaic virus (Pospieszny *et al*., 1991, Chirkov., 2002). On bean leaves, local infections produced by alfalfa mosaic virus (ALMV) were completely controlled with the highest concentration (0.1%) either sprayed or added to the inoculum (Pospiezny *et al*., 1991). Similar inhibition was reported on tomato leaves treated with chitosan at the same concentration and inoculated with potato spindle tuber viroid (Pospiezny, 1997).

In these studies, systemic resistance was induced by chitosan on various host virus combinations. In general, it was observed that previous chitosan treatments significantly reduced virus infection in various plants.

2.12.3 Application of chitosan in plant disease control

Chitosan used to control plant pathogens has been extensively explored with more or less success depending on the pathosystem, the used derivatives, concentration, degree of deacylation, viscosity, and the applied formulation (*i.e.*, soil amendment, foliar application; chitosan alone or in association with other treatments). For example, Muzzarelli *et al*., (2001) tested the effectiveness of five chemically modified chitosan derivatives in restricting the growth of *Saprolegnia parasitica*. Results indicated that methylpyrrolidinonechitosan, *N*-phosphonomethylchitosan, and *N*-carboxymethyl-chitosan, as opposed to *N*-dicarboxymethylchitosan, did not allow the fungus to grow normally. Substratum amendment with chitosan was reported to enhance plant growth and suppress some of the notorious soil-borne diseases. For example, in soilless tomato, root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was suppressed using chitosan amendments (Lafontaine and Benhamou., 1996). Similarly, in order to control post-harvest diseases, addition of chitosan stimulated microbial degradation of pathogens in a way resembling the application of a hyper-parasite (Benhamou, 2004). Recent investigations on coating tomatoes with chitosan have shown that it delayed ripening by modifying the internal atmosphere, which reduced decays due to pathogens (El Ghaouth *et al*., 1992, El Ghaouth *et al*., 2000). Nandeeshkumar *et al*., (2008) reported that sunflower seeds treated with 5 per cent chitosan resulted in decreased disease severity of downy mildew caused by *Plasmopara halstedii* and offered 46 and 52 per cent protection under green house and field conditions respectively. Postma *et al*., (2009) studied the biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Application of *L. enzymogenes* 3.1T8 in combination with chitosan reduced the number of diseased plants by 50–100 per cent to the *Pythium* control. Application of chitosan or the bacterial inoculant alone was not effective. Washed bacterial cells plus chitosan inhibited *Pythium*-induced disease, but the supernatant without bacterial cells combined with chitosan was not effective. Ting *et al*. (2007) reported that the combination of chitosan and a yeast antagonist *Cryptococcus laurentii* resulted in a synergistic inhibition of the blue mold rot caused by *Penicillium expansumin* apple fruit, being the most effective at the optimal concentration of 0.1% of chitosan with the lowest viscosity (12 cP). Various methods of application of chitosan and chitin are practiced to control or prevent the development of plant diseases or trigger plant innate defenses against pathogens.

2.12.4 Applied as seed coating agents

Guan *et al*. (2009) examined the use of chitosan to prime maize seeds. Although chitosan had no significant effect on germination under low temperatures, it enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines. In both tested lines, chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars and proline, and of peroxidase and catalase activities. In other studies, seed priming with chitosan improved the vigor of maize seedlings (Shao *et al*., 2005). It was also reported to increase wheat seed resistance to certain diseases and improve their quality and/or their ability to germinate (Reddy *et al*., 1999). Similarly, peanut seeds soaked in chitosan were reported to exhibit an increased rate of germination and energy, lipase activity, and gibberellic acid and indole acetic acid levels (Zhou *et al*., 2002). Ruan and Xue (2002) showed that rice seed coating with chitosan may accelerate their germination and improve their tolerance to stress conditions. In carrot, seed coating helps restrain further development of *Sclerotinia* rot (Cheah and Page, 1997). Chitosan has also been extensively utilized as a seed treatment to control *F. oxysporum* in many host species (Rabea *et al*., 2003). Manjula and Podile (2005) reported that as a seed treatment, chitin supplemented peat formulation of *B. subtilis* AF1 increased the emergence and dry weight of pigeon pea seedlings by 29 and 33 per cent , in comparison to an increase of 21 and 30 per cent, respectively by mid-exponential phase cells of *Bacillus subtilis* AF1.

2.12.5 Applied as foliar treatment agents

The foliar application of chitosan has been reported in many systems and for several purposes. For instance, foliar application of a chitosan pentamer affected the net photosynthetic rate of soybean and maize one day after application (Khan *et al*., 2002). Chitosan has also been extensively utilized as a foliar treatment to control the growth, spread and development of many diseases involving viruses, bacteria, fungi and pests (Rabea *et al*., 2003). It has also been used to increase yield and tuber quality of micropropagated greenhouse-grown potatoes (Kowalski *et al*., 2006). Similarly, Faoro *et al.* (2008) showed that the use of chitosan applied as a foliar spray on barley reduced locally and systemically the infection by powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*. Chirkov *et al*., (2001) reported that potato plants sprayed with a solution of chitosans (1mg/ml) induced resistance against potato virus X (PVX) in potato plants. Ben-Shalom *et al.* (2003) evaluated the effects of chitosan and chitin oligomers on gray mould caused by *Botrytis cinerea* in cucumber plants. It was found that chitosan effectively controlled the gray mould (0.45 disease index) compared to control (3.5 disease index). Chitin oligomers did not show any effect (3.5 disease index). Although chitin oligomers elicited chitosanase activity by 2.4 folds and peroxidase activity by 2.0 folds, chitosan elicited their activities only by 1.9 and 0 fold, respectively; disease control was not affected. Spraying chitosan 1 h before inoculation with *Botrytis* conidia decreased gray mould incidence by 65 per cent. Spraying chitosan 4 or 24 h before inoculation reduced disease development by 82 per cent and 87 per cent, respectively. However, spraying chitosan on the leaves 1 h after inoculation decreased gray mould only by 52 per cent. It is concluded that although a dual mode of action was involved in the control of gray mould by chitosan, the antifungal activity of the compound was an essential factor.

2.12.6 Applied as soil amendment

Chitosan utilized as a soil amendment was shown to control *Fusarium* wilt in many plant species (Rabea *et al*., 2003). Applied at an optimal concentration, this biomaterial was able to induce a delay in disease development, leading to a reduced plant wilting (Benhamou *et al*., 1994). Similar results were reported in forest nurseries suffering from *Fusarium acuminatum* and *Cylindrocladium floridanum* infections. These infections were dramatically reduced upon the use of chitosan as soil amendment (Laflamme *et al*., 1999). *Aspergillus flavus* was also completely inhibited in field-grown corn and peanut after soil treatment with chitosan (El Ghaouth *et al*., 1992). Part of the effect observed by chitosan on the reduction of soilborne pathogens comes from the fact that it enhances plant defense responses. The other part is linked to the fact that this biopolymer is composed of polysaccharides that stimulate the activity of beneficial microorganisms in the soil such as *Bacillus,* fluorescent *Pseudomonas*, actinomycetes, mycorrhiza and rhizobacteria (Bell *et al*., 1998, Murphy *et al*., 2000). This alters the microbial equilibrium in the rhizosphere disadvantaging plant pathogens. Beneficial organisms, on the other hand, are able to out compete them through mechanisms such as parasitism, antibiosis, and induced resistance (Uppal *et al*., 2008).

3. MATERIAL AND METHODS

The present investigations were carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad to evaluate the rhizobacterial collection for their ability to control ToLCV in Tomato. The materials and methods followed during the course of investigations are presented in this chapter.

3.1 Rhizobacterial isolates used in the study

A total of 50 rhizobacterial isolates were obtained from the culture collection of the Department of Agricultural Microbiology and Department of Biotechnology, University of Agricultural Sciences, Dharwad and used (Table 2). *Pseudomonas fluorescens* (NCIM 2099) obtained from National Collection of Industrial Microorganisms, Pune was used as the reference strain for comparison of biocontrol efficiency.

3.1.1 Maintenance of the isolates

Out of 50 isolates, 30 were earlier tentatively identified (Kirankumar, 2007). Of these, four belonged to fluorescent *Pseudomonas* and the remaining belonged to the non-fluorescent *Pseudomonas* group. Fluorescent bacteria were subcultured on King's B slants and non-fluorescent bacteria on nutrient agar slants and allowed to grow at 30^{o}C for 48 h. They were preserved in a refrigerator at 4^{o}C and revived once a month. These pure cultures were used for further studies.

3.2 Confirmation of ToLCV

Before start, the presence of ToLCV in the infected leaves was confirmed through transmission electron microscopy as well as by sequencing.

3.2.1 Transmission Electron Microscopy of ToLCV particles

The ToLCV particles in leaf samples were observed under a transmission electron microscope (TEM). This was done at RUSKA Lab, College of Veterinary Science, SVVU, R' Nagar, Hyderabad, India.

Procedure

3.2.1.1 Leaf dip method (John and Lonnie, 1998)

ToLCV infected leaf sample was ground in 0.1 M phosphate buffer, centrifuged at 4000 rpm for 5 min. One drop of supernatant was placed on para film mounted on a glass slide. Carbon coated grid was placed over the sample for 10 minutes, excess fluid was drained with the help of filter paper, then washed with distilled water, blot dried and stained with 2 per cent Uranyl acetate for 10 min. The grid was air dried and observed under a transmission electron microscope at various magnifications ("Hitachi",H-7500,Japan) and photographs taken.

3.2.1.2 Virus dimensions

The dimensions of ToLCV were calculated by the formula as given by Reddy (2000):

$$\text{Dimensions (nm)} = \frac{\text{Virus particle size (nm)}}{\text{Magnification}} \times 10{,}000{,}00$$

3.2.2 Cloning and sequencing for confirmation of ToLCV in tomato leaves

3.2.2.1 Polymerase chain reaction (PCR)

'Eppendorf' Master Cycler (5331) was used to run the PCR programme as described in 3.3.5.4.

Table 2: Sources of rhizobacterial isolates used in the study

Sl. No	Code No. of the isolate	Location	Crop	Source
1	JK-2	Garag	Chilli	Rhizosphere
2	JK-3	Garag	Tomato	Rhizosphere
3	JK-4	Garag	Tomato	Rhizosphere
4	JK-5	Garag	Tomato	Phylloplane
5	JK-6	Garag	Tomato	Phylloplane
6	JK-9	M.K.Hubli	Chilli	Rhizosphere
7	JK-10	M.K.Hubli	Tomato	Rhizosphere
8	JK-12	M.K.Hubli	Toamto	Phylloplane
9	JK-13	M.K.Hubli	Brinjal	Rhizosphere
10	JK-14	Kotur	Brinjal	Rhizosphere
11	JK-15	Kotur	Tomato	Phylloplane
12	JK-16	Mummigatti	Tomato	Phylloplane
13	JK-17	Mummigatti	Tomato	Rhizosphere
14	JK-18	Mummigatti	Chilli	Rhizosphere
15	JK-19	Mummigatti	Chilli	Rhizosphere
16	JK-20	Mummigatti	Chilli	Rhizosphere
17	JK-24	Narendra	Tomato	Phylloplane
18	JK-25	Jodihalli	Tomato	Rhizosphere
19	JK-27	Jodihalli	Chilli	Rhizosphere
20	JK-29	Jodihalli	Chilli	Rhizosphere
21	JK-32	Shingenhalli	Brinjal	Rhizosphere
22	JK-33	Shingenhalli	Tomato	Phylloplane
23	JK-36	Shingenhalli	Chilli	Rhizosphere
24	B-15	Shingenhalli	Tomato	Rhizosphere
25	B-25	Navalur	Tomato	Rhizosphere
26	B-32	Shingenhalli	Tomato	Phylloplane
27	B-40	Shingenhalli	Tomato	Rhizosphere
28	B-53	Navalur	Tomato	Phylloplane
29	B-67	Navalur	Tomato	Rhizosphere

Contd…

Sl. No	Code No. of the isolate	Location	Crop	Source
30	E-21	Tadas	Tomato	Rhizosphere
31	E-55	Tadas	Chilli	Rhizosphere
32	224(1)	Western Ghats, Uttara Kannada	-	Forest soil
33	223(2)	Western Ghats, Uttara Kannada	-	Forest soil
34	218(1)	Western Ghats, Uttara Kannada	-	Forest soil
35	206(2)	Western Ghats, Uttara Kannada	-	Forest soil
36	206(3)	Western Ghats, Uttara Kannada	-	Forest soil
37	206(4)	Western Ghats, Uttara Kannada	-	Forest soil
38	221(2)	Western Ghats, Uttara Kannada	-	Forest soil
39	226(1)	Western Ghats, Uttara Kannada	-	Forest soil
40	212(1)	Western Ghats, Uttara Kannada	-	Forest soil
41	212(4)	Western Ghats, Uttara Kannada	-	Forest soil
42	207(1)	Western Ghats, Uttara Kannada	-	Forest soil
43	205(3)	Western Ghats, Uttara Kannada	-	Forest soil
44	211(2)	Western Ghats, Uttara Kannada	-	Forest soil
45	216(2)	Western Ghats, Uttara Kannada	-	Forest soil
46	210(1)	Western Ghats, Uttara Kannada	-	Forest soil
47	221(1)	Western Ghats, Uttara Kannada	-	Forest soil
48	216(3)	Western Ghats, Uttara Kannada	-	Forest soil
49	221(3)	Western Ghats, Uttara Kannada	-	Forest soil
50	JK-30	Jodihalli	Tomato	Rhizosphere

3.2.2.2 Separation of amplified products

After the set programme was completed, the contents of the tube were electrophoresed to separate amplified products. About 10 μl of amplified products from each tube along with 2 μl of loading dye were separated on 1 per cent agarose gel (Appendix I) along with *Hind*III / *EcoR*I double digest (Bangalore Genei Pvt. Ltd, Bangalore). The gel was prepared using 1X TAE buffer from 50x TAE buffer and agarose (Appendix I). The electrophoresis was done at 60 V for 2 hrs. The gels were observed under a mid range UV trans-illuminator and gel image was documented using gel documentation system (UVitec, Cambridge, England).

3.2.2.3 Elution of PCR fragment

A large-scale amplification was done to elute the PCR fragment. The amplification fragment corresponding to approximately 1035 bp was excised from the 1 per cent low melting agarose gel using a sharp sterile scalpel blade by keeping the gel at low intensity UV trans-illuminator. The agarose gel piece containing the band was collected in the sterile pre-weighed 1.5 ml micro centrifuge tube. The Qiagen Mini Elute Kit (Qiagen, Germany) was used to elute the DNA from the agarose block as described in user's manual.

3.2.2.4 Cloning of PCR product

The ligation of purified PCR product to PTZ57R/T vector (2886 bp) was done as described in Ins T/A clone^{TM} PCR product cloning kit (k1214) from MBI, Fermentas USA. For ligation, an optimal molar ratio of 1:3 vector: insert (Appendix II) was calculated. The components of ligation mixture were mixed 0.5 ml micro centrifuge tubes and incubated overnight at 16°C in thermal cycler (Dyad engine,MJ research,USA).A control ligation reaction was performed using control PCR fragment provided in the kit by adding components.

3.2.2.5 Preparation of competent cells

The competent cells of *E. coli* DH5α were prepared following the protocol:

An isolated colony from *E. coli* DH5α plate was inoculated into 5 ml Luria broth and incubated at 37°C overnight at 200 rpm. The next day, the culture was diluted to 1:100 using Luria broth *i.e*., 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2 - 3 hours till it attained an OD of 0.3 to 0.4 at 600 nm. The culture was chilled in ice for 30 min and 25 ml of culture was dispensed into two centrifuge tubes of capacity 50 ml. The cells were pelleted at 6000 rpm for 5 min. The supernatant was discarded and pellet was suspended in 12.5 ml of ice-cold 0.1 M calcium chloride. The centrifuge tubes were again kept in ice for 45 min and later centrifuged at 4000 rpm for 10 min. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$ and to this 88 µl of dimethyl sulfoxide (DMSO) was added if intended for later use. About 200µl of cells were distributed to each chilled 1.5 ml micro centrifuge tubes and immediately used.

3.2.2.6 Transformation of *E. coli* DH5α

About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligation mixture was added and mixed gently. The mixture was chilled in ice for 45 min and heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2min. It was immediately transferred to ice to chill for 5 minutes. To this, 900 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 minutes to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. The culture was centrifuged at 13, 000 rpm for 1 min and about 900 μl of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the plates having Luria agar with Amp_{50}, Nal_{10} and incubated overnight at 37^0C.

The recombinant clones having recombinant vectors were picked up and streaked on plates having Luria agar with Amp_{100}

3.2.2.7 Confirmation of the clones

The transformed cells were picked up and streaked on Luria agar containing ampicillin (100 μg/ml). The plasmids were isolated from clones and the clones were confirmed through PCR amplification by using specific primers. Further, confirmation was also done by restriction analysis (Appendix III) using *EcoR*I / *Hind*III, which released app.1035 bp.

3.2.2.8 Sequencing

The clone was selected for sequencing.The insert in PTZ57R/T was sequenced using M13 F/R primer at Ocimum Biosolutions Ltd., Hyderabad. Homology search was done with BLAST algorithm available at http://www. ncbi.nlm.nih.gov.

3.3 *In vivo* screening of the rhizobacterial collection against ToLCV disease in tomato

All the isolates were assessed for their ability to suppress the ToLCV disease in tomato in pot cultures.

3.3.1 Pot culture study

Pot culture experiments were conducted during *kharif* season, 2010 in a special glasshouse facility created at Institute of Agril Biotechnology, UAS, Dharwad. It is equipped with humidifier, fans and fluorescent lamps in order to maintain favourable conditions for the multiplication of whiteflies as well as for the development of the virus (Plate 2)

3.3.1.1 Soil

The black sandy loamy soil was collected from the Main Agricultural Research Station, Dharwad and used for the pot experiment.

3.3.1.2 Potting

The plastic pots (10” diameter) having 10 kg capacity were filled with the above soil and FYM mixtures. Before planting, each pot received 3.96, 3.92 and 1.80 g of N, P and K, respectively as per the package of practices for tomato in the form of urea, single super phosphate (SSP) and muriate of potash (MOP), respectively.

3.3.1.3 Seeds

The tomato seeds of the variety, Pusa Ruby were obtained from M/s. National Seeds Corporation (NSC), Dharwad and used in the study. This variety is susceptible to ToLCV disease.

3.3.1.4 Method of application of the rhizobacterial isolates

The isolates were inoculated following seed treatment, soil application and foliar application method.

3.3.1.4.1 Seed treatment

Fluorescent bacteria grown in King’s B broth and non –fluorescent bacteria in nutrient broth medium were incubated on a rotary shaker (150 rpm) for two days and centrifuged at 10,000 rpm for 5 min. The pellet was mixed with sterile carboxy methyl cellulose (CMC) (Hi Media) suspension (1%).

Tomato seeds, surface sterilized with sodium hypochlorite solution, were placed in CMC cell suspension and air dried inside a laminar flow chamber (Jagadeesh, 2000). The biocoated seeds were sampled and bacteria adhering to seeds were determined. Control seeds were treated with CMC and sown. Seeds were directly sown in pots.

3.3.1.4.2 Soil application

Erlenmeyer’s flasks (100 ml) containing 50 ml nutrient broth were inoculated with a loopful of bacteria and incubated on a rotary shaker at 150 rpm for three days at 30°C. The broth was mixed with sterile lignite powder at 1:3 ratio and the formulation prepared. The lignite based culture was applied to soil @ 5kg/ha before sowing seeds and mixed.

3.3.1.4.3 Foliar application

The lignite based culture was sprayed @ 1% (w/v) at 15 DAS and 20 DAS, after filtering through a muslin cloth.

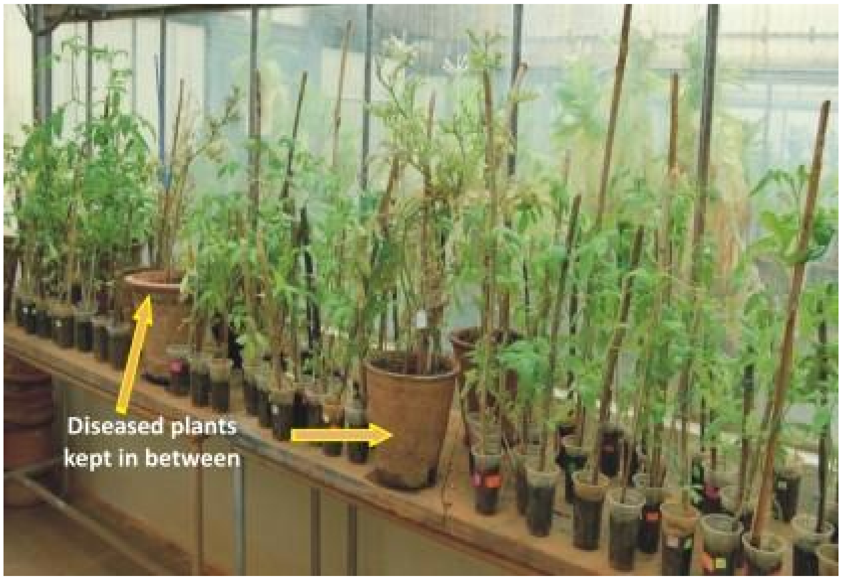


Plate 2. General view of the greenhouse experiment No. 1

Table 3a: Characterization of the soil used for pot experiment

A) Microbiological properties

Bacteria	8.8×10^6 CFU/g soil
Fungi	4.2×10^3 CFU/g soil
Actinomycetes	2.9×10^3 CFU/g soil

B) Chemical properties

Electrical conductivity (dS/m)	0.31	EC bridge (Jackson, 1973)
pH	7.26	pH meter (Jackson, 1967)

Table 3b: Characterization of the soil used for field experiment during *kharif* 2011

A) Microbiological properties

Bacteria	7.6×10^6 CFU/g soil
Fungi	6.1×10^3 CFU/g soil
Actinomycetes	2.7×10^3 CFU/g soil

B) Chemical properties

Electrical conductivity (dS/m)	0.18	EC bridge (Jackson, 1973)
pH	7.04	pH meter (Jackson, 1967)

Table 3c: Characterization of the soil used for field experiment during summer 2012

A) Microbiological properties

Bacteria	7.3× 10^6 CFU/g soil
Fungi	4.9 × 10^3 CFU/g soil
Actinomycetes	4.4 × 10^3 CFU/g soil

B) Chemical properties

Electrical conductivity (dS/m)	0.12	EC bridge (Jackson, 1973)
pH	6.27	pH meter (Jackson, 1967)

3.3.2 Maintenance of the ToLCV inoculum

3.3.2.1 Raising Tomato plants

Tomato seeds were sown in bigger plastic pots filled with soil and farmyard manure and seedlings raised. Twenty five days old seedlings were used for inoculation purposes.

3.3.2.2 Tomato Leaf Curl Virus Culture

The culture of ToLCV was obtained from the diseased tomato plants grown in and around Agricultural College, UAS, Dharwad and inoculated to healthy tomato plants using whiteflies (*Bemisia tabaci)* as the vector and these plants maintained in the glasshouse.

3.3.3 Vector Culture rearing

Whiteflies were collected from cotton and tomato plants with the help of an aspirator by slowly turning the leaves slightly upwards. Whiteflies were released on the ToLCV diseased tomato plants grown in insect proof rearing cages and continuously maintained by introducing new younger plants in to the rearing cage. Thus, the insects were made viruliferous (Plate 3).

3.3.3.1 Release of viruliferous insects

The viruliferous insects were collected from the diseased plants with the help of an aspirator and released on to healthy tomato seedlings on the top leaves. Immediately, these seedlings were placed in insect proof rearing cages and allowed insects for a week, to feed on all plants and bring about infection by the virus. Twenty five days old seedlings were used for releasing the viruliferous insects (Plate 4).

3.3.4 Treatments

The experiment was conducted in pots under greenhouse condition with 53 treatments and four replications following the Completely Randomized Block Design (CRBD).

Treatment Details

T_1 - Isolate No. JK-2 + ToLCV

T_2 - Isolate No. JK-3+ ToLCV

T_3 - Isolate No. JK-4 + ToLCV

T_4 - Isolate No. JK-5 + ToLCV

T_5 - Isolate No. JK-6 + ToLCV

T_6 - Isolate No. JK-9 + ToLCV

T_7 - Isolate No. JK-10 + ToLCV

T_8 - Isolate No. JK-12 + ToLCV

T_9 - Isolate No. JK-13 + ToLCV

T_{10} - Isolate No. JK-14 + ToLCV

T_{11} - Isolate No. JK-15 + ToLCV

T_{12} - Isolate No. JK-16 + ToLCV

T_{13} - Isolate No. JK-17 + ToLCV

T_{14} - Isolate No. JK-18+ ToLCV

T_{15} - Isolate No. JK-19 + ToLCV

T_{16} - Isolate No. JK-20 + ToLCV

T_{17} - Isolate No. JK-24 + ToLCV

T_{18} - Isolate No. JK-25 + ToLCV

T_{19} - Isolate No. JK-27 + ToLCV

Plate 3. **Rearing of white flies on different hosts**

Plate 4. ***Sufficient infestation of viruliferous whiteflies on tomato plants***

T_{20} - Isolate No. JK-29 + ToLCV
T_{21} - Isolate No. JK-32 + ToLCV
T_{22} - Isolate No. JK-33+ ToLCV
T_{23} - Isolate No. JK-36+ ToLCV
T_{24} - Isolate No. B-15+ ToLCV
T_{25} - Isolate No. B-25 + ToLCV
T_{26} - Isolate No. B-32 + ToLCV
T_{27} - Isolate No. B-40 + ToLCV
T_{28} - Isolate No. B-53 + ToLCV
T_{29} - Isolate No. B-67 + ToLCV
T_{30} - Isolate No. E-21 + ToLCV
T_{31} - Isolate No. E-55 + ToLCV
T_{32} - Isolate No. 224 (1) + ToLCV
T_{33} - Isolate No. 223 (2) + ToLCV
T_{34} - Isolate No. 218 (1) + ToLCV
T_{35} - Isolate No. 206 (2) + ToLCV
T_{36} - Isolate No. 206 (3) + ToLCV
T_{37} - Isolate No. 206 (4) + ToLCV
T_{38} - Isolate No. 221 (2) + ToLCV
T_{39} - Isolate No. 226 (1) + ToLCV
T_{40} - Isolate No. 212 (1) + ToLCV
T_{41} - Isolate No. 212 (4) + ToLCV
T_{42} - Isolate No. 207 (1) + ToLCV
T_{43} - Isolate No. 205 (3) + ToLCV
T_{44} - Isolate No. 211 (2) + ToLCV
T_{45} - Isolate No. 216 (2) + ToLCV
T_{46} - Isolate No. 210 (1) + ToLCV
T_{47} - Isolate No. 221 (1) + ToLCV
T_{48} - Isolate No. 216 (3) + ToLCV
T_{49} - Isolate No. 221 (3) + ToLCV
T_{50} - Isolate No. JK-30 + ToLCV
T_{51} - Diseased control (ToLCV)
T_{52} - Healthy control (no virus, no rhizobacteria)
T_{53} - Reference strain (*P.fluorescens* NCIM 2099) + ToLCV

The healthy control plants (T52) were placed in another glasshouse, devoid of ToLCV disease.

3.3.5 Monitoring of the disease

Disease incidence (% diseased plants) and symptom severity were recorded according to the disease severity scale described by Muniyappa *et al* (1991), as below.

Resistant (R) - No symptoms

Mild infection (M) - Light yellowing along margin but no curling and very few plants infected

Moderate infection (Mo) - Light yellowing along margin, slight curling and stunting

Severe infection (SI) - Severe curling, puckering, stunting, reduction in leaf size and reduced fruit formation

Very severe infection (VSI) - Very severe curling, puckering, stunting, great reduction in leaf size and barely produce small sized fruits.

The viral disease was monitored in all the treatments. Observations were made at 45 days after viral inoculation (DAI) and 75 DAI for ToLCV symptoms on tomato (Plate 5) and the percent disease severity was calculated using the formula,

$$\text{Per cent disease severity} = \frac{\text{Number of plants severly infected (SI)}}{\text{Total number of plants}} \times 100$$

3.3.6 Selection of the isolates

Ten promising isolates which effectively reduced the ToLCV disease severity were selected for further evaluation.

3.4 Elucidation of mechanisms of ToLCV biocontrol by the promising isolates

The selected efficient isolates were tested for their ability to supress ToLCV as well as growth promotion under greenhouse conditions through a pot culture study (Plate 6) as explained in 3.2.1 and the disease monitored as explained earlier (3.3.5).

3.4.1 Characterization of the selected promising isolates

Based on the morphological and biochemical tests, the selected isolates were characterized by referring to the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) as detailed below. And based on the results, the isolates were tentatively identified up to generic level.

3.4.1.1 Morphological characterization

The selected isolates were examined for their colony morphology, pigmentation, cell shape and gram reaction as per the standard procedures given by Anon. (1957) and Bartholomaw and Mittewar (1950).

3.4.1.2 Biochemical Characterization

The biochemical characterization was done as per the procedure outlined by the Cappacino and Sharman (1992). The tests conducted are detailed below.

3.4.1.2.1 Starch hydrolysis

The ability of the isolates to hydrolyze starch was examined (Eckford, 1927). Triplicate plates of starch agar were inoculated with the test cultures and incubated at 30°C for three days. After incubation, the plates were flooded with Lugol's iodine solution, allowed to stand for 15 to 30 minutes and observed for clear zone around the colony to indicate hydrolysis of starch.

Maintenance of disease plants

Rearing of whitefiles

Acquisition of virus by whiteflies inside the cage

Inoculation of healthy tomato plants by viruliferous whiteflies inside the cage

Symptom development (15 DAI)

Symptom development (30 DAI)

Plate 5 : Sequential events in the development of ToLCV disease in tomato

30 Days after inoculation (DAI)

75 DAI

Plate 6. General view of the greenhouse experiment No. 2

The starch agar was prepared by suspending one gram of starch powder in 10 ml of cold distilled water, mixed with 90 ml nutrient agar and autoclaved at 121°C for 20 minutes.

3.4.1.2.2 Casein Hydrolysis

Triplicate plates of skim milk agar inoculated with the test cultures were incubated at 30°C for two days and then observed for clear zone around the colony against the black background.

The skim milk agar was prepared by suspending 10 g of skim milk powder in 100 ml of distilled water and later heated, cooled and then mixed with 900 ml of sterilized nutrient agar before pouring into the plates.

3.4.1.2.3 Gelatin liquefaction

The gelatin liquefaction ability of the selected bacterial isolates was examined by the procedure of Blazeric and Ederer (1975). Plates of gelatin agar in triplicates inoculated with culture in one spot were incubated at 30°C for three days. After incubation, the plates were flooded with 12% HgCl2 solution and allowed to stand for 20 minutes and observed for clear zone around the growth of the organism to indicate gelatin liquefaction.

3.4.1.2.4 Oxidase test

To the trypticase soy agar plates, overnight grown culture of the test isolate was spotted and the plates incubated for 24 hours at 28 ± 2°C. After incubation, two to three drops of tetramethyl-phenylenediamine di hydrochloride was added to the surface of the growth of the test organism. The color change to maroon was taken as oxidase positive.

3.4.2 Treatments

The experiment was conducted in pots with thirteen treatments and six replicates following the Completely Randomized Block Design (CRBD).

3.4.2.1 Treatment details

T_1	*Pseudomonas* isolate no. B - 15+ ToLCV
T_2	*Pseudomonas* isolate no. B - 25+ ToLCV
T_3	*Pseudomonas* isolate no. JK-16+ ToLCV
T_4	*Pseudomonas* isolate no. JK-5+ ToLCV
T_5	Fluorescent *Pseudomonas* isolate no. JK-33 + ToLCV
T_6	*Pseudomonas* isolate no. 212 (1) + ToLCV
T_7	Fluorescent *Pseudomonas* isolate no. 207(1) + ToLCV
T_8	*Pseudomonas* isolate no. 206 (4) + ToLCV
T_9	Fluorescent *Pseudomonas* isolate no. 218 (1) + ToLCV
T_{10}	*Enterobacter* isolate no. E-21 + ToLCV
T_{11}	Diseased control (ToLCV)
T_{12}	Healthy control (no virus, no rhizobacteria)
T_{13}	Reference strain (*P. fluorescens* NCIM 2099) + ToLCV

Inoculation of rhizobacteria, rearing of the vector, release of the viruliferous insects etc. were undertaken as carried out previously. (3.2.1.4; 3.2.3; 3.2.3.1)

3.4.2.2 Disease monitoring

The disease was monitored up to harvest as explained earlier. (3.2.5)

3.4.3 Induction of systemic resistance by the strains

3.4.3.1 Assay of Peroxidase activity

The peroxidase activity in leaves was estimated at 45 and 75 DAS following the method of Mahadevan and Sridhar (1986).

3.4.3.1.1 Sample collection and enzyme extraction

The leaf samples were collected from different treatments and immediately homogenized with liquid nitrogen. The powder was extracted with 10 ml of chilled 0.1M phosphate buffer (pH 7.0) in a alcohol sterilized pestle and mortar. The mixture was centrifuged at 18,000 rpm at 5°C for 30 min. and the supernatant used as the enzyme source.

3.4.3.1.2 Measurement of peroxidase activity

Three ml of buffer solution, 0.05 ml guaicol solution 0.1 ml enzyme extract and 0.05 ml hydrogen peroxide solution were pipetted into a cuvette. The absorbance was adjusted to zero at 436 nm in a UV-visible spectrophotometer. The change in absorbance was noticed at an interval of 20 seconds after adding 0.5 ml of 2% H_2O_2 (Hydrogen peroxide) and inverting the cuvette. The enzyme activity was expressed as change in optical density per g protein per minute.

3.4.3.2 Assay of Polyphenol oxidase

The polyphenol oxidase activity in leaves was estimated at 45 and 75 DAS following the method of Mayer *et al*. (1965).

3.4.3.2.1 Sample collection and enzyme extraction

The leaf samples were collected from different treatments and one gram of leaf tissue was immediately homogenized with liquid nitrogen. The powder was extracted with 10 mM phosphate buffer (pH 6.0) (w/v; 1:1) in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant served as enzyme source for PPO assay.

3.4.3.2.2 Measurement of Polyphenol oxidase activity

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of 10 mM catechol were pipetted into a cuvette. The change in absorbance was noticed at an interval of 20 seconds after adding 200 µl of the enzyme extract in the cuvette. The rate of increase in absorbance was measured at 420 nm for one min. The activity was expressed as change in absorbance min^{-1} mg^{-1} protein.

3.4.3.3 Assay of phenylalanine ammonia lyase (PALase)

The phenylalanine ammonia lyase activity was estimated at 45 and 75 DAS, following the method described by Ross and Sederoff (1992)

3.4.3.3.1 Sample collection and enzyme extraction

The leaf samples were collected from different treatments and immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with two ml of Sodium phosphate buffer 0.1M (pH 7.0) at 4°C. The homogenate was centrifuged at 10,000 rpm for 20 min. The extract prepared was used for estimation of PALase activity.

3.4.3.3.2 Measurement of phenylalanine ammonia lyase (PALase)

The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCI (pH 8.8) and 600 µl of 1 mM Phenylalanine was incubated for 60 minutes. The reaction was arrested by adding 2N HCI. Later on, 1.5 ml of toluene was added, vortexed for 30 seconds, centrifuged (10,000 rpm for 5 min) and toluene fraction containing transcinnamic acid was separated. The toluene fraction containing transcinnamic acid was measured at 295 nm against the blank of toulene. A standard curve was drawn with graded amount of cinnamc acid in toluene as described earlier. The enzyme activity was expressed as change in cinnamic acid per min per g fresh weight of tissue.

3.4.3.4 Assay of chitinase

The chitinase activity was estimated at 45 and 75 DAS, following the method described by Miller (1959).

3.4.3.4.1 Sample collection and enzyme extraction

The leaf samples were collected from different treatments and one gm of leaf tissue was ground immediately with liquid nitrogen.

The powdered sample was extracted with two ml of Sodium acetate buffer 0.01M (pH 5.0) at 4°C. The homogenate was centrifuged at 12,000 rpm for 15 min at 4^0 C and the supernatant decanted into a clean tube. The protein content of the supernatant was measured using Lowry's method and the protein was appropriately diluted and equal quantity of protein was used for the enzyme assay.

3.4.3.4.2 Measurement of chitinase

The reaction mixture was prepared by mixing 0.4 ml of 0.03 per cent of glycol chitin substrate, 0.4 ml appropriately diluted crude enzyme solution and 0.4 ml of McIvaline buffer. This mixture was incubated at 50^0 C for 30 minutes. The reaction was arrested by adding 0.5 ml of dinitrosalicylic acid and immediately boiled for 15 min.After 15 min, the colour developed was diluted to 10 ml with distilled water.The intensity of the colour developed was measured using a spectrophotometer at 540 nm. A standard curve was prepared using graded amounts of N-acetyl glucose amine.

3.4.3.5 Phenol estimation

The total phenol content in leaves was estimated at 45 and 75 DAS by following Folin Cio-calteau method (Sadasivam and Manikam, 1991) in oven dried plant samples.

3.4.3.5.1 Sample collection and extraction

One gram of leaf tissue was weighed and cut into small pieces and boiled in 10 ml of 70 percent alcohol and then filtered. The tissue was crushed in a pestle and mortar and then filtered. The extracts were pooled and alcohol evaporated on a hot water bath and volume made upto 10 ml distilled water. This extracts was stored in a refrigerator at 4°C

One ml of alcohol extract was taken in a test tube to which one ml of FC reagent was added followed by 2 ml of 2 percent sodium carbonate. The tubes were shaken well and heated in a water bath for one hour and then cooled under running tap water. The blue colour developed was diluted to 25 ml with distilled water and its absorbance read at 650 nm in a UV- visible spectrophotometer. The amount of phenol present in the sample was calculated by referring to a standard curve prepared using catechol and the content was expressed as mg per gram dry weight.

3.4.4 Enumeration of the vector, *Bemisia tabaci*

The number of insects present on the lower surface of the leaf were counted by slowly turning the leaf without disturbing the insects. Enumeration was done in all the treatments at 30, 45 DAI and 75 DAI.

3.4.5 Detection of ToLCV by polymerase chain reaction (PCR)

PCR was performed in order to detect the level of ToLCV in tomato leaves of all the treatments.

3.4.5.1 DNA extraction procedure (Sambrook and Russell, 2001)

One hundred mg of infected leaf tissue was ground in liquid nitrogen and the powder was transferred to 2 ml microfuge tubes containing 600 µl DNA isolation buffer and both were mixed thoroughly and incubated at 65° C for 20 min. The DNA isolation buffer contained NaCI- 250 mM, Tris Cl- 200 mM, EDTA- 25 mM, SDS-0.5% It was centrifuged at 13,200 rpm for 10 min at 4° C. The supernatant was transferred to two ml microfuge tubes and equal volume of phenol: chloroform was added. It was mixed properly and again centrifuged at 13,200 rpm for 10 min at 4 °C. The upper aqueous layer was transferred to two ml microfuge tube and equal volume of chloroform: Isoamyl alcohol (24:1) was added and mixed properly. It was centrifuged at 13,200 rpm for 10 min at 4° C. The upper aqueous layer was transferred to 1.5 ml of microfuge tube and equal volume of chilled isopropanol was added. It was mixed properly and centrifuged at 13,200 rpm for 10 min at 4^0C. The supernatant was discarded and the pellet washed in 100 µl 70% alcohol. The alcohol was evaporated at 37°C for 20 minutes and the pellet was dissolved in 50 µl $T_{10}E_1$ buffer

3.4.5.2 Primers (Reddy, 2006)

Primers	Sequence
Forward(ToLCBV33F)	5’ GGT CCC CTC CAC TAA ATCAT 3’(20nt)
Reverse(ToLCBV1070R)	5’CAG TTG GTT ACA GAA TCG TAG AAG 3’(24nt)

3.4.5.3 PCR Mixture

The master mix required was prepared from components obtained from M/s Bangalore Genei, Bangalore and Eppendorf, Germany and was distributed into 0.2 ml PCR tubes and the volume was made upto 20 µl with sterile distilled water.

Components	Volume in µl/tube
Sterile distilled water	9.3
Taq buffer	2.0
MgCl2 (25 mM)	1.2
dNTP (1 mM)	2.0
Primers	
Forward (5 pM)	2.0
Reverse (5pM)	2.0
DNA template	1.0
Taq polymerase (3µ/µl)	0.5
Total	20

3.4.5.4 PCR programme

The following PCR amplification conditions were employed for amplification of ToLCV – coat protein gene

Stage	Step	Temp °C	Duration (min)	No. of cycles
1	Initial denaturation	95	5	1
2	Final denaturation	95	1	30cycles
3	Annealing	55	1	
4	Extension	72	1	
5	Final extension	72	10	1
6	Hold	4	Hold	

3.4.5.5 Semi quantitative analysis

One hundred nanograms of DNA from each treatment were used as template in a 20µl PCR reaction containing PCR ingredients. For the PCR reaction, the above conditions were used. The PCR reaction was performed for different reaction cycles of 10, 20 and 30 cycles with the same reaction conditions throughout. After the reaction, the samples were run on 1 per cent agarose gel for comparison.

3.4.6 Influence of rhizobacterial strains on growth and yield of Tomato

The following observations were recorded

3.4.6.1 Plant height

The plant height was measured from base of the plant upto the tip of the fully opened top leaf at 30, 45, 75DAS and at harvesting.

3.4.6.2 Total biomass content

Without disturbing roots, the plants were uprooted at 45 DAS, 75 DAS and at harvest and oven dried at 60°C to a constant weight. The dry biomass was recorded and expressed in grams per plant.

3.4.6.3 Yield parameters

Fruit number and fruit weight per plant

Tomato fruits were harvested periodically and the number of fruits per plant was recorded and simultaneously weight was also recorded.

3.4.6.4 Shelf life of tomato fruits

Ten randomly selected fruits in each treatment were kept in polyethylene bags with ventilation. Shelf life of the tomato fruits were assessed by recording the number of days up to which the fruits could be stored at room temperature without exhibiting spoilage.

3.4.6.5 Estimation of chlorophyll

The chlorophyll content was measured by using a SPAD (Soil Plant Analysis Device) meter at different stages of growth by selecting five leaves randomly at the centre of the branch and the average worked out.

3.5 To assess the effect of combination of PGPR strains and chitosan on the biocontrol of ToLCV under pot cultures

Three efficient isolates were selected based on their disease controlling performance in the previous experiment and combined with chitosan to increase the efficacy of biocontrol of ToLCV. The experiment was conducted in pots with twelve treatments and six replicates, following the Completely Randomized Block Design (CRBD). (Plate 7).

3.5.1 Treatment details

T_1 - *Pseudomonas* 206(4) + ToLCV
T_2 - *Pseudomonas* B-15+ ToLCV
T_3 - *Pseudomonas* JK-16+ ToLCV
T_4 - *Pseudomonas* B-15+JK-16+206(4) + ToLCV
T_5 - *Pseudomonas* 206(4)+ Chitosan + ToLCV
T_6 - *Pseudomonas* B-15+ Chitosan + ToLCV
T_7 - *Pseudomonas* JK-16+Chitosan + ToLCV
T_8 - *Pseudomonas* (B-15+JK-16+206(4)) +Chitosan + ToLCV
T_9 - Chitosan + ToLCV
T_{10} - Reference strain (*P. fluorescens* NCIM 2099) + ToLCV
T_{11} - Chemical control(confidor 2ml/l) + ToLCV
T_{12} - Diseased control (only ToLCV)

Rearing of the vector, release of the viruliferous insects etc. were undertaken as done previously.

3.5.2 Method of application of the rhizobacterial isolates

The isolates were inoculated following seed treatment, soil application and foliar application method.

3.5.2.1 Seed treatment

Fluorescent bacteria were grown in King's B broth and non –fluorescent bacteria in nutrient broth medium on a shaker (150 rpm) for two days and centrifuged at 10,000 rpm for 5 min. Chitosan was dissolved in 100 mM acetate buffer (pH 4.5) and the pH adjusted to 6.5 using 1 N NaOH. The cell pellet was mixed with chitosan solution (5%). Tomato seeds, surface sterilized with sodium hypochlorite solution, were soaked in chitosan cell suspension and kept at shaking condition for 3 h at 28^{o}C.

Plate 7. General view of the greenhouse experiment No. 3

The seeds were shaken in chitosan solution until they became fully coated. The biocoated seeds were dried inside a laminar flow chamber. Control seeds were treated with CMC and sown. Seeds were directly sown in pots.

3.5.2.2 Soil application

Erlenmeyer's flasks (100 ml) containing 50 ml of nutrient broth were inoculated with a loopful of bacteria and incubated on a rotary shaker at 150 rpm for three days at 30°C. And, the broth was mixed with sterile lignite powder at 1:3 ratio and the formulation prepared. The lignite based culture was applied to soil @ 5kg/ha before sowing seeds and mixed.

3.5.2.3 Foliar application

The lignite based culture was sprayed @ 1% (w/v) at 10 DAS and 20 DAS, after filtering through a muslin cloth. At 25 DAS , both upper and lower surfaces of the leaves were sprayed with the Chitosan solution (1 mg/ml) prepared in 100 mM acetate buffer (pH 4.5) and adjusted with 1 N NaOH to pH 6.5. The leaves of controlled plants were sprayed with only water.

3.5.3 Disease monitoring

The disease was monitored up to 75 DAI as explained earlier (3.3.5).

3.5.4 Induction of systemic resistance by the strains

Assay of defence molecules were done at 45 and 75 DAS as explained earlier (3.4.3).

3.5.5 Enumeration of the vector, *Bemisia tabaci*

The number of insects present on the lower surface of the leaf were counted by slowly turning the leaf without disturbing the insects. Enumeration was done in all the treatments at 30, 45 DAI and 75 DAI.

3.5.6 Detection of ToLCV by polymerase chain reaction (PCR)

Detection of ToLCV by polymerase chain reaction (PCR) was done at 45 DAI and 75 DAI in all the treatments as explained earlier (3.4.5).

3.5.7 Influence of rhizobacterial strains on growth and yield of Tomato

All observations like plant height, total biomass content, fruit number and fruit weight per plant, shelf life of tomato fruits and estimation of chlorophyll were done periodically as carried out previously (3.4.6).

3.6 Field evaluation of the selected PGPR strains and chitosan

A field experiment was conducted to assess the effect of selected PGPR strains and chitosan on the disease severity as well as on growth and yield of tomato. It was carried out at Main Agricultural Research Station, U A S, Dharwad, during Kharif (June-Dec 2011). The 30 days old seedlings raised in a glasshouse were transplanted in the main field with 75cm x 60cm spacing. In the chemical control treatment, confidor @2 ml/L was sprayed at weekly intervals to control the vector, as per the package of practices for tomato crop (Plate 8).

3.6.1 Details of the experiment:

3.6.1.1 Field Experiment 1

Treatments	12
Replications	3
Plot size	25 m x 12m
Design	Randomized complete block design (RCBD)
Cultivar	Pusa Ruby
Spacing	75cm x 60 cm

Afruiting stage

Plate 8. General view of the field experiment conducted during *kharif* 2011

At 60 DAT

At fruiting stage

Plate 9. General view of the field experiment conducted during summer 2012

Treatment details

T_1 - *Pseudomonas* 206(4) + ToLCV
T_2 - *Pseudomonas* B-15+ ToLCV
T_3 - *Pseudomonas* JK-16 + ToLCV
T_4 - *Pseudomonas* (B-15+JK-16+206(4)) + ToLCV
T_5 - *Pseudomonas* 206(4)+ Chitosan + ToLCV
T_6 - *Pseudomonas* B-15+ Chitosan + ToLCV
T_7 - *Pseudomonas* JK-16+Chitosan + ToLCV
T_8 - *Pseudomonas* (B-15+JK-16+206(4))+Chitosan + ToLCV
T_9 - Chitosan + ToLCV
T_{10} - Reference strain (*P. fluorescens* NCIM 2099) + ToLCV
T_{11} - Chemical control + ToLCV
T_{12} - Diseased control (only ToLCV)

3.6.2 Method of application of the rhizobacterial isolates

The isolates were inoculated following seed treatment, soil application and foliar application method as explained earlier (3.3.1.4)

3.6.3 Disease monitoring

The disease was monitored up to harvest as discussed earlier (3.3.5)

3.6.4 Influence of rhizobacterial strains on growth and yield of Tomato

All the observations such as plant height, total biomass content, fruit number and fruit weight per plant, shelf life of tomato fruits and chlorophyll content were made periodically as done previously (3.4.6)

3.7 Field evaluation of the further selected efficient combination of PGPR strains and Chitosan

Based on the earlier field experiment, the best treatment was selected and the experiment was conducted to assess the effect of different treatments on the disease severity as well as on growth and yield of tomato. It was carried out at Main Agricultural Research Station, U A S, Dharwad, during Summer (Jan- May 2012). The 30 days old seedlings raised in a glass house were transplanted in the main field with 75cm x 60cm spacing. In the chemical control treatment,confidor @2 ml/L was sprayed at weekly intervals to control the vector, as per the package of practices for tomato crop (Plate 9).

Treatments	6
Replications	5
Plot size	20 m X 10 m
Design	Randomized complete block design (RCBD)
Cultivar	Pusa Ruby
Spacing	75cm X 60 cm

Treatment details

T_1 - *Pseudomonas* B-15+ Chitosan + ToLCV
T_2 - *Pseudomonas* 206(4)+ Chitosan + ToLCV
T_3 - *Pseudomonas* (B-15+JK-16+206(4)) + Chitosan + ToLCV
T_4 - Reference strain (*P. fluorescens* NCIM 2099) + ToLCV
T_5 - Chemical control (confidor 2ml/l) + ToLCV
T_6 - Diseased control (only ToLCV)

3.7.1 Method of application of the rhizobacterial isolates

The isolates were inoculated following seed treatment, soil application and foliar application method as explained earlier (3.3.1.4).

3.7.2 Disease monitoring

The disease was monitored up to harvest as discussed earlier (3.3.5).

3.7.3 Induction of systemic resistance by the strains

Assay of defence molecules were done at 45 and 75 DAS as explained earlier (3.4.3).

3.7.4 Detection of ToLCV by polymerase chain reaction (PCR)

Detection of ToLCV by polymerase chain reaction (PCR) was done at 45 DAS and 75 DAS in all the treatments as explained earlier (3.4.5).

3.7.5 Influence of rhizobacterial strains on growth and yield of Tomato

All the observations such as plant height, total biomass content, fruit number and fruit weight per plant, shelf life of tomato fruits and chlorophyll content were made periodically as done previously. (3.4.6)

3.8 Field evaluation of the best isolate *Pseudomonas* 206(4) with chitosan

It was carried out at Main Agricultural Research Station, U A S, Dharwad, during Summer (Jan- May2012). The 30 days old seedlings raised in a glass house were transplanted in the main field with 75cm x 60cm spacing. In the chemical control treatment,confidor @2 ml/L was sprayed at weekly interval to control the vector, as per the package of practices for tomato crop (Plate 10).

Treatments	3
Replications	7
Plot size	13 m X 9 m
Design	Randomized complete block design (RCBD)
Cultivar	Pusa Ruby
Spacing	75cm X 60 cm

Treatment details

T_1- *Pseudomonas* 206(4) + chitosan + ToLCV

T_2 Chemical control (confidor 2ml/l) + ToLCV

T_3 Diseased control (only ToLCV)

3.8.1 Method of application of the rhizobacterial isolates

The isolates were inoculated following seed treatment, soil application and foliar application method as explained earlier (3.3.1.4).

3.8.2 Disease monitoring

The disease was monitored up to harvest as discussed earlier (3.3.5).

3.8.3 Induction of systemic resistance by the strains

Assay of defence molecules were done at 45 and 75 DAS as explained earlier (3.4.3).

3.8.4 Detection of ToLCV by polymerase chain reaction (PCR)

Detection of ToLCV by polymerase chain reaction (PCR) was done at 45 DAS and 75 DAS in all the treatments as explained earlier (3.4.5).

Disease control

Chemical control

206(4) + Chitoson

Plate 10. **Field assessment of the best strain 206(4) with chitosan on plant growth and disease severity of ToLCV (under severe disease pressure condition, during summer 2012)**

3.8.5 Influence of rhizobacterial strains on growth and yield of Tomato

All the observations such as plant height, total biomass content, fruit number and fruit weight per plant, shelf life of tomato fruits and chlorophyll content were made periodically as done previously (3.4.6).

3.9 Transmission electron microscopy of ToLCV infected leaves (Spurr, 1969)

Many sub cellular changes do take place in tomato leaves infected by ToLCV. These were studied using TEM. Leaves of healthy, treated by *Pseudomonas* 206(4) + chitosan and diseased plants were taken for the study and compared. This was performed at RUSKA Lab, College of Veterinary Science, SVVU, R' Nagar, Hyderabad, India.

Cleaning the surface of the specimen

The surface of 30 days old tomato leaves infected at 10 DAS by ToLCV were cleaned from contaminants by carefully rinsing them three times for 10 min in 0.1 M phosphate buffer (pH 7.2) at room temperature.

Primary fixation of the specimens

The samples were fixed in a 2.5 % glutaraldehyde solution (0.1 M phosphate buffer-pH- 7.2) and stored at 4^0C for 24 h.

Washing of the specimens

To remove the fixative from the interstitium, all the samples were washed with 0.1 M phosphate buffer (pH 7.2) for three times 45 min each change.

Secondary fixation of the specimens

Secondary fixation has been done with 2% aqueous osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.2) for 1h at room temperature.

Dehydrating the specimens

Then, the samples were dehydrated with graded ethanol (30 % to 100% - two changes) for 45 min each.

Infiltration of the specimens with a transitional solvent

The specimens were shifted to mixed solution of Araldite 6005 Resin and ethanol in the following manner:-

1:2 (Araldite 6005 Resin: ethanol) for 1h followed by

2:1 (Araldite 6005 Resin: ethanol) for 1h followed by

Pure araldite 6005 resin for overnight.

Infiltration with resin and embedding the specimens

The samples were infiltrated and embedded in araldite 6005 resin and incubated for 72 h at 70-80^0C in an incubator.

Sectioning and staining of the specimens

Semi thin sections (1000 – 1200 nm) were made with a glass knife on ultra microtome (Lecia Ultra cut UCT-GA-D/E-1/00) mounted on a glass slide stained with toludene blue and observed under a light microscope for identification of specific area. Then ultra thin sections were (50-70 nm) made and mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate.

The stained samples were observed under TEM ("Hitachi", H-7500, Japan) at different magnifications and photographs taken.

3.10 Studies for assessing the root colonization ability of the most efficient PGPR strain by Scanning Electron Microscopy

Preparation of samples

To determine the location of *Pseudomanas* 206(4) on the roots and if morphological differences occur due to application of chitosan , Scanning Electron Microscopy (SEM) pictures were taken. This was done at RUSKA Lab, College of Veterinary Science, SVVU, R' Nagar, Hyderabad, India.

Tomato seeds were surface sterilized by rinsing for 30 sec in 96% ethanol, followed by 3 min in 5% NaHClO and three washes in sterile tap water. These seeds were allowed to germinate in cleaned plastic cups filled with soil and sand.

The cups were incubated in the glass house at optimum conditions. Ten days after sowing, plants were inoculated with *Pseudomanas* 206(4) grown in nutrient broth with or without chitosan (5%)

Plants were incubated in a glasshouse at optimum conditions and roots were sampled five days later. The following steps were followed for the specimen preparation to examine the plant roots under scanning electron microscope. (John and Lonnie, 1998)

1. Fixation : Root parts of 5 mm were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) at 4°C for 24 h and post fixed in 2% aqueous osmium tetraoxide for 1 h.
2. Washing and Dehydration : The steps followed are as follows
    (i) Three buffer washing was done.
    (ii) Then the specimens were treated with series of graded ethanol in a sequential manner starting at 30% to 100% (two changes) ethanol for 45 min each.
    (iii) The specimens were loaded in critical point dryer (EMS-850) for complete drying.
    (iv) Then, the specimens were mounted over the silver stubs (metallic holder) fixed with double sided carbon conductivity tape and subjected for gold sputtering.

Sputter coating of specimen and observation under SEM

1. Coating of specimen: All the dried samples were coated with gold palladium with automated sputter coater (JEOL JFC-1600) for 3 min at 40 psi.
2. The specimens were observed under a Scanning Electron Microscope ("JEOL"-JSM 5600, Japan) as per the standard procedure under various magnifications and photographs taken.

3.11 Histological and histochemical changes due to ToLCV virus

Histological changes in tomato due to ToLCV were studied. Leaves of healthy plants, PGPR treated plants with and without chitosan and diseased plants were taken for the study and compared. This was performed at Karnataka University (KUD), Department of Botany, Dharwad, India.

Preparation of samples

Fixation

Leaf bits (1.0×0.5 cm) were fixed in standard FAA fixative (1:1:18 of Formalin: Acetic acid: 70% Alcohol by v/v) for 24 h to fix the tissues, *i.e.*, to arrest the cytological changes in plant tissues.

Dehydration

Fixed samples were washed in 70 per cent ethyl alcohol and dehydrated using ethanol and n-butanol solution as herein below:

Sl. No.	Water (%)	Ethanol (%)	n-butanol (%)	Time (h)
1.	30	70	-	2
2.	20	80	-	2
3.	10	90	-	2
4.	-	100	-	1
5.	-	100	-	1
6.	-	75	25	2
7.	-	50	50	2
8.	-	25	75	2
9.	-	-	100	2

Infiltration and embedding

Paraffin wax melted at 58-60^0 C was successively added to the medium of n- butanol containing dehydrated samples until the medium reached a saturation point at room temperature.The samples were subsequently placed in an oven maintained at 60^0 C.Changes with fresh molten paraffin wax were given at every 24 h interval to replace the last traces of butanol with paraffin.The leaf bits were subsequently embedded in paraffin wax of 58-60^0 C using paper boat technique (Jensen, 1962).

Sectioning and affixing the sections to the slides

Ten micron size uniform sections of embedded samples were taken using Leica microtome. An adhesive was prepared using gelatin at 1.5 g/100 ml distilled water and a little quantity of potassium dichromate to prevent fungal growth.A few drops of gelatin were added on to the surface of clean microslide.Sections were carefully placed on adhesive and slides were warmed over hot plate maintained at 50^0 C for 1-2 min to facilitate flattening and stretching of section ribbons.The excess adhesive was drained off and the slides were dried for 24 h at room temperature.

Deparaffinising and hydrating the sections

The sections were deparaffinised using xylene and were then treated with different grades of alcohol for gradual dehydration. Later, the sections were subjected to staining either directly or after hydration depending on the reqirement.The steps followed for deparaffinising were as follows:

Pure Xylene - 5 min

Xylene + Absolute alcohol - 5 min

Absolute alcohol - 5 min

90 per cent alcohol - 5 min

70 per cent alcohol - 5 min

50 per cent alcohol - 5 min

Distilled water - 5 min

After each step, slides were blot dried to remove excess chemical adhered to the slides.

Staining, dehydration and mounting the sections

The sections were subjected to histological and histochemical staining for localization of different cellular chemical compounds namely insoluble polysaccharides, proteins and nucleic acids.

Structural staining

To observe anatomical changes in diseasd leaves, sections were passed through safranin and fast green stains.

Steps for structural staining

1. Sections were stained in one per cent safranin (1 g of safranin in 100 ml of 50 per cent alcohol) for 2 h.
2. Dehydrated the sections in 50, 70 and 90 per cent alcohol for 5 min each.
3. Stained with 0.5 per cent fast green (0.5 g of fast green in 100 ml of 95 per cent alcohol) for 5 min.
4. Dehydrated with 95 per cent alcohol for 5 min.
5. Cleaned in xylene and mounted in DPX.

Staining for total insoluble polysaccharides

Assessment of total insoluble polysaccharides was made by periodic acid Schiff's (PAS) method (Hatchkiss, 1948). It is an ideal test for insoluble polysaccharides,besides it facilitates the measurement of cell size and different tissues. The reagents were prepared as suggested by Longley (1952).The stain was prepared by mixing 1 g of basic fuchsin,1.8 g of potassium metabisulphite and 100 ml of 0.15 N HCL. The solution was poured in airtight container and kept on shaker for 24 h. A straw yellow coloured solution, thus, obtained was mixed with activated charcoal, filtered and stored in amber coloured bottle.

Steps for staining polysaccharides

1. Sections were placed in 0.5% periodic acid for 15 min.
2. Washed in running tap water for 5-10 min.
3. Stained in Schiff's reagent for 30 min.
4. Rinsed in water for differentiation and to remove excess stain.
5. Dehydrated by passing through n-butanol (two changes of 3 min each) and xylene (two changes of 3 min each) and mounted in DPX.
6. The intensity of deep magenta colour in cell content was measured for total insoluble polysaccharide content.

Staining for total proteins

Assessment of total proteins was made by Amido Black 10 B method (O' Brien and Mc Cully, 1981).This method was employed to localize total proteins.

Steps for staining proteins:

1. Sections were deparaffinised and hydrated.
2. Hydrated sections were kept in 7% acetic acid for 2-3 min.
3. Then incubated in 0.25% Amido black (10 B) for 5-10 min.
4. Sections were then differentiated in 7% acetic acid.
5. Washed,air dried,cleaned in xylene and mounted in DPX.

The sites in the cell containing protein stain is deep blue.The intensity of the blue colour is the measure of amount of protein content in the tissue.

Staining for nucleic acids

Assessment of nucleic acid was made by Toludine Blue O method (Chayen *et al*., 1973) .The dye Toludine blue was used for the purpose of detecting the richness of RNA and DNA in cells.This being a metachromatic stain, offers an excellent method for staining both RNA and DNA with clear contrast. The DNA appears as green blue, while RNA appears purple or dark blue.

Steps for staining nucleic acids:

1. Sections were deparaffinised and hydrated.
2. Hydrated sections were treated with 0.5% Toludine Blue for 5 min.
3. Washed in running tap water.
4. Air dried, cleaned in xylene and mounted with DPX.

Histochemical assessment and microphotography

Based on visual observations on the degree of histochemical reactions with specific stain,the qualitative grading was done as detailed below:

a. Very rich ++++
b. Rich +++
c. Medium ++
d. Low +
e. Absent/Negligible -

The slides were photographed using Axiostar plus ("Carl Zeiss") Bright field microscope with Canon power shot G2 digital camera attachment.

3.12 Statistical analysis

The data generated in pot experiments were subjected to Completely Randomized Block Design analysis and interpretation of the data was carried out in accordance with Panse and Sukhatme (1985). The level of significance used in the 'F' and't' test was P=0.01. The critical difference values were calculated, whenever the F test values were significant.

The data obtained from field experiments were subjected to Randomized Complete Block Design analysis as described by Gomez and Gomez (1984). The level of significance used in the 'F' test was P=0.05. The critical difference values were calculated whenever the F test values were significant.

4. EXPERIMENTAL RESULTS

The experiments were carried out to evaluate the biocontrol potential of the rhizobacterial collection against ToLCV in Tomato. Investigations were also conducted to find out the mechanism of biocontrol by the promising isolates besides evaluating their plant growth promotional activity. The results obtained during the experimentation are presented in this chapter.

At start, the presence of ToLCV was confirmed through TEM studies and sequencing.

4.1 Transmission Electron Microscopy of ToLCV particles

4.1.1 Detection of geminivirus in ToLCV affected leaves

The ToLCV particles in leaf samples were observed under a transmission electron microscope the electron microscopic examination of the partially purified preparation of ToLCV revealed the presence of isometric and pentagonal, with single and paired Gemini virus particles, (monomers and dimmers) with a dimension of 20 × 30 nm to 24 × 30 nm (Plate 11). The particles exhibited characteristic shape of geminiviruses.

4.2 Cloning and Sequencing

The transformed cells which have taken ToLCV coat specific gene insert were picked up and streaked on Luria agar containing ampicillin (100 μg/ml). The plasmids were isolated from clones and the clones were confirmed through PCR amplification (Plate 12c) by using specific primers. Further, confirmation was also done by restriction analysis (Appendix III) using *EcoR*I / *Hind*III, which released app.1035 bp (Plate 12b).The clone was selected for sequencing. The insert in PTZ57R/T was sequenced using M13 F/R primer at Ocimum Biosolutions Ltd., Hyderabad. Homology search was done with BLAST algorithm available at http://www. ncbi.nlm.nih.gov. The insert fragment was sequenced and subjected to BLAST search at NCBI, which showed 98% similarity with Tomato Leaf Curl Banglore Virus Isolate (ToLBV-AVT1 segment DNA A) (Table 4a, 4b)

4.3 *In vivo* screening of the rhizobacterial isolates against ToLCV

I*n vivo* screening of the rhizobacterial isolates was carried out in pots, under conditions of high vector - virus pressure. As many as 50 isolates were evaluated. The results of the experiments are shown in Tables 5, Plate 14. At 30 days after inoculation (30 DAI) of the pathogen, it was interesting to note that almost all the isolates controlled the disease severity varying from 50 to 100 per cent.Out of 50 isolates, as many as 11 isolates controlled the severity of disease completely. Nineteen strains controlled the disease severity moderately by 50 percent. The remaining twenty strains showed a 75 per cent disease severity control at 30 DAI. In the disease control, there was cent percent disease severity occurrence, while in the healthy control, there was no disease. At 60 DAI, 18 isolates controlled the disease severity by 75 percent. Twenty isolates controlled the disease severity moderately by 50 percent. Twelve strains could bring about a disease severity control by 25 per cent. Based on the percent disease severity control by the isolates and their effects on visual plant growth, as many as ten isolates were selected for further characterization and biocontrol studies. These were B-15, B-25, 212 (1), 206(4), 207 (1), JK-33, JK-5, JK-16, E-21 and 218 (1).

4.4 Evaluation of the selected rhizobacterial isolates for the biocontrol of ToLCV as well as growth promotion of Tomato

4.4.1 Characterization of the selected promising rhizobacterial isolates

The morphological and biochemical characterization of the selected efficient isolates was carried out and the isolates were tentatively identified up to the generic level. Out of the ten selected isolates, three isolates 218(1), JK-33 and 207(1) belonged to fluorescent *Pseudomonas* and the remaining six (B-15, B-25, 212 (1), 206(4), JK-5, and JK-16 belonged to non-fluorescent *Pseudomonas* (Table 5) and E-21 was identified as *Enterobacter* (Tables 5 and Plate 13).

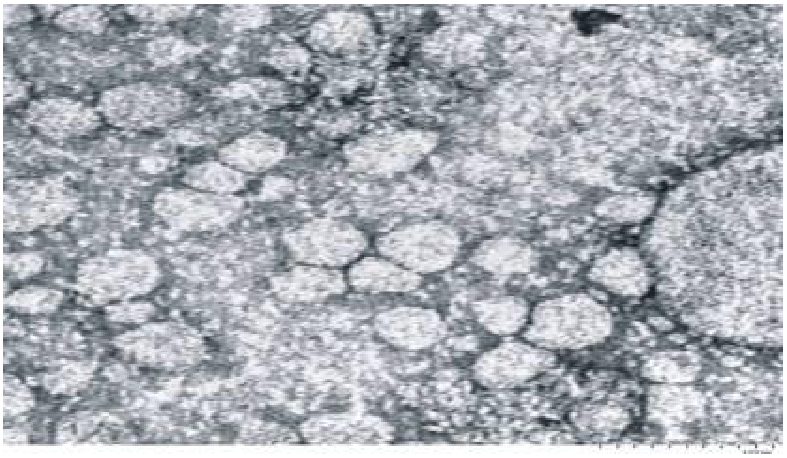


Plate 11. TEM picture showing partially purified ToLCV particles extracted from diseased plants (40,000X)

4.4.2 Biocontrol potential of the selected rhizobacterial isolates

The selected ten isolates (B-15, B-25, 212 (1), 206(4), 207 (1),JK-33, JK-5, JK-16,E-21 and 218 (1)) were further tested for their ability to control ToLCV severity in Tomato in pots under green house condition with high level of vector virus pressure conditions and the results are furnished in Table7, Plate 15, Fig.1. Inoculation of tomato seeds with rhizobacterial isolates revealed that these isolates were significantly effective in reducing ToLCV disease. At 45 and 75 DAI, all the isolates showed higher disease severity control ranging from 14.29 to 85.72 percent. At 45 DAI, the highest viral disease severity control (85.72 %) was noticed in the plants inoculated with 206(4), which was followed by B-15 and JK-16 with 71.43 percent disease control. And, the least was by 212(1) with 28.58 percent control. At 75 DAI also, similar trend was observed. (Table 7 and Plate 16)

4.4.3 Ability of the rhizobacteria to induce systemic resistance against ToLCV in tomato plants

The ISR activity of the isolates was tested by estimating defense enzymes such as peroxidase, chitinase, polyphenoloxidase, phenylalanine ammonia lyase (PALase) and phenol content in plants at different intervals of time.

4.4.3.1 Phenol content

The phenol content of leaves, at different intervals *viz.*, 45 and 75 DAS, as influenced by inoculation with rhizobacteria are presented in Table 8a and Fig. 2a.

At 45 DAS, the treatment receiving *Pseudomonas* 206(4) strain recorded the maximum phenol content (0.51mg/g), followed by *Pseudomonas* strain B-15 and the reference strain (0.49 mg/g and 0.48 mg/g respectively). The least phenol content was recorded in the healthy control (0.34 mg/g).

At 75 DAS, phenol content was decreased slightly in all the treatments as compared to 45 DAS. The highest phenol content was recorded in the plants inoculated with *Pseudomonas* strain 206(4) (0.49 mg/g).

This was followed by *Pseudomonas* strain B-15 and the reference strain (0.47 mg/g and 0.46 mg/g respectively). Healthy control recorded significantly lower amount of phenol content (0.32 mg/g).

4.4.3.2 Phenylalanine ammonia lyase (PALase) activity

The results on biosynthesis of Phenylalanine ammonia lyase (PALase) as influenced by inoculationn with rhizobacterial strains in Tomato at two intervals *viz.*, 45 DAS and 75 DAS are presented in Table 8b and Fig. 2e.It was observed that PALase activity was significantly increased in all the bacteria inoculated treatments.

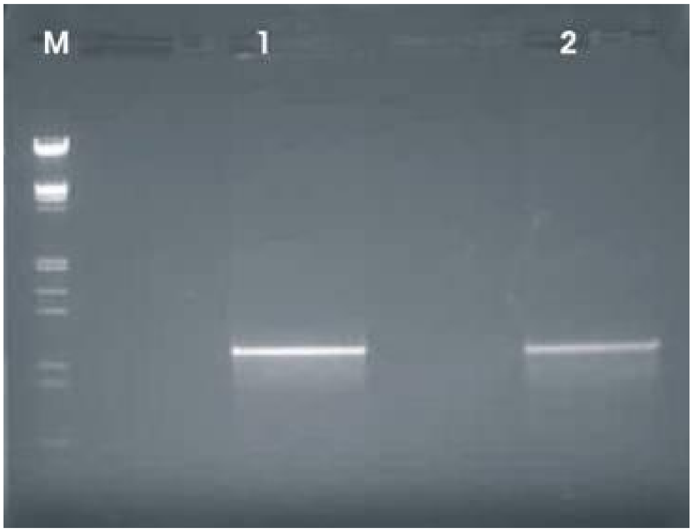


Plate 12 a. PCR product in bulk for elution

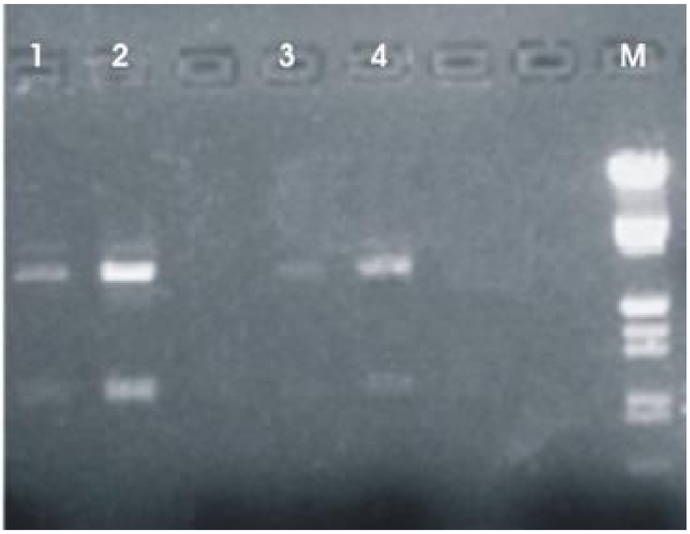


Plate 12 b. Restriction confirmation of ToLCV coat specific gene

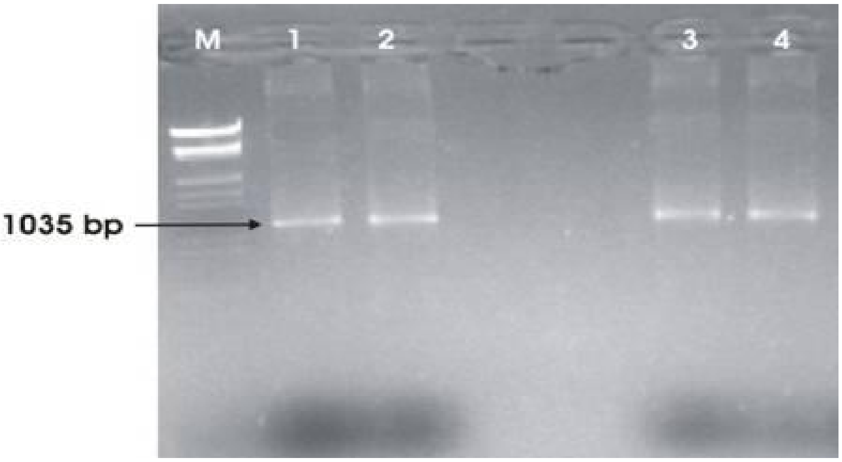
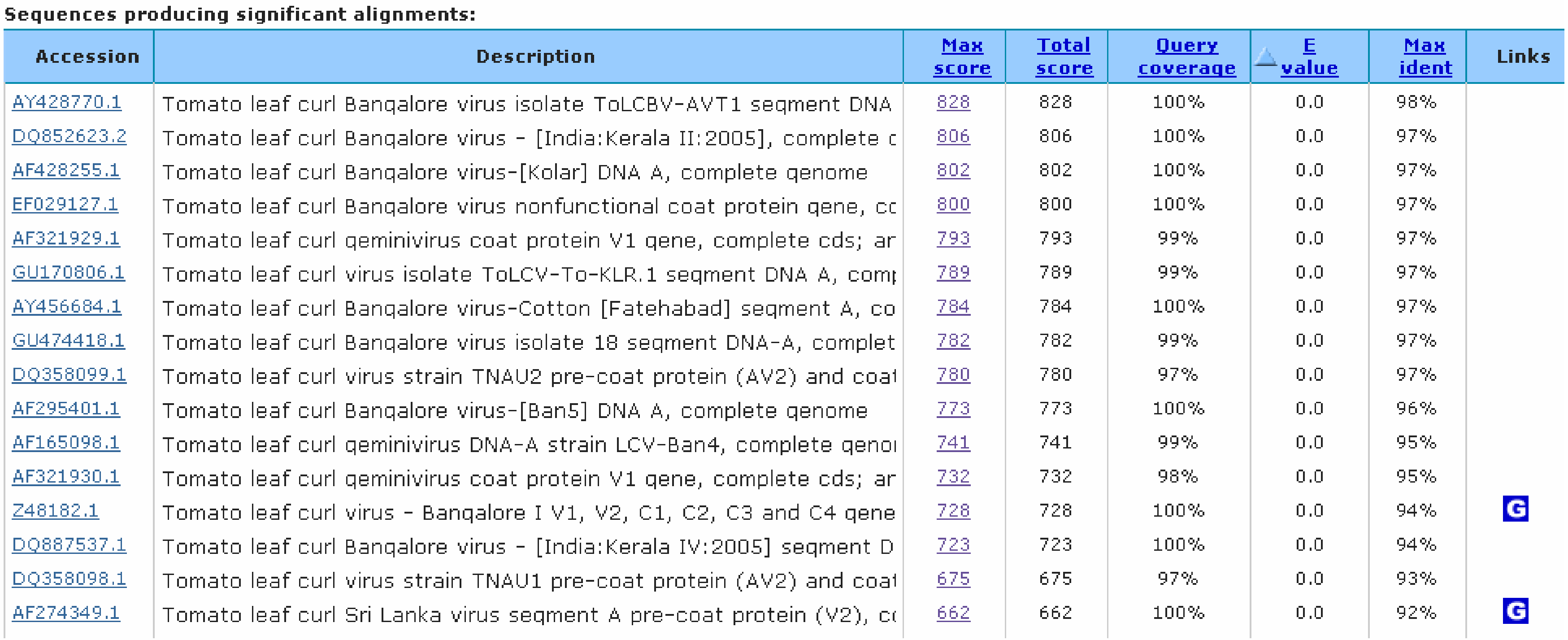


Plate 12 c. PCR confirmation of ToLCV coat specific gene

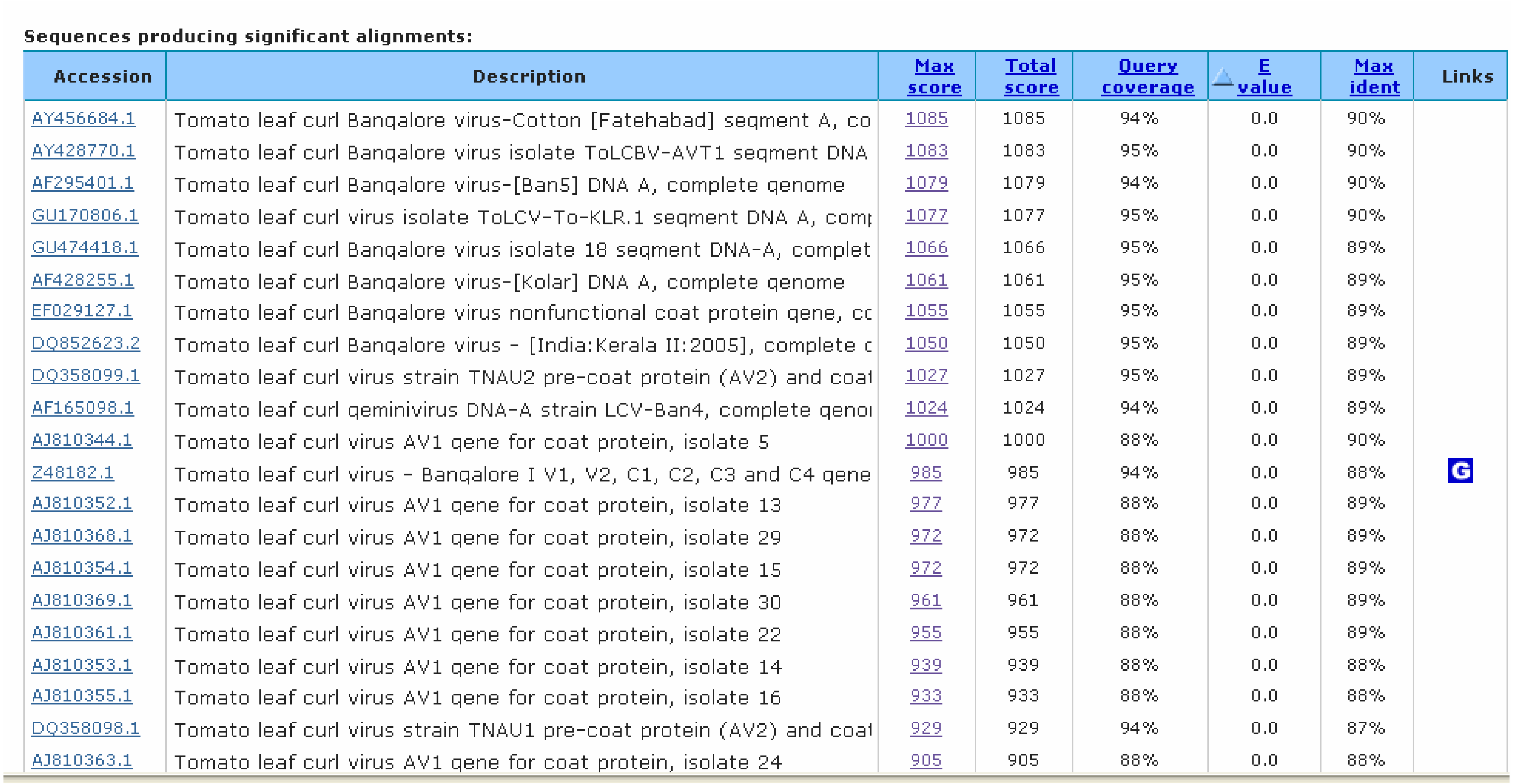
Table 4a: Blast analysis for ToLCV (Forward sequence)



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AY428770.1	Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment DNA	828	828	100%	0.0	98%	
DQ852623.2	Tomato leaf curl Bangalore virus - [India:Kerala II:2005], complete c	806	806	100%	0.0	97%	
AF428255.1	Tomato leaf curl Bangalore virus-[Kolar] DNA A, complete genome	802	802	100%	0.0	97%	
EF029127.1	Tomato leaf curl Bangalore virus nonfunctional coat protein gene, cc	800	800	100%	0.0	97%	
AF321929.1	Tomato leaf curl geminivirus coat protein V1 gene, complete cds; ar	793	793	99%	0.0	97%	
GU170806.1	Tomato leaf curl virus isolate ToLCV-To-KLR.1 segment DNA A, comp	789	789	99%	0.0	97%	
AY456684.1	Tomato leaf curl Bangalore virus-Cotton [Fatehabad] segment A, co	784	784	100%	0.0	97%	
GU474418.1	Tomato leaf curl Bangalore virus isolate 18 segment DNA-A, complet	782	782	99%	0.0	97%	
DQ358099.1	Tomato leaf curl virus strain TNAU2 pre-coat protein (AV2) and coat	780	780	97%	0.0	97%	
AF295401.1	Tomato leaf curl Bangalore virus-[Ban5] DNA A, complete genome	773	773	100%	0.0	96%	
AF165098.1	Tomato leaf curl geminivirus DNA-A strain LCV-Ban4, complete genom	741	741	99%	0.0	95%	
AF321930.1	Tomato leaf curl geminivirus coat protein V1 gene, complete cds; ar	732	732	98%	0.0	95%	
Z48182.1	Tomato leaf curl virus - Bangalore I V1, V2, C1, C2, C3 and C4 gene	728	728	100%	0.0	94%	G
DQ887537.1	Tomato leaf curl Bangalore virus - [India:Kerala IV:2005] segment D	723	723	100%	0.0	94%	
DQ358098.1	Tomato leaf curl virus strain TNAU1 pre-coat protein (AV2) and coat	675	675	97%	0.0	93%	
AF274349.1	Tomato leaf curl Sri Lanka virus segment A pre-coat protein (V2), cc	662	662	100%	0.0	92%	G

Table 4b: Blast analysis for ToLCV (Reverse sequence)



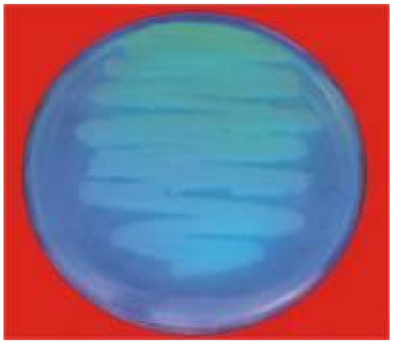
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AY456684.1	Tomato leaf curl Bangalore virus-Cotton [Fatehabad] segment A, co	1085	1085	94%	0.0	90%	
AY428770.1	Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment DNA	1083	1083	95%	0.0	90%	
AF295401.1	Tomato leaf curl Bangalore virus-[Ban5] DNA A, complete genome	1079	1079	94%	0.0	90%	
GU170806.1	Tomato leaf curl virus isolate ToLCV-To-KLR.1 segment DNA A, comp	1077	1077	95%	0.0	90%	
GU474418.1	Tomato leaf curl Bangalore virus isolate 18 segment DNA-A, complet	1066	1066	95%	0.0	89%	
AF428255.1	Tomato leaf curl Bangalore virus-[Kolar] DNA A, complete genome	1061	1061	95%	0.0	89%	
EF029127.1	Tomato leaf curl Bangalore virus nonfunctional coat protein gene, cc	1055	1055	95%	0.0	89%	
DQ852623.2	Tomato leaf curl Bangalore virus - [India:Kerala II:2005], complete c	1050	1050	95%	0.0	89%	
DQ358099.1	Tomato leaf curl virus strain TNAU2 pre-coat protein (AV2) and coat	1027	1027	95%	0.0	89%	
AF165098.1	Tomato leaf curl geminivirus DNA-A strain LCV-Ban4, complete genoi	1024	1024	94%	0.0	89%	
AJ810344.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 5	1000	1000	88%	0.0	90%	
Z48182.1	Tomato leaf curl virus - Bangalore I V1, V2, C1, C2, C3 and C4 gene	985	985	94%	0.0	88%	G
AJ810352.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 13	977	977	88%	0.0	89%	
AJ810368.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 29	972	972	88%	0.0	89%	
AJ810354.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 15	972	972	88%	0.0	89%	
AJ810369.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 30	961	961	88%	0.0	89%	
AJ810361.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 22	955	955	88%	0.0	89%	
AJ810353.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 14	939	939	88%	0.0	88%	
AJ810355.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 16	933	933	88%	0.0	88%	
DQ358098.1	Tomato leaf curl virus strain TNAU1 pre-coat protein (AV2) and coat	929	929	94%	0.0	87%	
AJ810363.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 24	905	905	88%	0.0	88%	

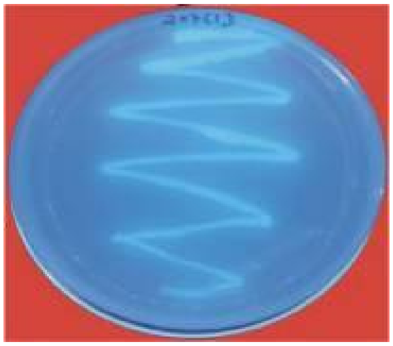
Table 5: Morphological and biochemical characterization of the selected rhizobacterial isolates

Strain code No.	Morphological tests			Biochemical tests								Probable genus
	Colony morphology	Fluorescence under UV	Gram reaction and cell shape	Oxidase test	Urease test	Starch hydrolysis	Gelatin liquefaction	Casein hydrolysis	Catalase activity	Acid production	Gas production	
B-15	Whitish, circular	-	-ve rod	+ve	-ve	-	+	-	+	-	+	*Pseudomonas* sp.
212 (1)	Creamy slimy	-	-ve rod	+ve	-ve	-	+	-	+	-	-	*Pseudomonas* sp.
206 (4)	Whitish, circular	-	-ve rod	+ve	-ve	-	+	-	+	-	+	*Pseudomonas* sp.
207 (1)	Creamy, circular,slimy	+	-ve rod	+ve	-ve	-	+	-	+	+	+	Fluorescent *Pseudomonas* sp.
218 (1)	Creamy circular, slimy	+	-ve rod	+ve	-ve	-	+	-	+	+	+	Fluorescent *Pseudomonas* sp.
B- 25	Creamy slimy	-	-ve rod	+ve	-ve	-	+	-	+	+	-	*Pseudomonas* sp.
JK-16	Whitish,circular,wrinkled	-	-ve rod	+ve	-ve	-	+	-	+	-	+	*Pseudomonas* sp.
JK-5	Creamish, circular	-	-ve rod	+ve	-ve	-	+	-	+	-	+	*Pseudomonas* sp.
JK-33	Whitish, circular,slimy	+	-ve rod	+ve	-ve	-	+	-	+	+	+	Fluorescent *Pseudomonas* sp.
E-21	Whitish, creamy,circular	-	-ve rod	+ve	-ve	+	-	-	+	+	+	*Enterobacter* sp.

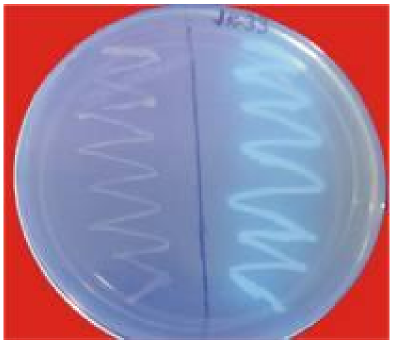
Reference strain



218(1)



207(1)



212(1)and JK – 33

B – 15 and B – 25

E – 21 and JK -33

206(4) and 207(1)

JK -16 and JK - 5

Plate 13 . Selected rhizobacterial isolates

Table 6: Screening of the rhizobacterial collection against ToLCV of tomato under pot culture (greenhouse experiment no. 1)

Sl. No	Code No. of the isolate	30 DAI		60 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
01	JK-2 +ToLCV	50	50	50	50
02	JK-3+ToLCV	50	50	75	25
03	JK-4+ToLCV	25	75	25	75
04	JK-5+ToLCV	0	100	25	75
05	JK-6+ToLCV	50	50	50	50
06	JK-9+ToLCV	50	50	50	50
07	JK-10+ToLCV	50	50	75	25
08	JK-12+ToLCV	50	50	75	25
09	JK-13+ToLCV	25	75	50	50
10	JK-14+ToLCV	50	50	75	25
11	JK-15+ToLCV	50	50	75	25
12	JK-16+ToLCV	0	100	25	75
13	JK-17+ToLCV	50	50	75	25
14	JK-18+ToLCV	25	75	25	75
15	JK-19+ToLCV	50	50	50	50
16	JK-20+ToLCV	50	50	75	25
17	JK-24+ToLCV	50	50	50	50
18	JK-25+ToLCV	25	75	50	50
19	JK-27+ToLCV	25	75	50	50
20	JK-29+ToLCV	25	75	50	50
21	JK-32+ToLCV	25	75	25	75
22	JK-33+ToLCV	0	100	25	75
23	JK-36+ToLCV	50	50	75	25
24	B-15+ToLCV	0	100	25	75
25	B-25+ToLCV	0	100	25	75
26	B-32+ToLCV	50	50	50	50
27	B-40+ToLCV	25	75	25	75
28	B-53+ToLCV	50	50	50	50
29	B-67+ToLCV	25	75	50	50
30	E-21+ToLCV	0	100	25	75
31	E-55+ToLCV	25	75	75	25

Contd...

Sl. No	Code No. of the isolate	30 DAI		60 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
32	224(1) +ToLCV	50	50	50	50
33	223(2) +ToLCV	25	75	50	50
34	218(1) +ToLCV	0	100	25	75
35	206(2) +ToLCV	50	50	75	25
36	206(3) +ToLCV	50	50	25	75
37	206(4) +ToLCV	0	100	25	75
38	221(2) +ToLCV	25	75	50	50
39	226(1) +ToLCV	25	75	50	50
40	212(1) +ToLCV	0	100	25	75
41	212(4) +ToLCV	0	100	50	50
42	207(1) +ToLCV	0	100	25	75
43	205(3) +ToLCV	25	75	75	25
44	211(2) +ToLCV	25	75	25	75
45	216(2) +ToLCV	25	75	50	50
46	210(1) +ToLCV	25	75	25	75
47	221(1) +ToLCV	25	75	50	50
48	216(3) +ToLCV	25	75	25	75
49	221(3) +ToLCV	50	50	50	50
50	JK-30+ToLCV	25	75	75	25
51	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	0.00	100.00	25	75
52	Diseased control (onlyToLCV)	100.00	-	100.00	-
53	Healthy control (no rhhizobacteria, noToLCV)	0.00	-	0.00	-

Note: DAI = Days after inoculation

Plate 14. Effect of rhizobacterial strains on incidence of ToLCV in tomato

At 45 DAS, the maximum PALase activity was recorded in the treatment receiving *Pseudomonas* strain 206(4) (0.264 change in cinnamic acid/min/g) followed by the plants inoculated with *Pseudomonas* B-15 and reference strain (0.255 change in Cinnamic acid/min/g and 0.251 change in Cinnamic acid/min/g) respectively. The least PALase activity was recorded in the healthy control (0.150 change in Cinnamic acid/min/g each).

At 75 DAS, PALase activity was found decreasing in all the treatments when compared to 45 DAS. The highest activity was seen in plants inoculated with *Pseudomonas* 206(4) (0.247 change in Cinnamic acid/min/g each), which was followed by B-15 and the reference strain (0.238 change in Cinnamic acid/min/g and 0.230 change in Cinnamic acid/min/g respectively). The least was exhibited by the healthy control (0.130 change in Cinnamic acid/min/g).

4.4.3.3 Peroxidase activity

The data pertaining to peroxidase activity as influenced by rhizobacterial strains in Tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table 8a, Fig.2b.

In general, there was a tremendous increase in peroxidase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest peroxidase activity was recorded in the plant leaves receiving *Pseudomonas* 206(4) strain (2.68 ΔOD/g protein/ min). *Pseudomonas* B-15 was the next best strain with 2.58 ΔOD/g protein/ min, which was on par with the plants treated with the reference strain (2.56 ΔOD/g protein/ min). The disease control treatment exhibited 1.45 ΔOD/g protein/ min. And, the least peroxidase activity was recorded in the healthy control (1.20 ΔOD/g protein/ min).

At 75 DAS, the peroxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest peroxidase activity was seen in leaves inoculated with *Pseudomonas* 206(4) (2.59 ΔOD/g protein/ min), followed by *Pseudomonas* B-15 (2.32 ΔOD/g protein/ min) and the reference strain (2.29 ΔOD/g protein/ min). The least peroxidase activity was recorded in the healthy control (1.06 ΔOD/g protein/ min). The disease control plants showed an activity of 1.12 ΔOD/g protein/ min.

4.4.3.4 Chitinase activity

Results on biosynthesis of chitinase activity as influenced by rhizobacterial strains in Tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table8a, Fig.2c.

In general, there was a tremendous increase in chitinase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest chitinase activity was recorded in the plant leaves receiving *Pseudomonas* 206(4) strain (2.73 µgGlc NAc/ µg protein/ min). *Pseudomonas* B-15 was the next best strain with 2.66 µgGlc NAc/ µg protein/ min, which was on par with the plants treated with the reference strain (2.83 µgGlc NAc/ µg protein/ min). The disease control treatment exhibited 1.93 µgGlc NAc/ µg protein/ min. And, the least chitinase activity was recorded in the healthy control (1.82 µgGlc NAc/ µg protein/ min).

At 75 DAS, the chitinase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest chitinase activity was seen in leaves inoculated with *Pseudomonas* 206(4) (1.62 µgGlc NAc/ µg protein/ min), followed by *Pseudomonas* B-15 (1.56 µgGlc NAc/ µg protein/ min) and the reference strain (1.55 µgGlc NAc/ µg protein/ min). The least chitinase activity was recorded in the healthy control (1.07 µgGlc NAc/ µg protein/ min). The disease control plants showed an activity of 1.16 µgGlc NAc/ µg protein/ min.

4.4.3.5 Polyphenol oxidase activity

Data pertaining to polyphenol oxidase activity as influenced by rhizobacterial strains in Tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table 8b, Fig.2d.

In general, there was a substantial increase in polyphenol oxidase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest polyphenol oxidase activity was recorded in the plant leaves receiving *Pseudomonas* 206(4) strain (1.53 ΔOD/g protein/ min).

Table 7: Disease severity of ToLCV as influenced by the selected rhizobacterial strains (greenhouse experiment no. 2)

SL. No.	Treatments	45 DAI		75 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
1	*Pseudomonas* JK-33 +ToLCV	42.5	57.5	71.42	28.58
2	*Pseudomonas* B-25 +ToLCV	57.14	42.86	71.42	28.58
3	*Pseudomonas* 207(1) +ToLCV	42.85	57.15	71.42	28.58
4	*Pseudomonas* 218 +ToLCV	42.85	57.15	71.42	28.58
5	*Pseudomonas* JK-5 +ToLCV	42.85	57.15	71.42	28.58
6	*Pseudomonas* 206(4) +ToLCV	14.28	85.72	42.85	57.15
7	*Pseudomonas* JK-16 +ToLCV	28.57	71.43	57.14	42.86
8	*Pseudomonas* 212(1) +ToLCV	71.42	28.58	85.71	14.29
9	*Pseudomonas* B-15 +ToLCV	28.57	71.43	57.14	42.86
10	*Enterobacter* E-21 +ToLCV	42.5	57.5	57.14	42.86
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	28.57	71.43	57.14	42.86
12	Healthy control (No rhizobacteria , no ToLCV)	0	100	0	100
13	Diseased control (only ToLCV)	100	0	100	0

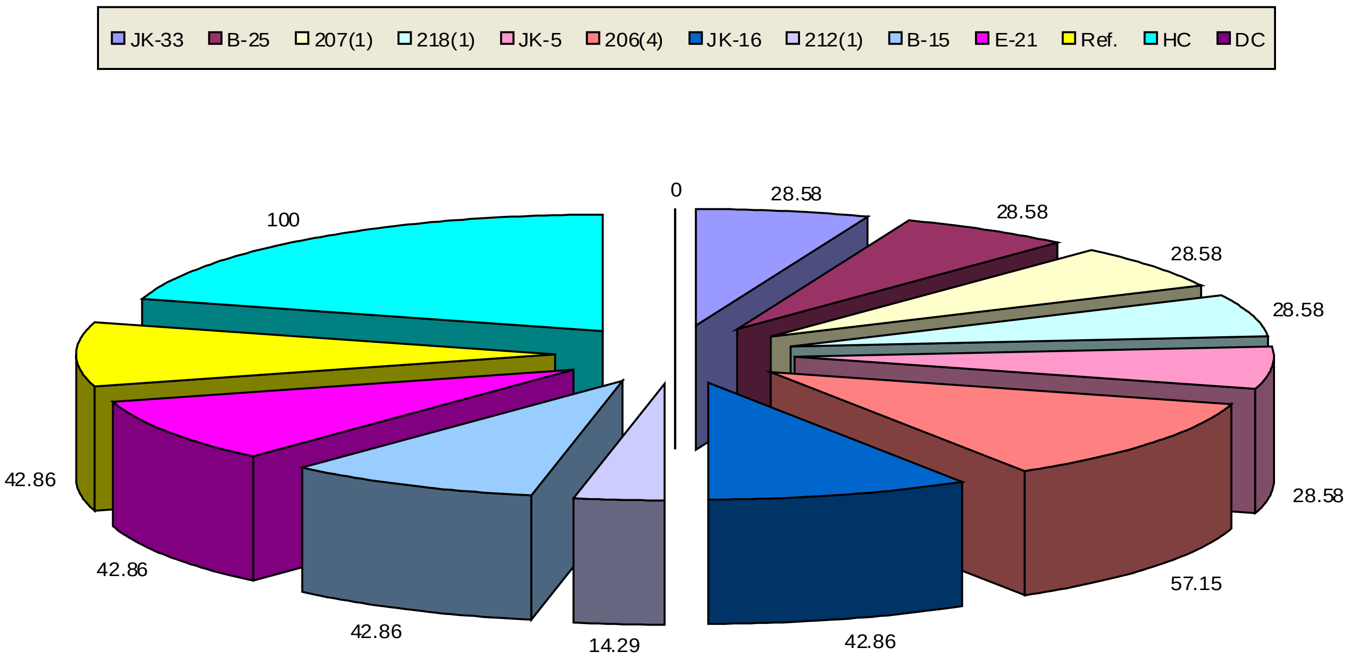


Fig. 1: Reduction in disease severity of ToLCV by the selected rhizobacterial strains at 75 DAI

Fig. 1: Reduction in disease severity of ToLCV by the selected rhizobacterial strains at 75 DAI

Plate 15. Reduction in disease severity of ToLCV in tomato due to inoculation with different rhizobacterial isolates (30 DAI)

Plate 15. Contd………..

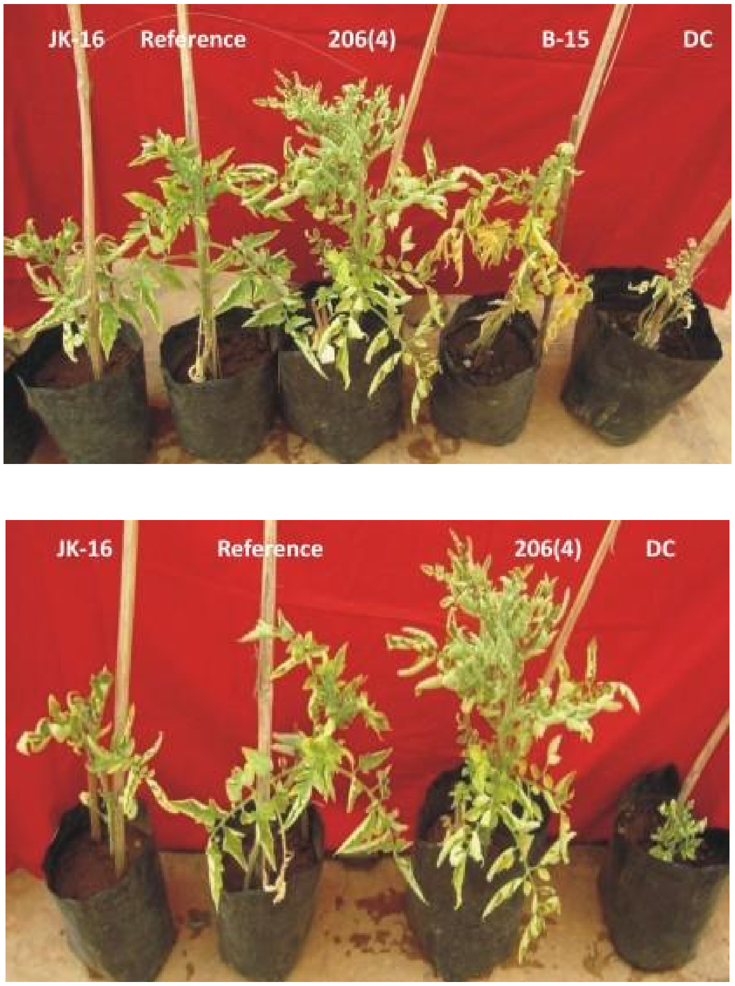


Plate 16. **Effect of rhizobacterial isolates on the plant growth and ToLCV severity (90 DAI)**

Table 8a: Defense molecules as triggered by inoculation with selected rhizobacteria

SL. No.	Treatments	Phenol(mg/g dry weight)		Peroxidase(∆OD/g protein/min)		Chitinase (µgGlc NAc/ µg protein/ min)	
		45 DAS	75 DAS	45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* JK-33 +ToLCV	0.44	0.43	1.83	1.62	2.49	1.25
2	*Pseudomonas* B-25 +ToLCV	0.46	0.45	1.76	1.48	2.32	1.20
3	*Pseudomonas* 207(1) +ToLCV	0.41	0.39	1.75	1.50	2.08	1.26
4	*Pseudomonas* 218 +ToLCV	0.42	0.40	1.61	1.25	2.19	1.42
5	*Pseudomonas* JK-5 +ToLCV	0.43	0.41	1.69	1.58	2.31	1.46
6	*Pseudomonas* 206(4) +ToLCV	0.51	0.49	2.68	2.59	2.73	1.62
7	*Pseudomonas* JK-16 +ToLCV	0.48	0.47	2.49	2.10	2.62	1.53
8	*Pseudomonas* 212(1) +ToLCV	0.48	0.45	2.41	2.03	2.09	1.21
9	*Pseudomonas* B-15 +ToLCV	0.49	0.47	2.58	2.32	2.66	1.56
10	*Enterobacter* E-21 +ToLCV	0.41	0.40	1.92	1.55	2.55	1.51
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	0.48	0.46	2.56	2.29	2.83	1.55
12	Healthy control (No rhizobacteria , no ToLCV)	0.34	0.32	1.20	1.06	1.82	1.07
13	Diseased control (only ToLCV)	0.37	0.35	1.45	1.12	1.93	1.16
	Sem±	0.01	0.02	0.06	0.05	0.05	0.03
	CD @1%	0.05	0.06	0.23	0.20	0.22	0.13

Contd…

LEGEND

JK-33 = *Pseudomonas* JK-33 + ToLCV

B-25 = *Pseudomonas* B-25 + ToLCV

207(1) = *Pseudomonas* 207(1) + ToLCV

218 = *Pseudomonas* 218 + ToLCV

JK-5 = *Pseudomonas* JK-5 + ToLCV

206(4) = *Pseudomonas* 206(4) + ToLCV

JK-16 = *Pseudomonas* JK-16 + ToLCV

212(1) = *Pseudomonas* 212(1) + ToLCV

B-15 = *Pseudomonas* B-15 + ToLCV

E-21 = *Enterobacter* E-21 + ToLCV

Ref = Reference strain (*P. fluorescens* NCIM 2099) +ToLCV

HC = Healthy control (No rhizobacteria , no ToLCV)

DC = Diseased control (only ToLCV)

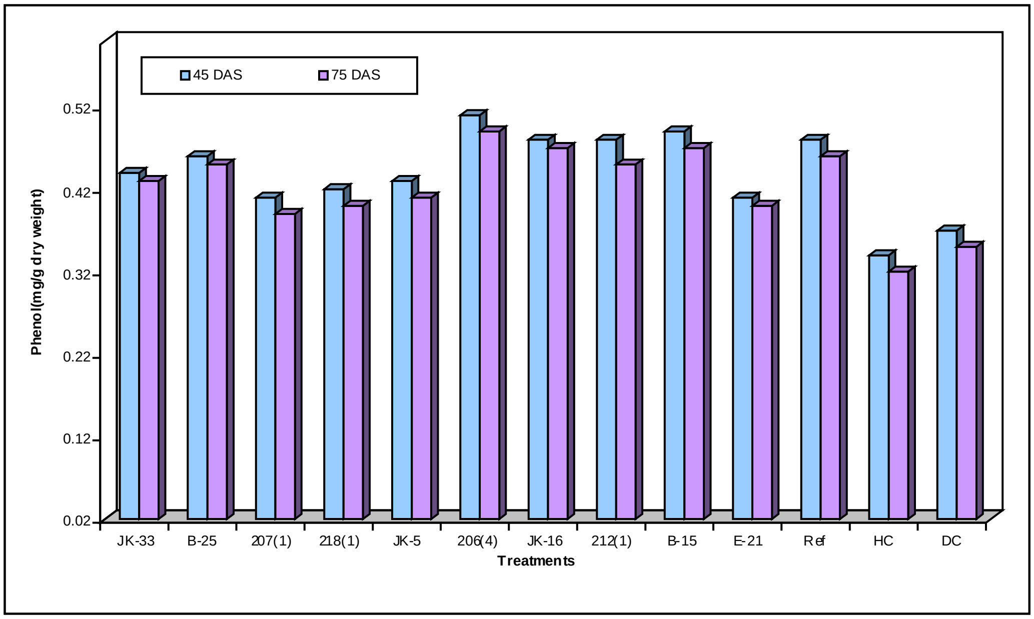


Fig. 2a: Effect of the selected rhizobacteria on defense molecules activity

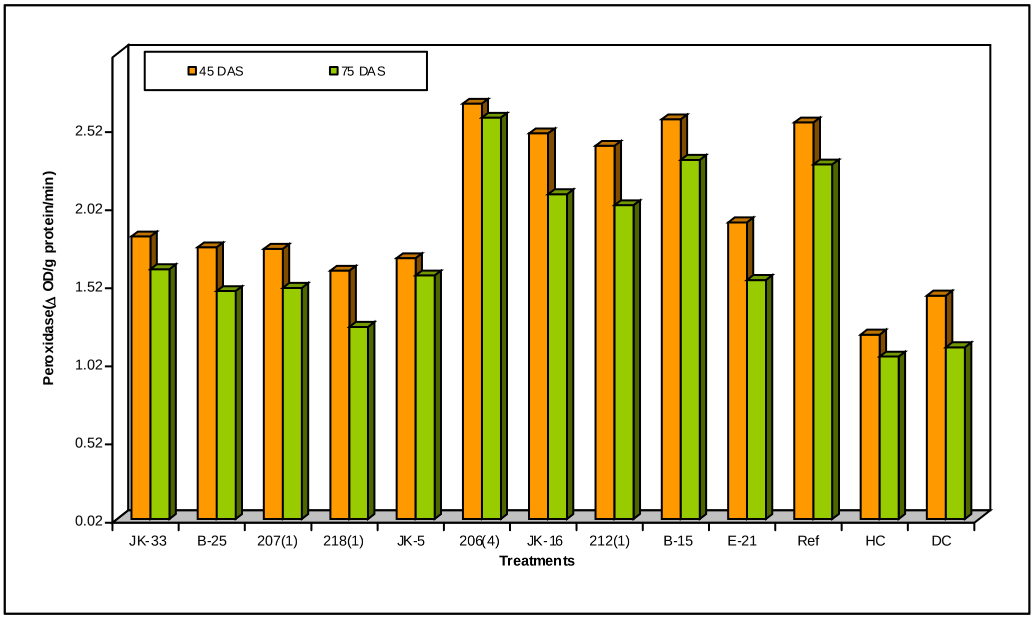


Fig. 2b: Effect of the selected rhizobacteria on defense molecules activity

Table 8b: Defense molecules as triggered by inoculation with selected rhizobacteria

SL. No.	Treatments	Polyphenol oxidase (ΔOD/g protein/min)		Phenylammonia lyase(changes in cinnamic acid/min/g)	
		45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* JK-33 +ToLCV	1.22	1.14	0.189	0.160
2	*Pseudomonas* B-25 +ToLCV	1.26	1.11	0.164	0.151
3	*Pseudomonas* 207(1) +ToLCV	1.20	1.09	0.173	0.161
4	*Pseudomonas* 218 +ToLCV	1.42	1.19	0.190	0.162
5	*Pseudomonas* JK-5 +ToLCV	1.34	1.13	0.184	0.178
6	*Pseudomonas* 206(4) +ToLCV	1.53	1.30	0.264	0.247
7	*Pseudomonas* JK-16 +ToLCV	1.42	1.25	0.241	0.222
8	*Pseudomonas* 212(1) +ToLCV	1.31	1.10	0.184	0.160
9	*Pseudomonas* B-15 +ToLCV	1.52	1.28	0.255	0.238
10	*Enterobacter* E-21 +ToLCV	1.32	1.18	0.231	0.215
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	1.87	1.57	0.251	0.230
12	Healthy control (No rhizobacteria , no ToLCV)	1.08	0.96	0.150	0.130
13	Diseased control (only ToLCV)	1.14	1.03	0.161	0.148
	SEm±	0.03	0.02	0.01	0.01
	CD @1%	0.12	0.06	0.03	0.04

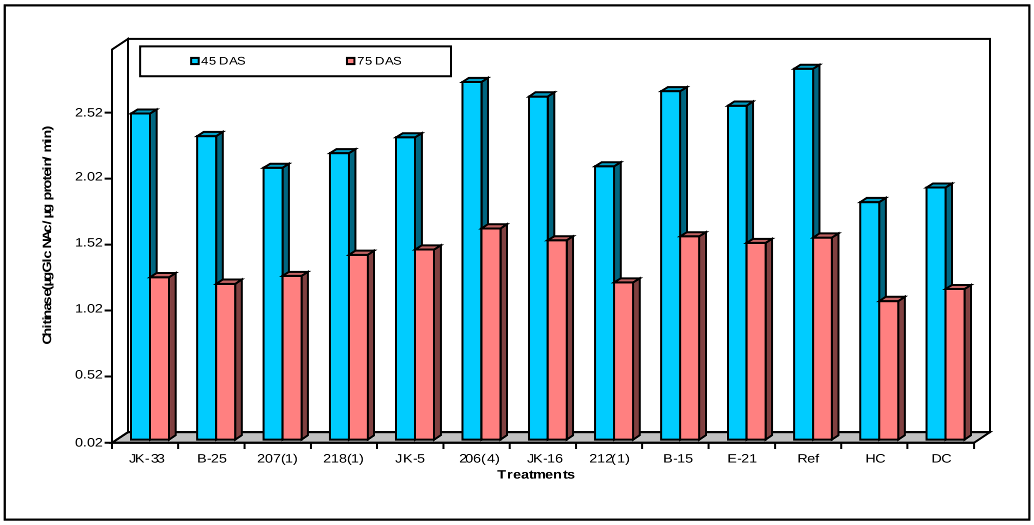


Fig. 2c: Effect of the selected rhizobacteria on defense molecules activity

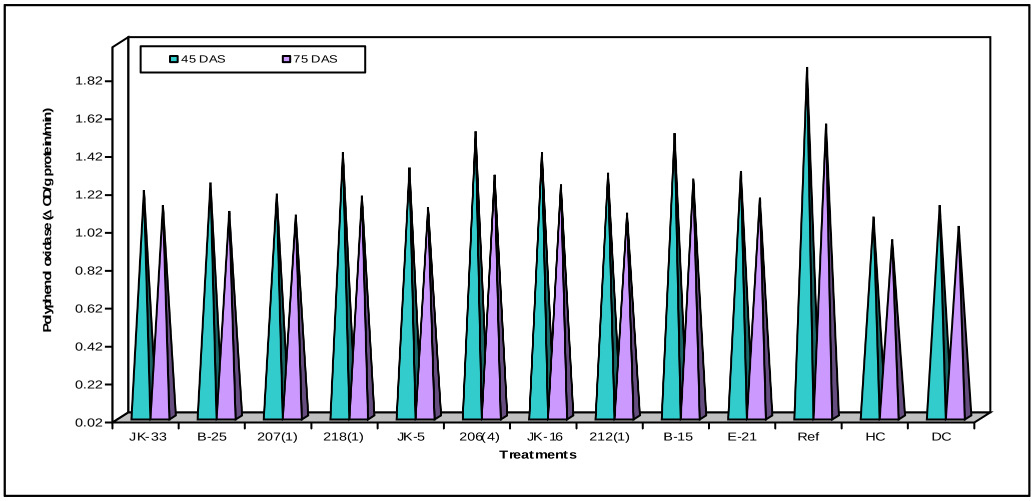


Fig. 2d: Effect of the selected rhizobacteria on defense molecules activity

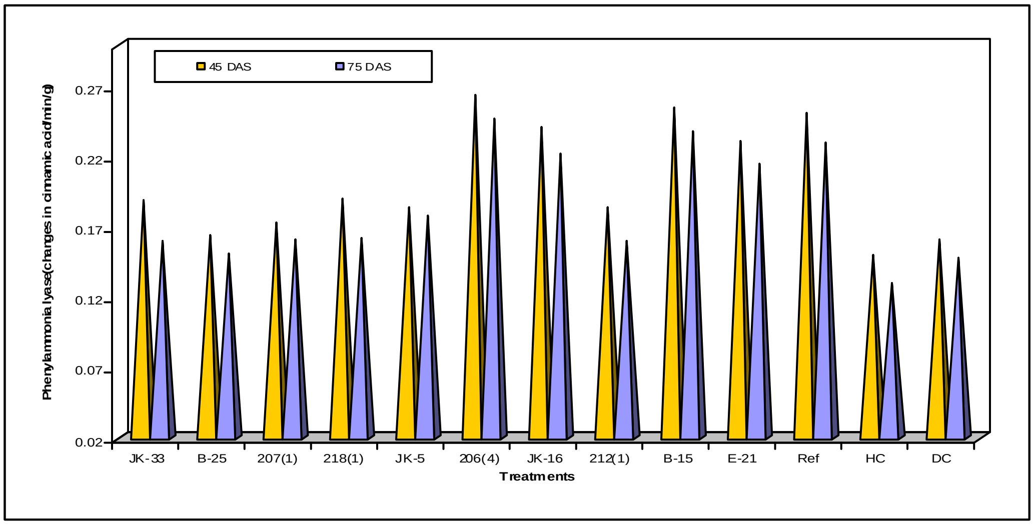


Fig. 2e: Effect of the selected rhizobacteria on defense molecules activity

Pseudomonas B-15 was the next best strain with 1.52 ∆OD/g protein/ min, which was on par with the plants treated with the reference strain (1.87 ∆OD/g protein/ min). The disease control treatment exhibited 1.14 ∆OD/g protein/ min. And, the least polyphenol oxidase activity was recorded in the healthy control (1.08 ∆OD/g protein/min).

At 75 DAS, the polyphenol oxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest polyphenol oxidase activity was seen in leaves inoculated with the reference strain (1.57 ∆OD/g protein/ min) followed by *Pseudomonas* 206(4) (1.30 ∆OD/g protein/ min) and *Pseudomonas* B-15 (1.28 ∆OD/g protein/ min. The least polyphenol oxidase activity was recorded in the healthy control (0.96 ∆OD/g protein/ min). The disease control plants showed an activity of 1.03 ∆OD/g protein/ min.

4.4.4 Effect of inoculation of rhizobacteria on the population of *Bemisia tabaci* on tomato plants

Influence of rhizobacteria on the population of whiteflies was studied. The insects present on the lower side of the leaf were counted by slowly turning the leaf without disturbing them. At 30 DAI, the treatment receiving *Pseudomonas* 206(4) strain recorded the least population of *Bemisia tabaci* (7.67 per leaf). This accounts to 74.99 per cent decrease over diseased control (Table 8). This treatment was followed by *Pseudomonas* B-15 (7.80 per leaf), which was on par with the plants treated with the reference strain (7.4 per leaf). The highest population of *Bemisia tabaci* was recorded in the diseased control (30.67 per leaf) (Table 9, Fig.3, Plate 17)

Similarly, at 45 DAI, also, *Pseudomonas* sp. 206(4) treatment showed the least population of *Bemisia tabaci* (2.33 per leaf), which accounts to 79.43 per cent decrease over diseased control. This was followed by B-15 (2.40 per leaf). The pathogen inoculated check exhibited the highest population of whiteflies (11.33 per leaf).

At 75 DAI, also, *Pseudomonas* sp. 206(4) treatment showed the least population of *Bemisia tabaci* (1.33 per leaf), which accounts to 71.52 per cent decrease over diseased control. This was followed by B-15 (1.40 per leaf). The pathogen inoculated check showed the highest population of whiteflies (4.67 per leaf).

4.4.5 Detection of ToLCV inoculum in leaves

In order to quantify the viral inoculum in tomato plants treated with rhizobacterial isolates, a semi quantitative PCR analysis was carried out. This analysis detects and estimates the viral DNA accumulation in the challenge inoculated plants.

All the rhizobacterial treated plants and the disease control plants were analyzed by means of semi quantitative PCR.

Equal amount of total DNA (100ng) isolated from rhizobacterial treated plants and from the check were used. PCR reaction using coat protein gene specific primers were performed for different number of cycles *viz*., 10, 20 and 30. PCR products were detected on agarose gel electrophoresis, and the threshold cycle was determined for each treatment. In general, a slight reduction in the viral inoculum was observed in leaves inoculated with rhizobacteria.Almost all the treated plants showed the viral inoculum with low amount of viral load except the diseased control plant whicn showed the highest amount of viral load after 20th cycle of the PCR at 45 DAS.

At 75 DAS, the reference strain, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed the least viral inoculum and remaining strains with higher amount of viral load.However,the the diseased control plant whicn showed the highest amount of viral load after 10th cycle of the PCR (Tables 10a and 10b, Plate 18).

4.4.6 Plant growth promotional potential of the selected rhizobacterial strains

The effect of the selected rhizobacteria on growth and yield parameters of Tomato was studied under high vector virus pressure conditions. The following parameters were recorded.

Table 9: Effect of inoculation of rhizobacteria on the population of *Bemisia tabaci* on tomato plants

Sl. No.	Treatments	30 DAI		45 DAI		75 DAI	
		Insect population(No./ leaf)	Per cent decrease over disease control	Insect population (No./ leaf)	Per cent decrease over disease control	Insect population (No./ leaf)	Per cent decrease over disease Control
1	*Pseudomonas* JK-33 +ToLCV	11.67	61.94	3.00	73.52	2.50	46.47
2	*Pseudomonas* B-25 +ToLCV	10.00	67.39	3.33	70.60	1.67	64.23
3	*Pseudomonas* 207(1) +ToLCV	11.20	63.48	2.80	75.28	2.20	52.89
4	*Pseudomonas* 218 +ToLCV	9.33	69.57	4.33	61.78	1.80	61.45
5	*Pseudomonas* JK-5 +ToLCV	10.50	65.76	3.50	69.10	2.33	50.10
6	*Pseudomonas* 206(4) +ToLCV	7.67	74.99	2.33	79.43	1.33	71.52
7	*Pseudomonas* JK-16 +ToLCV	8.00	73.91	2.67	76.43	1.67	64.23
8	*Pseudomonas* 212(1) +ToLCV	9.50	69.02	2.50	77.93	2.30	50.74
9	*Pseudomonas* B-15 +ToLCV	7.80	74.56	2.40	78.81	1.40	70.02
10	*Enterobacter* E-21 +ToLCV	8.67	71.73	2.67	76.43	2.00	57.17
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	7.40	75.87	2.30	79.69	1.33	71.52
12	Healthy control (No rhizobacteria , no ToLCV)	_	_	_	_	_	_
13	Diseased control (only ToLCV)	30.67	_	11.33	_	4.67	_

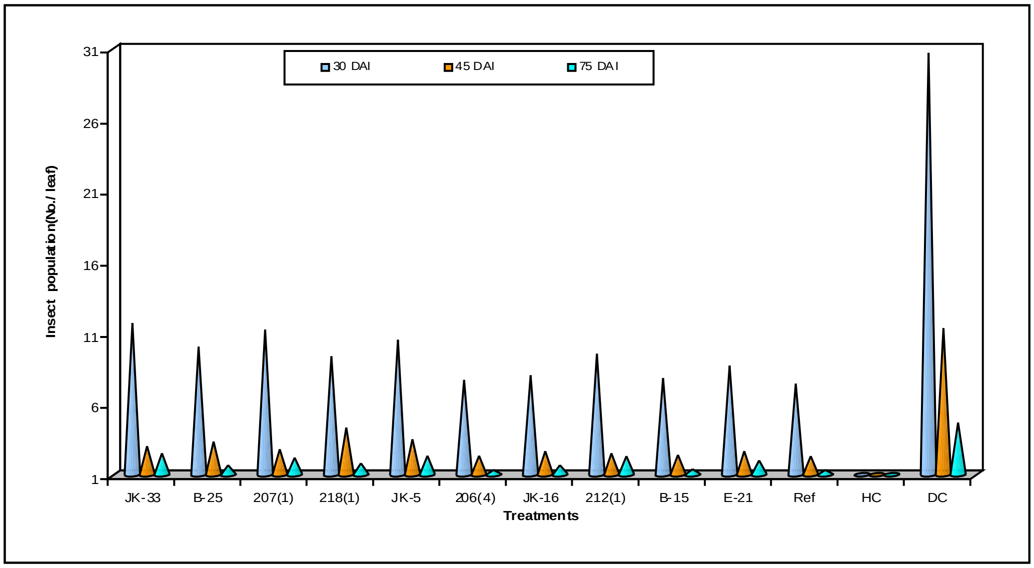


Fig. 3: Effect of inoculation of rhizobacteria on the population of Bemisia tabaci on tomato plants

Pseudomonas 206(4) treated

Disease control (UIC)

Plate 17. Comparison of whiteflies population in tomato with and without rhizobacterial inoculation

Table 10a: Quantification of viral load in the rhizobacteria treated tomato plants at 45 DAS

Sl. No	Treatments	AT 45 DAS	
		Threshold cycle	Viral load
1	*Pseudomonas* JK-33 +ToLCV	20	+
2	*Pseudomonas* B-25 +ToLCV	20	+
3	*Pseudomonas* 207(1) +ToLCV	20	++
4	*Pseudomonas* 218 +ToLCV	20	+
5	*Pseudomonas* JK-5 +ToLCV	20	+
6	*Pseudomonas* 206(4) +ToLCV	20	+
7	*Pseudomonas* JK-16 +ToLCV	20	+
8	*Pseudomonas* 212(1) +ToLCV	20	+
9	*Pseudomonas* B-15 +ToLCV	20	+
10	*Enterobacter* E-21 +ToLCV	20	+
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	20	+
12	Diseased control (only ToLCV)	20	+++

+ - indicates visible band appeared at 20th cycle; low amount of viral load
++ - indicates visible band appeared at 20th cycle; higher amount of viral load
+++ - indicates visible band appeared at 20th cycle; the highest amount of viral load

LEGEND

M Double digest marker(ECoRI+Hind III)

Lane 1: ToLCV amplicon after 10,20 and 30 cycles(Positive control)

Lane 2-12: PGPR treated plants after 10,20 and 30 cycles

1 – Disese control

2- Enterobacter E-21

3- *Pseudomonas* 207(1)

4- *Pseudomonas* 212(1)

5- *Pseudomonas* JK-33

6- *Pseudomonas* B-25

7- *Pseudomonas* JK-5

8 - *Pseudomonas* JK-16

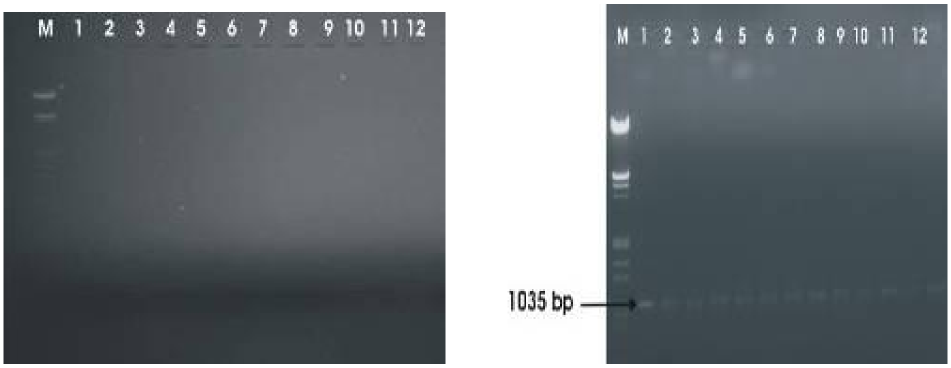
9 - *Pseudomonas* B-15

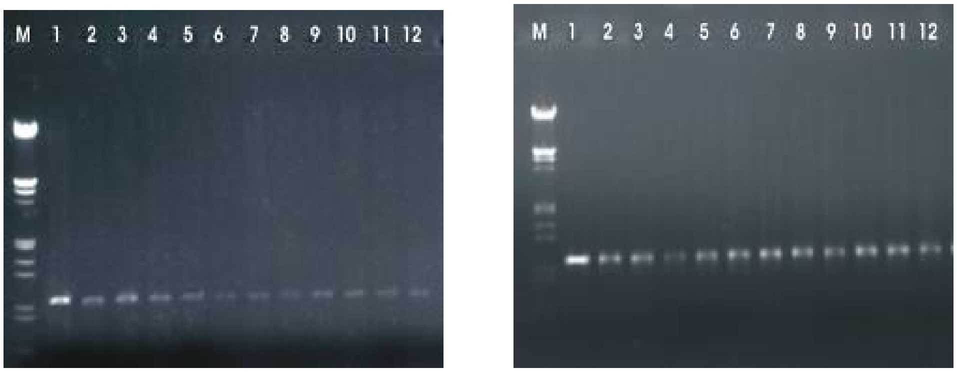
10 - *Pseudomonas* 206(4)

11 - *Pseudomonas* 218(1)

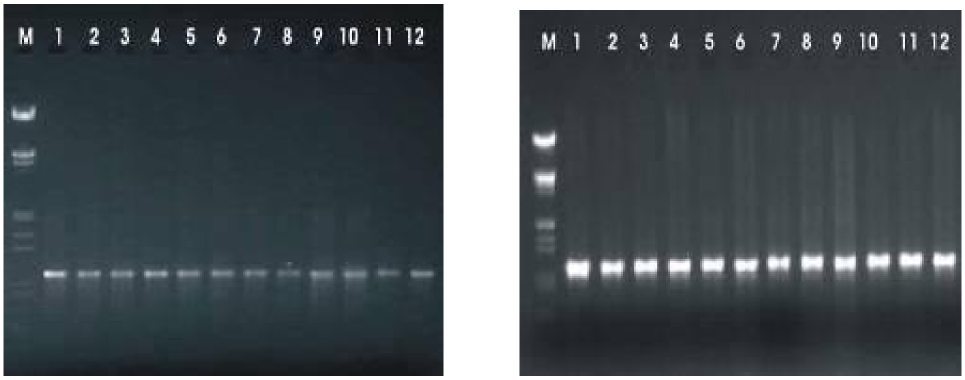
12 - Reference strain (*P. fluorescens* NCIM 2099)

45 DAS **75 DAS**





20 Cycles



30 Cycles

Plate 18. Quantification of viral load in the rhizobacteria treated tomato plants

Table 10b: Quantification of viral load in the rhizobacteria treated tomato plants at 75 DAS

Sl. No	Treatments	AT 75 DAS	
		Threshold cycle	**Viral load**
1	*Pseudomonas* JK-33 +ToLCV	10	++
2	*Pseudomonas* B-25 +ToLCV	10	++
3	*Pseudomonas* 207(1) +ToLCV	10	++
4	*Pseudomonas* 218 +ToLCV	10	++
5	*Pseudomonas* JK-5 +ToLCV	10	++
6	*Pseudomonas* 206(4) +ToLCV	10	+
7	*Pseudomonas* JK-16 +ToLCV	10	++
8	*Pseudomonas* 212(1) +ToLCV	10	++
9	*Pseudomonas* B-15 +ToLCV	10	+
10	*Enterobacter* E-21 +ToLCV	10	++
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	10	+
12	Diseased control (only ToLCV)	10	+++

+ - indicates visible band appeared at 10th cycle; low amount of viral load
++ - indicates visible band appeared at 10th cycle; higher amount of viral load
+++ - indicates visible band appeared at 10th cycle; the highest amount of viral load

4.4.6.1 Plant height

The data pertaining to effect on plant height of Tomato plants at different intervals of time *viz.*, 30, 45, 60, 75 DAS and at harvest are given in Table 11,Fig.4., Plate 19. At 30 DAS, plant height was significantly improved due to bacterial inoculation. The highest length (25.5 cm / plant) was observed in the plants inoculated with *Pseudomonas* 206(4) which was on par with the plants inoculated with the reference strain (24.90 cm). The least height was observed in the diseased control treatment (19.40 cm / plant)

At 45 DAS, again *Pseudomonas* 206(4) inoculated plants resulted in the highest plant height (38.10 cm/plant). The next best strain was *Pseudomonas* B-15 which showed the plant height (36.90 cm / plant). This was followed by the reference strain (36.60 cm / plant), and the least plant height was in the challenge inoculated control treatment (26.05 cm / plant).

At 75 DAS also, *Pseudomonas* 206(4) inoculated plants resulted in the highest plant height (48.50 cm/plant). This was followed by *Pseudomonas* B-15 (48.30 cm / plant) and reference strain with 45.70 cm/plant.

At harvest, similar trend was observed. The highest plant height was observed in the treatment inoculated with *Pseudomonas* 206(4*)*. It produced a height of 51.80 cm per plant. This was followed by the *Pseudomonas* B-15 (49.70 cm / plant) and the reference strain with 48.10 cm/plant. The viral pathogen inoculated check exhibited the least plant height (33.50 cm/plant).

4.4.6.2 Plant biomass

In general, bacterized plants produced significantly higher biomass than the uninoculated plants. And, *Pseudomonas* 206(4) produced consistently higher biomass throughout the growth period of tomato. At 45 DAS, the plants inoculated with *Pseudomonas* 206(4) produced the highest biomass (32.48 g/plant) (Table 12,Fig.5). This was followed by the reference strain (31.86 g/plant) and *Pseudomonas* B-15 (31.44 g/plant).The least biomass was recorded in the viral pathogen inoculated treatment (24.24 g/plant).

At 75 DAS, significantly higher biomass was obtained in the treatment receiving *Pseudomonas* 206(4) (34.98 g/plant). This was followed by the reference strain (34.46 g/plant) and *Pseudomonas* B-15 (34.12 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (26.14 g/plant).

At the time of harvesting, the maximum biomass was recorded in *Pseudomonas* 206(4) treatment (36.90 g/plant), which was followed by the reference strain (36.06 g/plant) and *Pseudomonas* B-15 treatment (35.94 g/plant). However, the least biomass was recorded in the viral pathogen inoculated treatment (26.34 g/plant).

4.4.6.3 Chlorophyll content

In general, chlorophyll content of tomato plants was significantly higher in rhizobacteria inoculated plants. The data are presented in Table 13, Fig.6. Chlorophyll content was increased up to 45 DAS and declined thereafter.

At 30 DAS, *Pseudomonas* 206(4) resulted in significantly higher chlorophyll content (24.70 SPAD value). This was followed by the healthy control (24.30 SPAD value), reference strain (23.60 SPAD value) and *Pseudomonas* B-15 (23.40 SPAD value). The least chlorophyll content was recorded in the diseased control (21.00 SPAD value).

At 45 DAS, *Pseudomonas* 206(4) inoculation resulted in significantly higher chlorophyll content (38.60 SPAD value), which was on par with the reference strain (37.90 SPAD value) and *Pseudomonas* B-15 (38.10 SPAD value). The least chlorophyll content was recorded in the diseased control (26.50 SPAD value).

At 75 DAS, the maximum chlorophyll content was recorded in the leaves of plants inoculated with *Pseudomonas* 206(4) (32.80 SPAD value). The next best strain was *Pseudomonas* B-15 (30.50 SPAD value), which was on par with the reference strain (30.90 SPAD value). The least chlorophyll content was recorded in the diseased control (18.50 SPAD value).

Table 11: Effect of the selected rhizobacteria on plant height of tomato

Sl. No	Treatments	Plant height (cm)			
		30 DAS	**45 DAS**	**75 DAS**	**At Harvest**
1	*Pseudomonas* JK-33 +ToLCV	22.2	30.9	35.9	38.4
2	*Pseudomonas* B-25 +ToLCV	22.3	31.0	35.5	38.0
3	*Pseudomonas* 207(1) +ToLCV	23.3	32.4	37.1	41.1
4	*Pseudomonas* 218 +ToLCV	22.4	31.1	36.6	40.8
5	*Pseudomonas* JK-5 +ToLCV	23.9	33.1	39.8	42.7
6	*Pseudomonas* 206(4) +ToLCV	25.5	38.1	48.5	51.8
7	*Pseudomonas* JK-16 +ToLCV	24.6	36.8	45.3	49.3
8	*Pseudomonas* 212(1) +ToLCV	22.6	33.7	38.7	41.1
9	*Pseudomonas* B-15 +ToLCV	24.7	36.9	48.3	49.7
10	*Enterobacter* E-21 +ToLCV	22.3	30.7	45.0	47.1
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	24.9	36.6	45.7	48.1
12	Healthy control (No rhizobacteria , no ToLCV)	19.7	28.2	33.8	36.2
13	Diseased control (only ToLCV)	19.4	26.05	31.3	33.5
	SEm±	0.42	0.66	0.67	0.79
	CD @1%	1.59	2.51	2.54	2.99

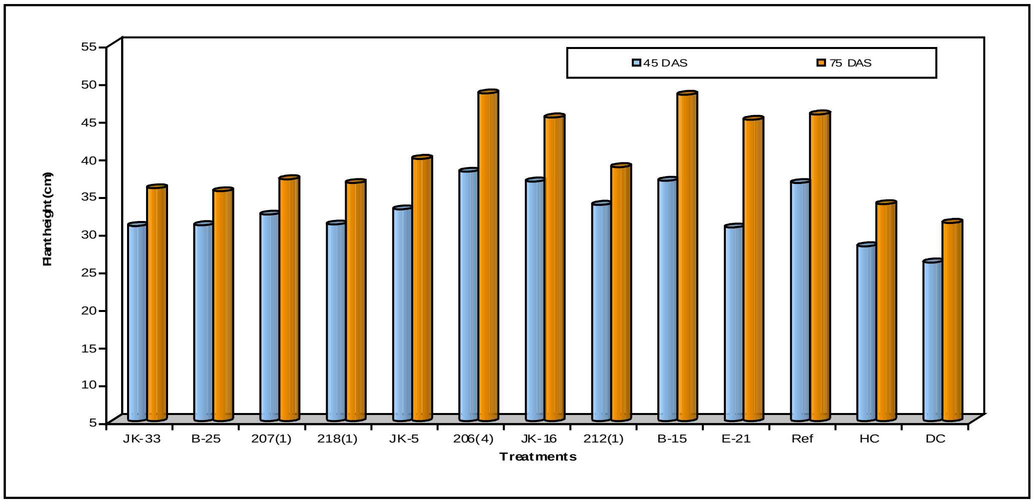


Fig. 4: Effect of the selected rhizobacteria on plant height of tomato

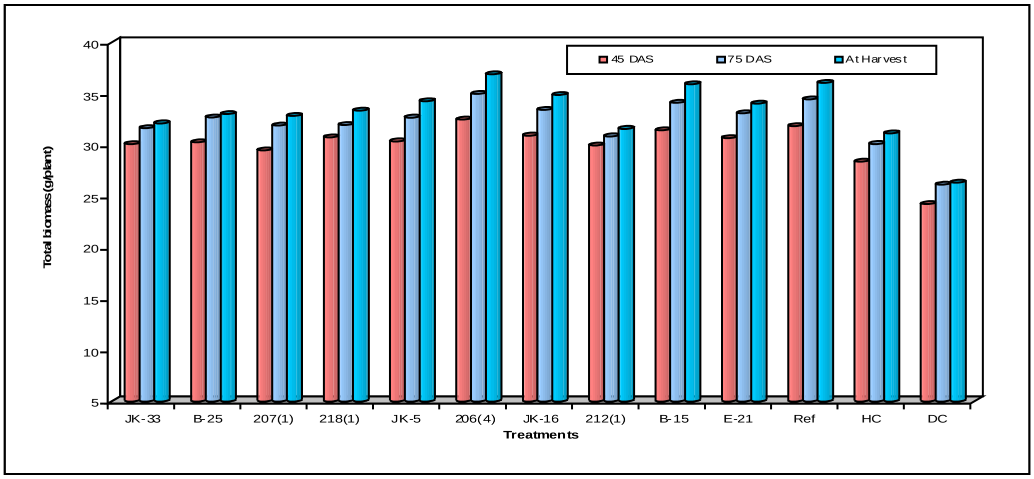


Fig. 5: Influence of inoculation of the selected rhizobacteria on total biomass of tomato plants

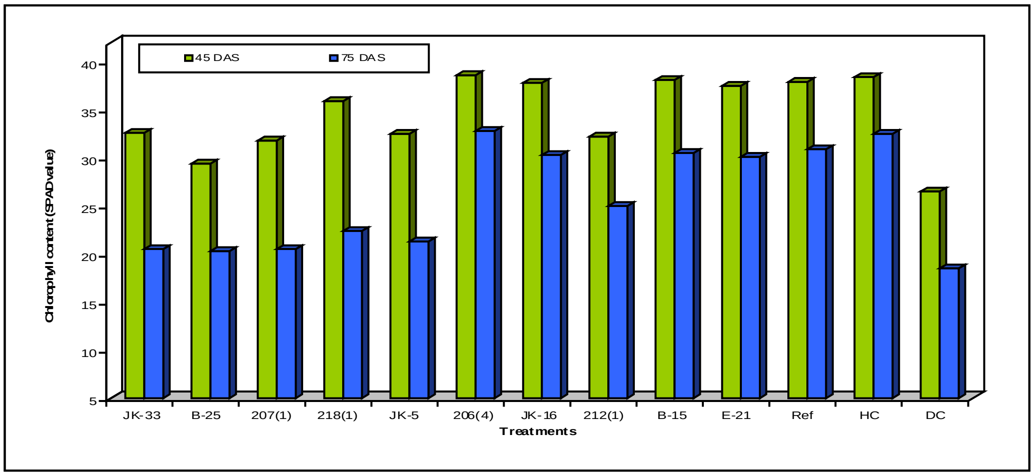


Fig. 6: Effect of the selected rhizobacterial inoculation on chlorophyll content in tomato plants

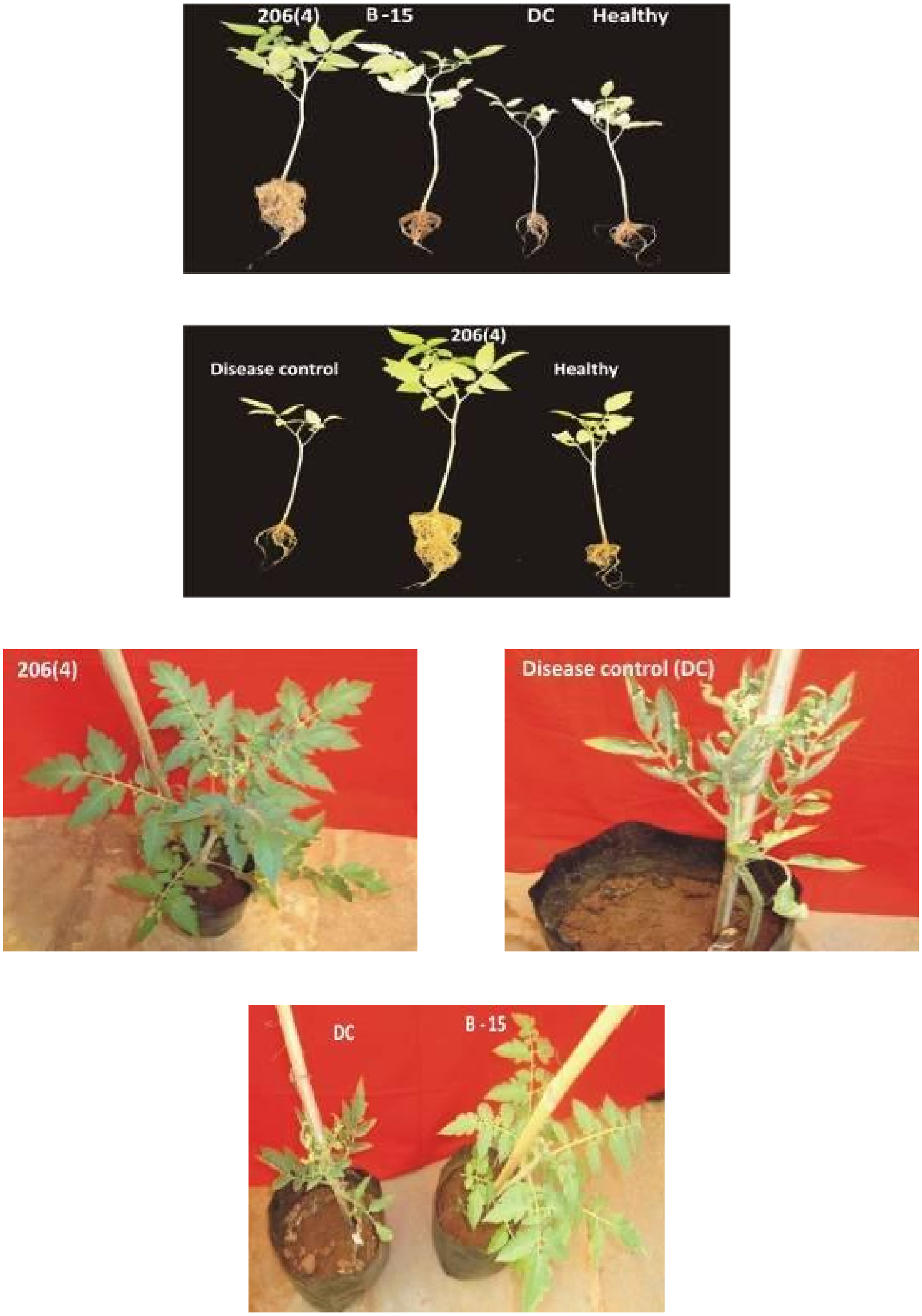


Plate 19. Plant growth promotion by the selected rhizobacterial isolates

Table 12: Influence of inoculation of the selected rhizobacteria on total biomass of tomato plants

Sl. No.	Treatments	Total biomass (g/plant)		
		45DAS	75 DAS	At Harvest
1	*Pseudomonas* JK-33 +ToLCV	30.094	31.65	32.12
2	*Pseudomonas* B-25 +ToLCV	30.26	32.7	33.02
3	*Pseudomonas* 207(1) +ToLCV	29.48	31.9	32.84
4	*Pseudomonas* 218 +ToLCV	30.76	31.96	33.36
5	*Pseudomonas* JK-5 +ToLCV	30.36	32.68	34.28
6	*Pseudomonas* 206(4) +ToLCV	32.48	34.98	36.9
7	*Pseudomonas* JK-16 +ToLCV	30.92	33.44	34.88
8	*Pseudomonas* 212(1) +ToLCV	29.96	30.84	31.58
9	*Pseudomonas* B-15 +ToLCV	31.44	34.12	35.94
10	*Enterobacter* E-21 +ToLCV	30.68	33.1	34.04
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	31.86	34.46	36.06
12	Healthy control (No rhizobacteria , no ToLCV)	28.38	30.1	31.14
13	Diseased control (only ToLCV)	24.24	26.14	26.34
	SEm±	0.41	0.30	0.32
	CD @1%	1.54	1.13	1.20

Table 13: Effect of the selected rhizobacterial inoculation on chlorophyll content in tomato plants

Sl. No	Treatments	Chlorophyll content (SPAD value)			
		30 DAS	45 DAS	75 DAS	At harvest
1	*Pseudomonas* JK-33 +ToLCV	22.1	32.6	20.5	15.3
2	*Pseudomonas* B-25 +ToLCV	22.2	29.4	20.3	13.8
3	*Pseudomonas* 207(1) +ToLCV	23.1	31.8	20.5	18.9
4	*Pseudomonas* 218 +ToLCV	22.6	35.9	22.4	20.5
5	*Pseudomonas* JK-5 +ToLCV	22.3	32.5	21.3	20.4
6	*Pseudomonas* 206(4) +ToLCV	24.7	38.6	32.8	22.3
7	*Pseudomonas* JK-16 +ToLCV	22.7	37.8	30.3	21.6
8	*Pseudomonas* 212(1) +ToLCV	22.4	32.2	25.0	18.4
9	*Pseudomonas* B-15 +ToLCV	23.4	38.1	30.5	21.8
10	*Enterobacter* E-21 +ToLCV	21.6	37.5	30.1	21.1
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.6	37.9	30.9	21.6
12	Healthy control (No rhizobacteria , no ToLCV)	24.3	38.4	32.5	23.1
13	Diseased control (only ToLCV)	21.0	26.5	18.5	10.0
	SEm±	0.43	0.57	0.46	0.39
	CD @1%	1.61	2.17	1.75	1.46

At harvest, the chlorophyll content was decreased in all the treatments when compared to at 45 DAS. The maximum chlorophyll content was recorded in the healthy control plants (23.10 SPAD value), which was followed by plants treated with *Pseudomonas* strain 206(4) (22.30 SPAD value), *Pseudomonas* strain B-15 (21.80 SPAD value) and the reference strain (21.60 SPAD value). The least chlorophyll was synthesized in the leaves of diseased control plants (10.00 SPAD value).

4.4.6.4 Fruit yield

Although plants inoculated with rhizobacterial strains have shown good physiological attributes including early flowering compared to uninoculated control plants, there was no fruiting because all the flowers got withered due to high vector virus pressure which was maintained throughout the growth period under glasshouse condition.

4.5 Evaluation of the combination of rhizobacterial isolates and chitosan for the biocontrol of ToLCV as well as growth promotion of Tomato

4.5.1 Biocontrol potential of the combination of selected rhizobacterial isolates and chitosan

Based on the previous pot experiment, three isolates (B-15, 206(4) and JK-16) were selected for further investigation.These rhizobacterial isolates were further tested for their ability to control ToLCV in tomato in pots under green house condition in combination with and without chitosan at high level of vector virus pressure conditions and the results are furnished in Table 14 and Fig. 7. Inoculation of these rhizobacterial isolates along with chitosan significantly reduced ToLCV severity both at 45 DAI and 75 DAI (Plate 20 and 21). At 45 DAI, all the isolates showed higher disease severity control ranging from 50 to 100 percent whereas at 75 DAI, the highest viral disease severity control (87.50%) was noticed in the plants inoculated with rhizobacterial mixture (206(4) +B-15+ JK-16) +Chitosan, which was followed by 206(4) + Chitosan and B-15+Chitosan treatment with 75.00 percent disease severity control. And, the least severity control was noticed when *Pseudomonas* JK-16 and chitosan were applied alone (25 % control).

4.5.2 Ability of the rhizobacteria and chitosan to induce systemic resistance against ToLCV in tomato plants

The ISR activity of the isolates along with chitosan was tested by assaying for defense enzymes such as peroxidase, chitinase, polyphenoloxidase, phenylalanine ammonia lyase (PALase) and phenol content in plants at different intervals of time.

4.5.2.1 Phenol content

Phenol content of leaves, at different intervals *viz.*, 45 and 75 DAS, as influenced by inoculation with rhizobacteria are presented in Table 15 a, Fig.8a.

At 45 DAS, the treatment receiving rhizobacterial mixture (206(4) +B-15+ JK-16) + Chitosan recorded the maximum phenol content (0.68mg/g), which was followed by the *Pseudomonas* 206(4) +Chitosan treatment (0.58 mg/g). However, the least phenol content was recorded in the chemical control treatment (0.37 mg/g).

At 75 DAS, phenol content was slightly decreased in all the treatments as compared to 45 DAS. The highest phenol content was recorded in the plants inoculated with rhizobacterial mixture with chitosan (0.64 mg/g). This was followed by the *Pseudomonas* 206(4) +Chitosan treatment (0.55 mg/g). Chemical control recorded significantly lower amount of phenol content (0.36 mg/g).

4.5.2.2 Phenylalanine ammonia lyase (PALase) activity

The results on biosynthesis of PALase activity as influenced by rhizobacterial strains in Tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in Table 15b, Fig.8e.

Table 14: Biocontrol of ToLCV disease by the selected rhizobacterial strains and chitosan

SL. No.	Treatments	45 DAI		75 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
1	*Pseudomonas* B-15 +ToLCV	37.5	62.5	62.5	37.5
2	*Pseudomonas* JK-16 +ToLCV	50.0	50	75.0	25.0
3	*Pseudomonas* 206(4) +ToLCV	25.0	75	50.0	50.0
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	12.5	87.5	37.5	62.5
5	*Pseudomonas* B-15+ Chitosan +ToLCV	12.5	87.5	25.0	75.0
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	25.0	75	37.5	62.5
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	12.5	87.5	25.0	75.0
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	-	100	12.5	87.5
9	Chitosan +ToLCV	37.5	62.5	75.0	25.0
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	25	75	50.0	50.0
11	Chemical control +ToLCV	37.5	62.5	62.5	37.5
12	Diseased control (only ToLCV)	100	0	100	0

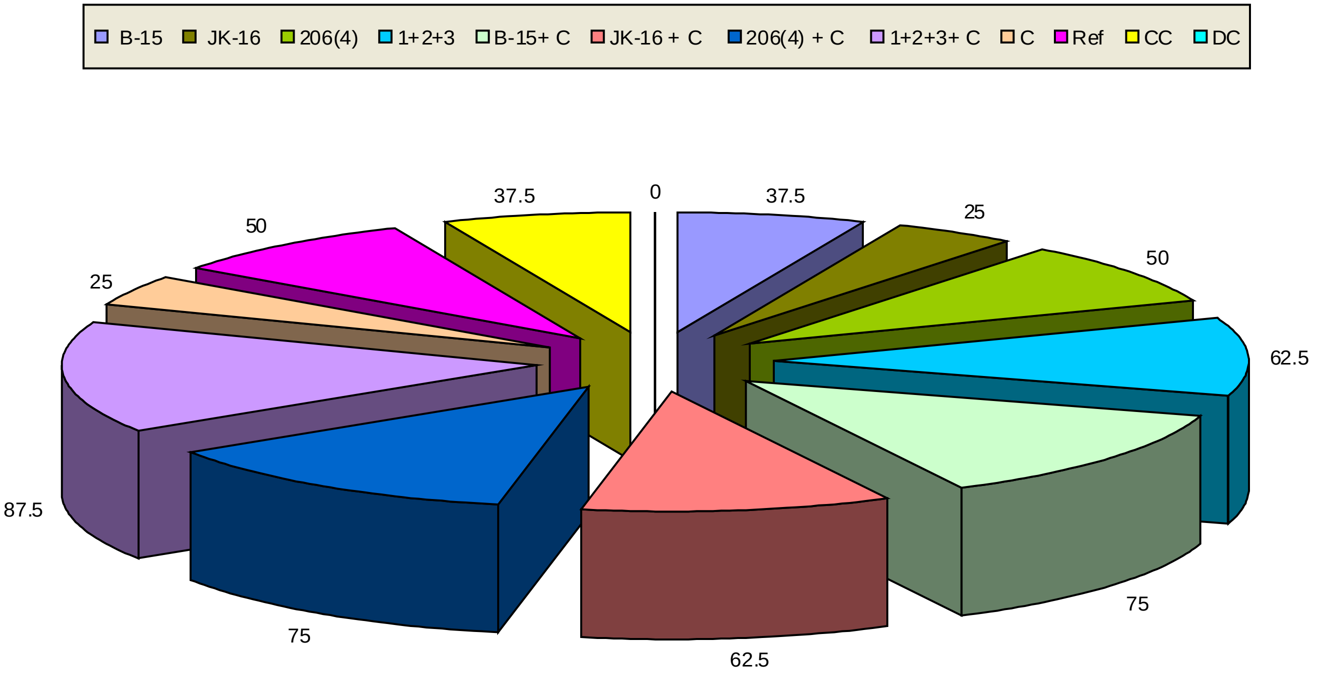


Fig. 7: Biocontrol of ToLCV disease by the selected rhizobacterial strains and chitosan at 75 DAI

Fig. 7: Biocontrol of ToLCV disease by the selected rhizobacterial strains and chitosan at 75 DAI

Plate 20. Plant growth and disease severity of ToLCV as influenced by inoculation with rhizobacterial isolates with and without chitosan (30 DAI)

Plate 20 Contd…..

Plate 21. Plant growth and disease severity of ToLCV as influnced by inoculation with rhizobacterial isolates with and without chitosan (90 DAI)

late 21 Contd,,,, (90 DAI)

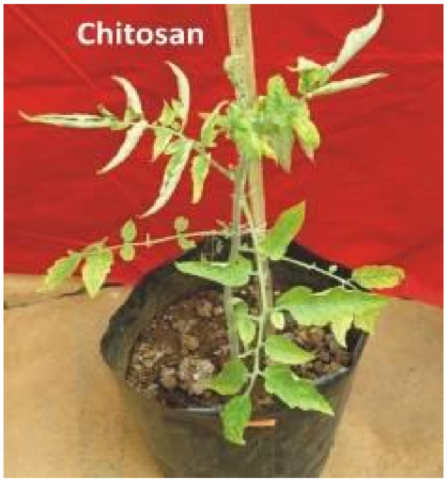
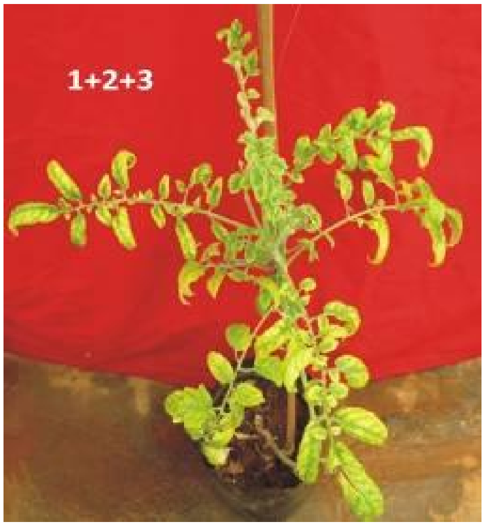


Plate 21 Contd,,,, (120 DAI)



1+2+3+B -15JK +16 + 206(4)

C – Chitosan, Dc – Disease control

Table 15a: Defense molecules as triggered by inoculation with selected rhizobacteria and chitosan

SL. No.	Treatments	Phenol(mg/g dry weight)		Peroxidase(ΔOD/g protein/min)		Chitinase (µgGlc NAc/ µg protein/ min)	
		45 DAS	75 DAS	45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* B-15 +ToLCV	0.46	0.43	2.32	1.96	2.84	1.97
2	*Pseudomonas* JK-16 +ToLCV	0.44	0.40	2.22	1.88	2.67	1.92
3	*Pseudomonas* 206(4) +ToLCV	0.47	0.45	2.64	2.08	2.95	2.00
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	0.51	0.49	2.88	2.18	3.05	2.11
5	*Pseudomonas* B-15+ Chitosan +ToLCV	0.55	0.51	2.82	2.15	3.63	2.23
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	0.52	0.48	2.49	2.05	3.56	2.17
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	0.58	0.55	2.94	2.66	3.70	2.27
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	0.68	0.64	3.29	2.82	3.91	2.52
9	Chitosan +ToLCV	0.43	0.38	1.85	1.62	2.50	1.85
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	0.49	0.47	2.28	1.92	2.75	1.97
11	Chemical control +ToLCV	0.37	0.36	1.45	1.34	2.17	1.53
12	Diseased control (only ToLCV)	0.39	0.37	1.52	1.45	2.31	1.61
	SEm±	0.02	0.01	0.09	0.08	0.04	0.02
	CD @1%	0.06	0.06	0.35	0.30	0.17	0.10

LEGEND

B-15= *Pseudomonas* B-15 +ToLCV

JK-16=*Pseudomonas* JK-16 +ToLCV

206(4) = *Pseudomonas* 206(4) +ToLCV

1+2+3 = *Pseudomonas* (206(4) +B-15+JK-16) +ToLCV

B-15+C = *Pseudomonas* B-15 + Chitosan +ToLCV

JK-16+C =*Pseudomonas* JK-16 + Chitosan +ToLCV

206(4) +C = *Pseudomonas* 206(4) + Chitosan +ToLCV

1+2+3+C = *Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV

C = Chitosan +ToLCV

Ref = Reference strain (*P. fluorescens* NCIM 2099) +ToLCV

CC = Chemical control +ToLCV

DC = Diseased control (only ToLCV)

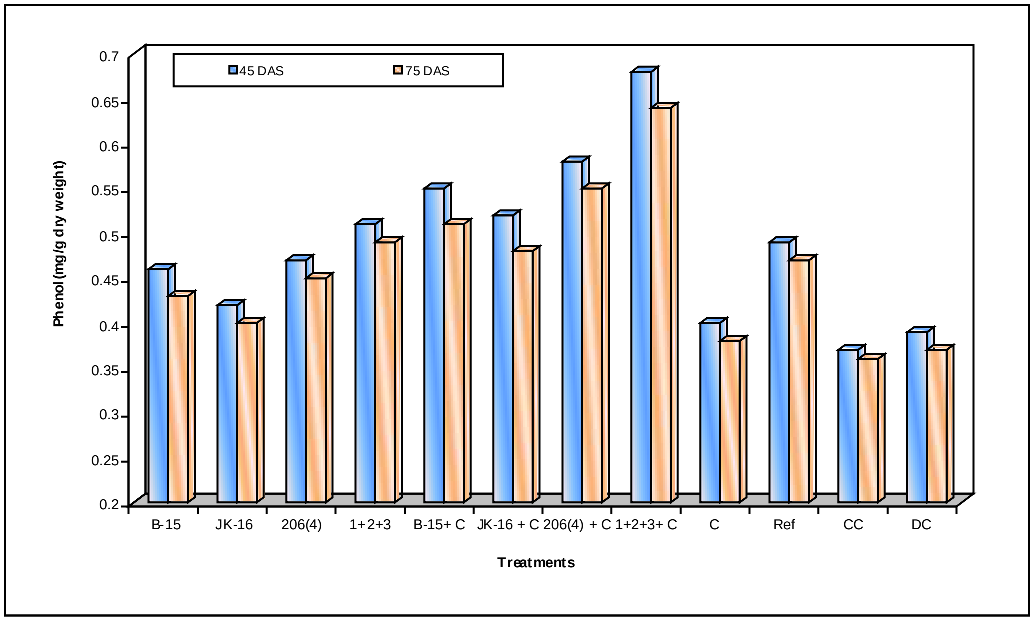


Fig. 8a: Effect of the selected rhizobacteria and chitosan on defense molecules activity

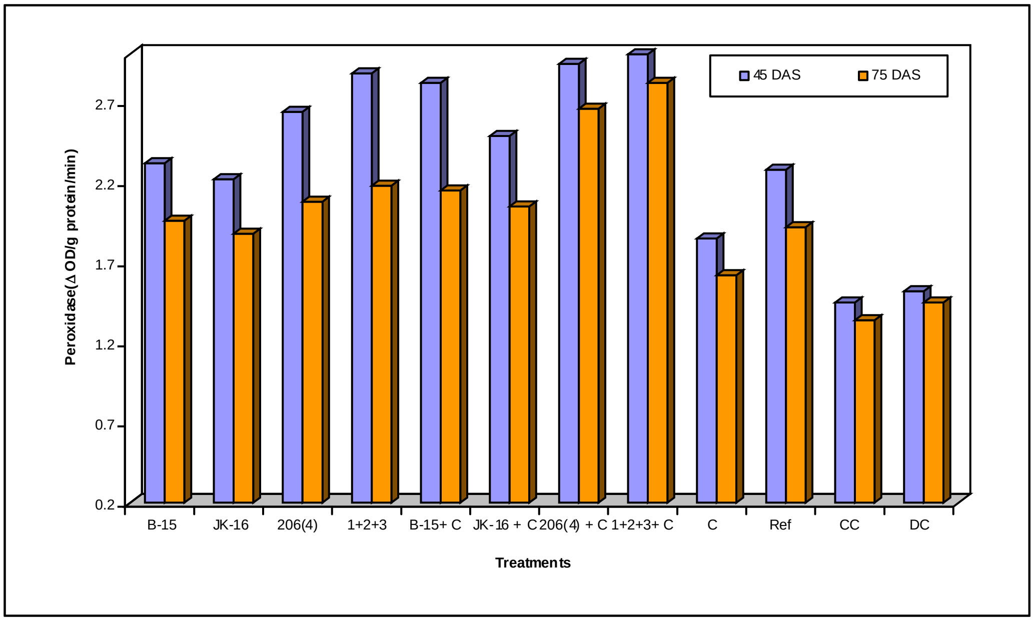


Fig. 8b: Effect of the selected rhizobacteria and chitosan on defense molecules activity

It was observed that PALase activity was substantially increased in all the rhizobacteria and chitosan inoculated treatments. At 45 DAS, the maximum PALase activity was recorded in the treatment receiving rhizobacterial mixture + chitosan (0.290 change in cinnamic acid/min/g) followed by the plants inoculated with rhizobacterial mixture alone (0.270 change in Cinnamic acid/min/g). The least PALase activity was recorded in the chemical control (0.160 change in Cinnamic acid/min/g).

At 75 DAS, PALase activity was found decreasing in all the treatments when compared to 45 DAS. The highest activity was seen in plants inoculated with rhizobacterial mixture + chitosan (0.270 change in cinnamic acid/min/g) followed by the plants inoculated with rhizobacterial mixture alone (0.260 change in Cinnamic acid/min/g).The least was exhibited by the chemical control (0.150 change in Cinnamic acid/min/g).

4.5.2.3 Peroxidase activity

The data pertaining to peroxidase activity as influenced by rhizobacterial combination with chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in Table 15 a, Fig.8b.

There was a tremendous increase in peroxidase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest peroxidase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (3.29 ∆OD/g protein/ min). *Pseudomonas* 206(4) +Chitosan was the next best treatment with 2.94 ∆OD/g protein/ min.The disease control treatment exhibited 1.52 ∆OD/g protein/ min. And, the least peroxidase activity was recorded in the chemical control (1.45 ∆OD/g protein/ min).

At 75 DAS, the peroxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest peroxidase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (2.82 ∆OD/g protein/ min), followed by *Pseudomonas* 206(4) +Chitosan (2.66 ∆OD/g protein/ min). The least peroxidase activity was recorded in the healthy control (1.34 ∆OD/g protein/ min). The disease control plants showed an activity of 1.45 ∆OD/g protein/ min.

4.5.2.4 Chitinase activity

The results on biosynthesis of chitinase activity as influenced by rhizobacterial strains with and without chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in Table 15a, Fig.8c.

At 45 DAS, the highest chitinase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (3.91 µgGlc NAc/ µg protein/ min). *Pseudomonas* 206(4) +Chitosan was the next best treatment with 3.70 µgGlc NAc/ µg protein/ min.The disease control treatment exhibited 2.31 µgGlc NAc/ µg protein/ min. And, the least chitinase activity was recorded in the chemical control (2.17 µgGlc NAc/ µg protein/ min).

At 75 DAS, the chitinase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest chitinase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (2.52 µgGlc NAc/ µg protein/ min), followed by *Pseudomonas* 206(4) +Chitosan (2.27 µgGlc NAc/ µg protein/ min). The least chitinase activity was recorded in the chemical control (1.53 µgGlc NAc/ µg protein/ min). The disease control plants showed an activity of 1.61 µgGlc NAc/ µg protein/ min.

4.5.2.5 Polyphenol oxidase activity

The polyphenol oxidase activity as influenced by rhizobacterial strains and chitosan treatment in tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table 15 b, Fig.8d.

In general, there was a tremendous increase in polyphenol oxidase activity in both the stages due to rhizobacterial and chitosan treatment. At 45 DAS, the highest polyphenol oxidase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (2.56 ∆OD/g protein/ min). *Pseudomonas* 206(4) +Chitosan was the next best treatment with 2.39 ∆OD/g protein/ min. The disease control treatment exhibited 1.15 ∆OD/g protein/ min. And, the least polyphenol oxidase activity was recorded in thechemical control (1.07 ∆OD/g protein/ min).

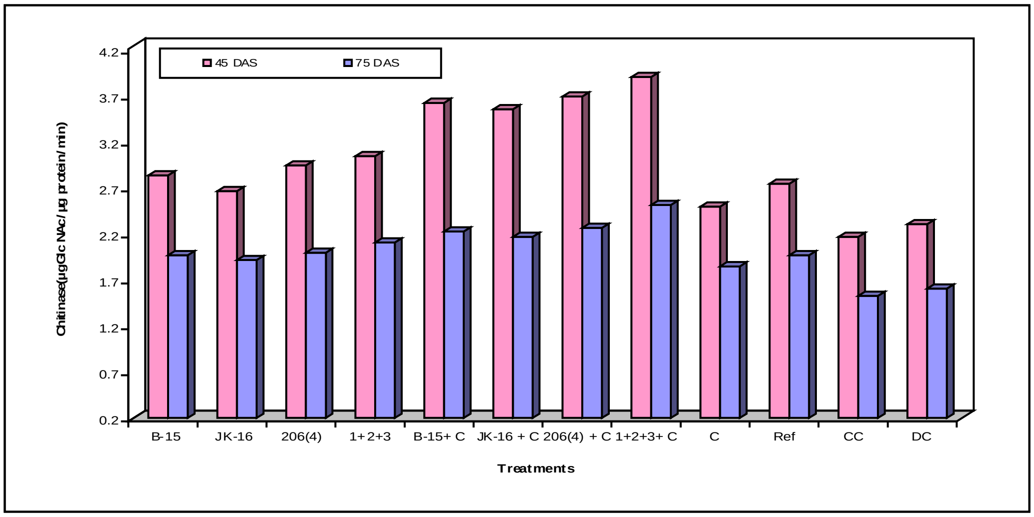


Fig. 8c: Effect of the selected rhizobacteria and chitosan on defense molecules activity

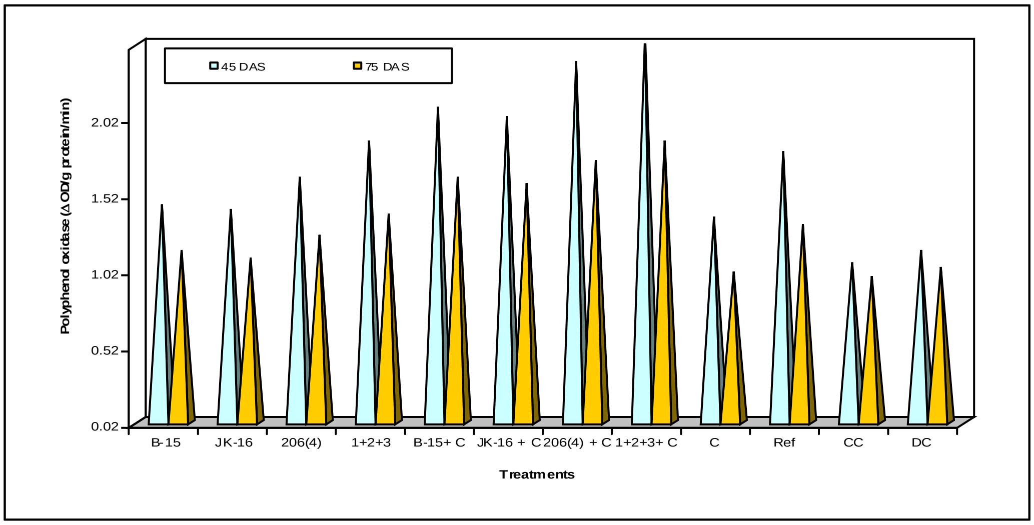


Fig. 8d: Effect of the selected rhizobacteria and chitosan on defense molecules activity

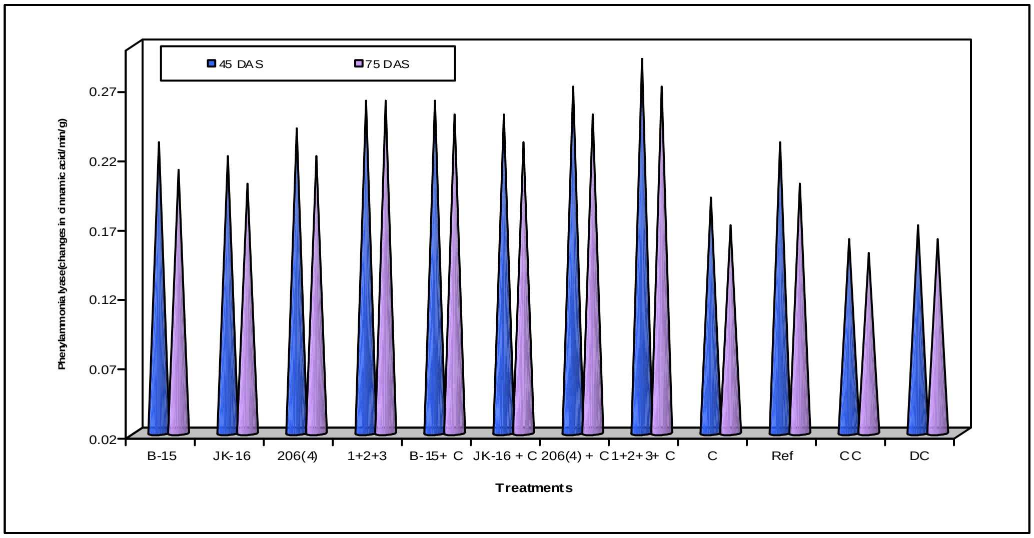


Fig. 8e: Effect of the selected rhizobacteria and chitosan on defense molecules activity

Table 15b: Defense molecules as triggered by inoculation with selected rhizobacteria and chitosan

SL. No.	Treatments	Polyphenol oxidase (∆OD/g protein/min)		Phenylammonia lyase (changes in cinnamic acid/min/g)	
		45 DAS	75 DAS	45 DAS	45 DAS
1	*Pseudomonas* B-15 +ToLCV	1.45	1.15	0.230	0.210
2	*Pseudomonas* JK-16 +ToLCV	1.42	1.10	0.220	0.200
3	*Pseudomonas* 206(4) +ToLCV	1.63	1.25	0.240	0.220
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	1.87	1.39	0.260	0.260
5	*Pseudomonas* B-15+ Chitosan +ToLCV	2.09	1.63	0.260	0.250
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	2.03	1.59	0.250	0.230
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	2.39	1.74	0.270	0.250
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	2.56	1.87	0.290	0.270
9	Chitosan +ToLCV	1.37	1.01	0.190	0.170
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	1.80	1.32	0.230	0.200
11	Chemical control +ToLCV	1.07	0.98	0.160	0.150
12	Diseased control (only ToLCV)	1.15	1.04	0.170	0.160
	SEm±	0.05	0.05	0.01	0.01
	CD @1%	0.21	0.20	0.03	0.04

Table 16: Effect of inoculation of rhizobacteria and chitosan on the population of *Bemisia tabaci* on tomato plants

Sl. No.	Treatments	30 DAI		45 DAI		75 DAI	
		Insect population(No./ leaf)	Per cent decrease over disease control	Insect population (No./ leaf)	Per cent decrease over disease control	Insect population (No./ leaf)	Per cent decrease over disease Control
1	*Pseudomonas* B-15 +ToLCV	9.65	48.31	5.5	50.89	3.5	59.30
2	*Pseudomonas* JK-16 +ToLCV	10.00	46.43	6.2	44.64	4.2	51.16
3	*Pseudomonas* 206(4) +ToLCV	9.20	50.72	4.5	59.82	3.3	61.62
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	8.33	55.38	4.3	61.60	2.8	67.44
5	*Pseudomonas* B-15+ Chitosan +ToLCV	7.40	60.36	3.6	67.85	2.5	70.93
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	7.67	58.91	5.4	51.78	2.7	68.60
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	6.60	64.65	3.5	68.75	2.4	72.09
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	6.20	66.79	3.2	71.42	2.2	74.41
9	Chitosan +ToLCV	9.80	47.50	6.5	41.96	5.2	39.53
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	8.57	54.09	5.6	50.0	4.0	53.48
11	Chemical control +ToLCV	3.40	81.78	2.3	79.46	1.8	79.06
12	Diseased control (only ToLCV)	18.67	_	11.2	_	8.6	_

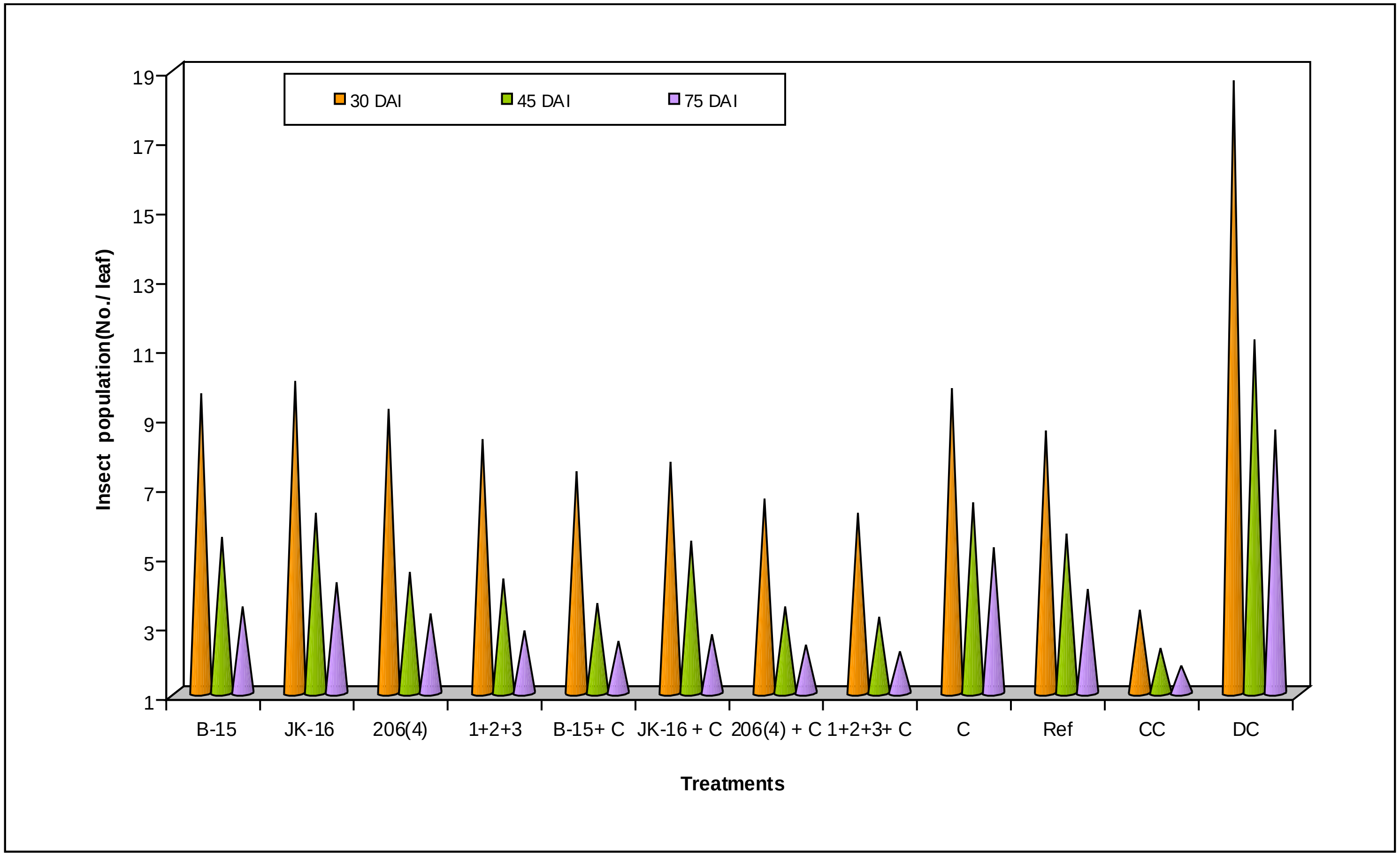


Fig. 9: Effect of inoculation of rhizobacteria and chitosan on the population of Bemisia tabaci on tomato plants

At 75 DAS, the polyphenol oxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest polyphenol oxidase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (1.87 ∆OD/g protein/ min) followed by *Pseudomonas* 206(4) + chitosan (1.74 ∆OD/g protein/ min).The least polyphenol oxidase activity was recorded in the healthy control (0.98 ∆OD/g protein/ min). The disease control plants showed an activity of 1.04 ∆OD/g protein/ min.

4.5.3 Effect of inoculation of rhizobacteria and chitosan on the population of *Bemisia tabaci* on tomato plants

The influence of rhizobacteria and chitosan inoculation on the population of whiteflies was studied. The insects present on the lower side of the leaf were counted by slowly turning the leaf without disturbing the insects. At 30 DAI, the rhizobacterial mixture + chitosan treatment recorded the least population of *Bemisia tabaci* (6.20 per leaf). This accounts to 66.79 per cent decrease over diseased control (Table 16,Fig.9). This treatment was followed by *Pseudomonas* 206(4) + chitosan (6.60 per leaf). The highest population of *Bemisia tabaci* was recorded in the diseased control (18.67 per leaf) and lowest population was observed in chemical control (3.40 per leaf).

Similarly, at 45 DAI also, rhizobacterial mixture + chitosan treatment showed the least population of *Bemisia tabaci* (3.20 per leaf), which accounts to 71.42 per cent decrease over diseased control. The pathogen inoculated check exhibited the highest population of whiteflies (11.20 per leaf) and the lowest population was observed in chemical control (2.30 per leaf).

At 75 DAI also, rhizobacterial mixture + chitosan treatment showed the least population of *Bemisia tabaci* (2.2 per leaf), which accounts to 74.41 per cent decrease over diseased control. This was followed by *Pseudomonas* 206(4) + chitosan (2.40 per leaf). The pathogen inoculated check exhibited the highest population of whiteflies (8.60 per leaf) whereas in chemical control only 1.8 whiteflies per leaf was noticed.

4.5.4 Detection of ToLCV in rhizobacteria and chitosan treated leaves

In order to quantify the viral inoculum in tomato plants treated with rhizobacterial isolates in combination with chitosan, a semi quantitative PCR analysis was carried out. This analysis detects and estimates the viral DNA accumulation in the challenge inoculated plants.

All the rhizobacterial treated plants and the disease control plants were analyzed by means of semi quantitative PCR.

Equal amount of total DNA (100ng) isolated from rhizobacterial treated plants and from the check were used. PCR reaction using coat protein gene specific primers were performed for different number of cycles *viz*., 10, 20 and 30. PCR products were detected on agarose gel electrophoresis, and the threshold cycle was determined for each treatment. In general, a slight reduction in the viral inocula was observed in all most all the treated plants except disease control. All the plants treated with rhizobacterial isolates in combination with chitosan showed the viral inoculum lower than the plants received rhizobacterial treatment alone.The diseased control plant whicn showed the highest amount of viral load after 20th cycle of the PCR at 45 DAS.

At 75 DAS, all the treatments exhibited a significant amount of viral load after 10th cycle of the PCR, except rhizobacterial mixture + chitosan, *Pseudomonas* sp. 206(4) and chemical control treatment which showed the lowest amont of viral titre after 20th cycle of the PCR.The diseased control plant exhibited highest amount of viral load after 10th cycle of the PCR (Tables 17a, 17b and Plate 22).

4.5.5 Plant growth promotional potential of the selected rhizobacterial strains in combination with chitosan

The effect of rhizobacterial combination with chitosan on growth and yield parameters of tomato was studied. The following parameters were recorded.

Table 17a: Quantification of viral load in the rhizobacteria and chitosan treated tomato plants at 45 DAS

Sl. No	Treatments	AT 45 DAS	
		Threshold cycle	Viral load
1	*Pseudomonas* B-15 +ToLCV	20	++
2	*Pseudomonas* JK-16 +ToLCV	20	++
3	*Pseudomonas* 206(4) +ToLCV	20	++
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	20	+
5	*Pseudomonas* B-15+ Chitosan +ToLCV	20	+
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	20	+
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	20	+
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	20	+
9	Chitosan +ToLCV	20	++
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	20	+
11	Chemical control +ToLCV	20	+
12	Diseased control (only ToLCV)	20	+++

+ - indicates visible band appeared at 20th cycle; low amount of viral load
++ - indicates visible band appeared at 20th cycle; higher amount of viral load
+++ - indicates visible band appeared at 20th cycle; the highest amount of viral load

LEGEND

Double digest marker (ECoRI+Hind III)

Lane 1: ToLCV amplicon after 10,20 and 30 cycles(Positive control)

Lane 2-12: PGPR and chitosan treated plants after 10,20 and 30 cycles

1 – Disease control

2 – *Pseudomonas* B-15

3 - *Pseudomonas* 206(4)

4 - *Pseudomonas* JK-16

5 - Chitosan

6 - *Pseudomonas* B-15+JK-16+206(4)

7 - Reference strain (*P. fluorescens* NCIM 2099)

8 - *Pseudomonas* B-15+Chitosan

9 - *Pseudomonas* JK-16+Chitosan

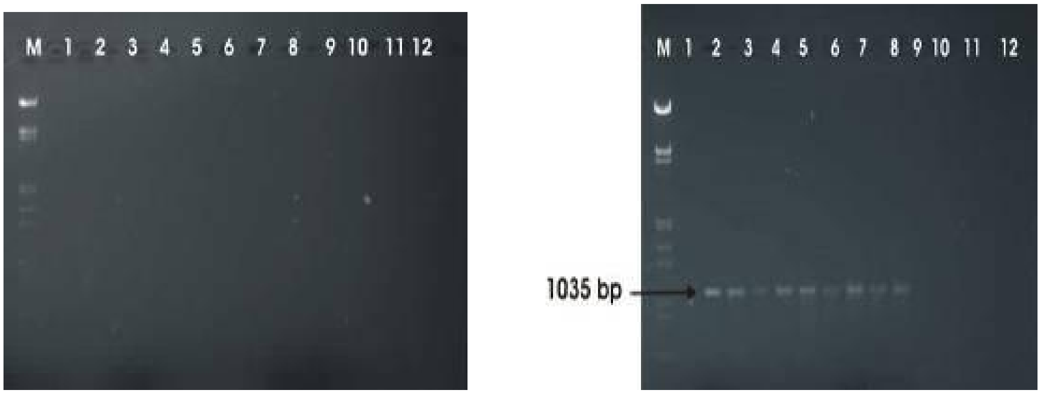
10 - *Pseudomonas* 206(4)+Chitosan

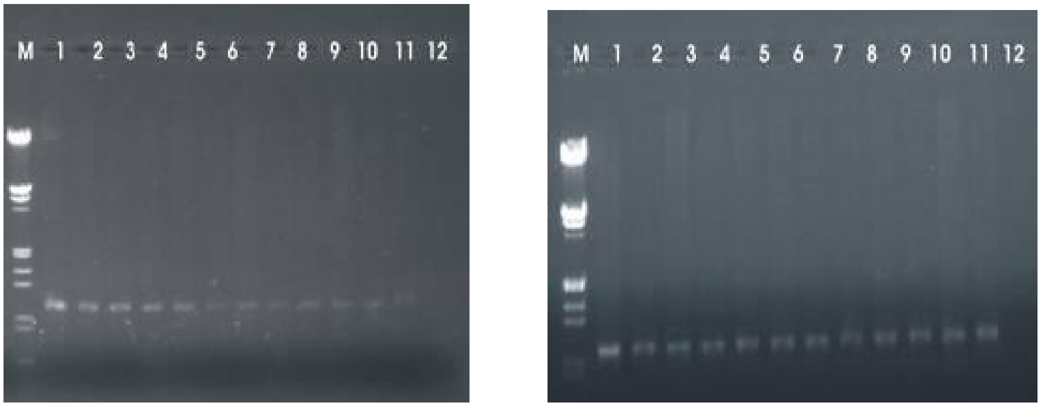
11 - *Pseudomonas* B-15+JK-16+206(4) + Chitosan

12 - Chemical control

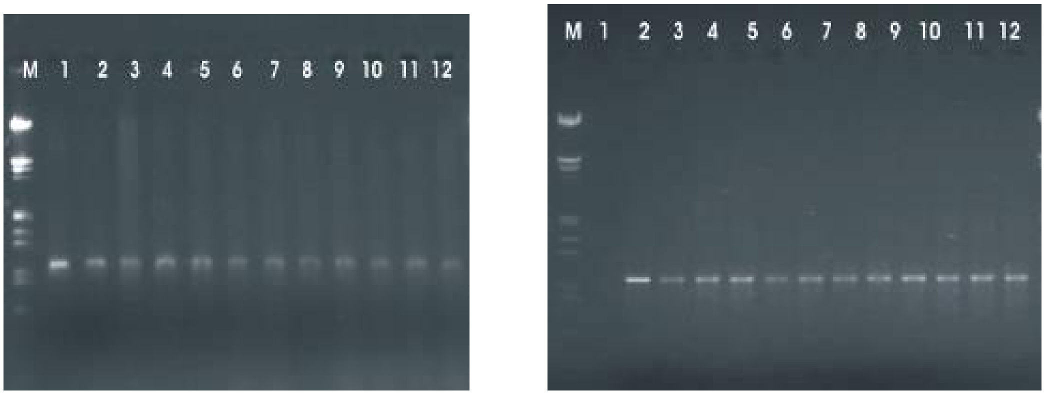
45 DAS

75 DAS





20 Cycles



30 Cyles

Plate 22. Quantification of viral load in the rhizobacteria and chitosan treated tomato plants

Table 17b: Quantification of viral load in the rhizobacteria and chitosan treated tomato plants at 75 DAS

Sl. No	Treatments	AT 75 DAS	
		Threshold cycle	Viral load
1	*Pseudomonas* B-15 +ToLCV	10	+++
2	*Pseudomonas* JK-16 +ToLCV	10	+++
3	*Pseudomonas* 206(4) +ToLCV	10	++
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	10	++
5	*Pseudomonas* B-15+ Chitosan +ToLCV	10	++
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	10	++
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	20	+
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	20	+
9	Chitosan +ToLCV	10	+++
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	10	+++
11	Chemical control +ToLCV	20	+
12	Diseased control (only ToLCV)	10	++++

+ - indicates visible band appeared at 20th cycle; lowest amount of viral load
+ + - indicates visible band appeared at 10th cycle; lower amount of viral load
+++ - indicates visible band appeared at 10th cycle; higher amount of viral load
++++ - indicates visible band appeared at 10th cycle; the highest amount of viral load

4.5.5.1 Plant height

The data pertaining to effect on plant height of Tomato plants at different intervals of time *viz.*, 30, 45, 60, 75 DAS and at harvest are given in Table 18, Fig. 10 and Plate 20.

At 30 DAS, plant height was significantly improved due to various combination of chitosan and rhizobacterial inoculation. The highest length (27.4 cm / plant) was observed in the plants inoculated with rhizobacterial mixture + chitosan. The least height was observed in the diseased control treatment (18.70 cm / plant)

At 45 DAS, again rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (44.10 cm/plant). The next best treatment was 206(4)+chitosan which showed the plant height (42.70 cm / plant). The least plant height was in the disease control treatment (23.20 cm / plant).

At 75 DAS also, rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (55.90 cm/plant). This was followed by the 206(4)+chitosan (53.80 cm / plant) and the reference strain with 42.90 cm/plant.

At harvest also, similar trend was observed. The highest plant height was observed in the treatment inoculated with rhizobacterial mixture + chitosan. It produced a height of 58.50 cm per plant. This was followed by the 206(4) + chitosan (55.50 cm / plant). The viral pathogen inoculated check exhibited the least plant height (25.10 cm/plant).

4.5.5.2 Plant biomass

In general, bacterized plants alone or in combination with chitosan produced significantly higher biomass than the uninoculated plants. And, rhizobacterial mixture + chitosan produced consistently higher biomass throughout the growth period of tomato. At 45 DAS, the plants inoculated with rhizobacterial mixture + chitosan produced the highest biomass (30.16 g/plant) (Table 19 and Fig. 11). This was followed by 206(4) + chitosan (29.48 g/plant).The least biomass was recorded in the viral pathogen inoculated treatment (20.48 g/plant).

At 75 DAS, significantly higher biomass was obtained in the treatment receiving rhizobacterial mixture + chitosan (33.62 g/plant). This was followed by the 206(4)+chitosan treatment (31.98 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (22.14 g/plant).

At the time of harvesting, the maximum biomass was recorded in rhizobacterial mixture + chitosan treatment (35.06 g/plant), which was followed by the 206(4)+chitosan treatment (34.04 g/plant).However, the least biomass was recorded in the viral pathogen inoculated treatment (23.82 g/plant).

4.5.5.3 Chlorophyll content

The chlorophyll content of tomato plants was significantly higher in rhizobacteria and chitosan treated plants. The data are presented in Table 20 and Fig.12. Chlorophyll content was increased up to 45 DAS and declined thereafter.

At 30 DAS, rhizobacterial mixture + chitosan resulted in significantly higher chlorophyll content (25.90 SPAD value). This was followed by the rhizobacterial mixture treatment alone (25.5 SPAD value). The least chlorophyll content was recorded in the diseased control (20.90 SPAD value).

At 45 DAS, rhizobacterial mixture + chitosan treatment resulted in significantly higher chlorophyll content (29.30 SPAD value), which was followed by rhizobacterial mixture treatment alone (28.70 SPAD value). The least chlorophyll content was recorded in the diseased control (15.30 SPAD value).

At 75 DAS, the maximum chlorophyll content was recorded in the leaves of chemical control treated plants (24.20 SPAD value) followed by rhizobacterial mixture + chitosan treated leaves (23.70 SPAD value). The least chlorophyll content was recorded in the diseased control (12.90 SPAD value).

Table 18: Effect of the selected rhizobacteria and chitosan on plant height of tomato

Sl. No	Treatments	Plant height (cm)			
		30 DAS	45 DAS	75 DAS	At Harvest
1	*Pseudomonas* B-15 +ToLCV	23.5	35.8	40.5	42.4
2	*Pseudomonas* JK-16 +ToLCV	21.7	33.3	39.0	41.9
3	*Pseudomonas* 206(4) +ToLCV	24.2	39.6	47.9	49.4
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	26.7	42.1	48.5	50.2
5	*Pseudomonas* B-15+ Chitosan +ToLCV	24.5	38.3	49.9	52.2
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	23.6	36.5	47.3	51.9
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	24.7	42.7	53.8	55.5
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	27.4	44.1	55.9	58.5
9	Chitosan +ToLCV	22.3	32.9	37.8	40.1
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.8	36.2	42.9	44.3
11	Chemical control +ToLCV	20.7	30.6	36.1	38.0
12	Diseased control (only ToLCV)	18.7	23.2	24.5	25.1
	SEm±	.62	.76	.68	.74
	CD @1%	2.34	2.87	2.60	2.79

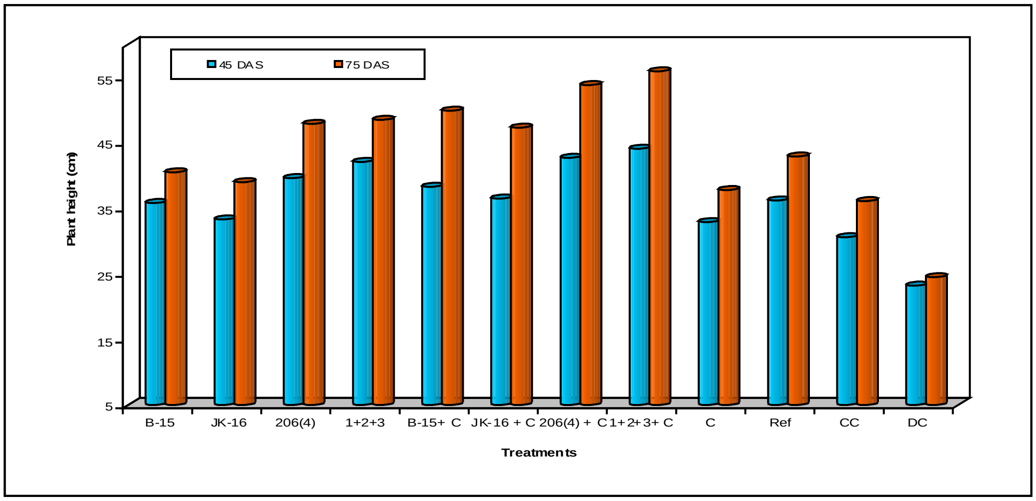


Fig. 10: Effect of the selected rhizobacteria and chitosan on plant height of tomato

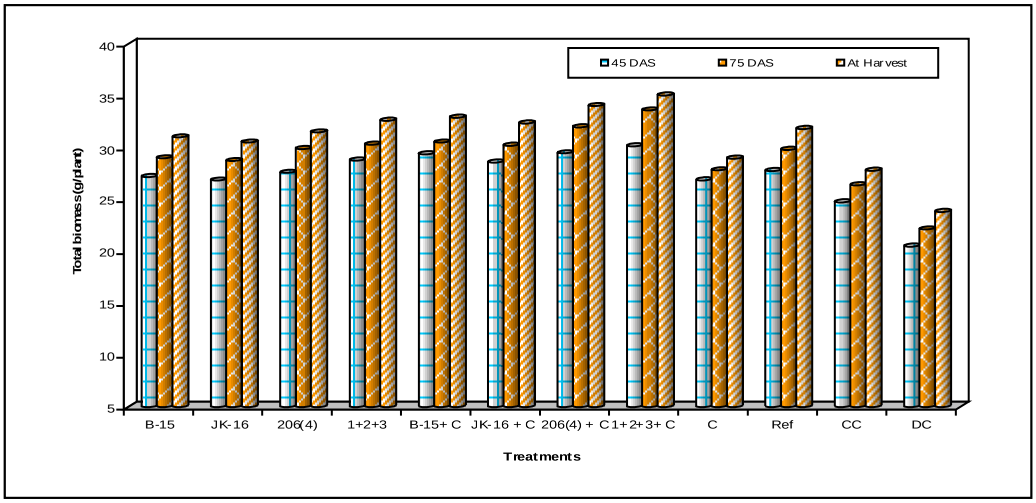


Fig. 11: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants

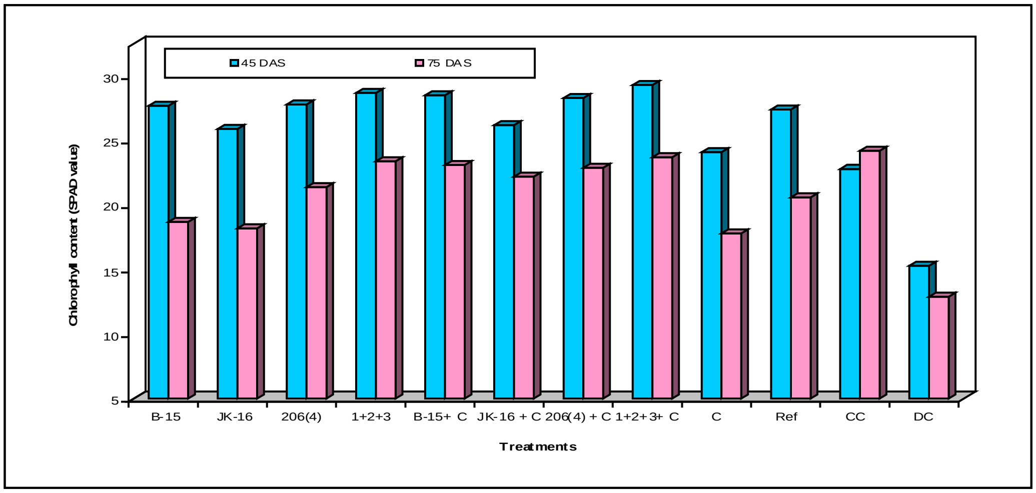


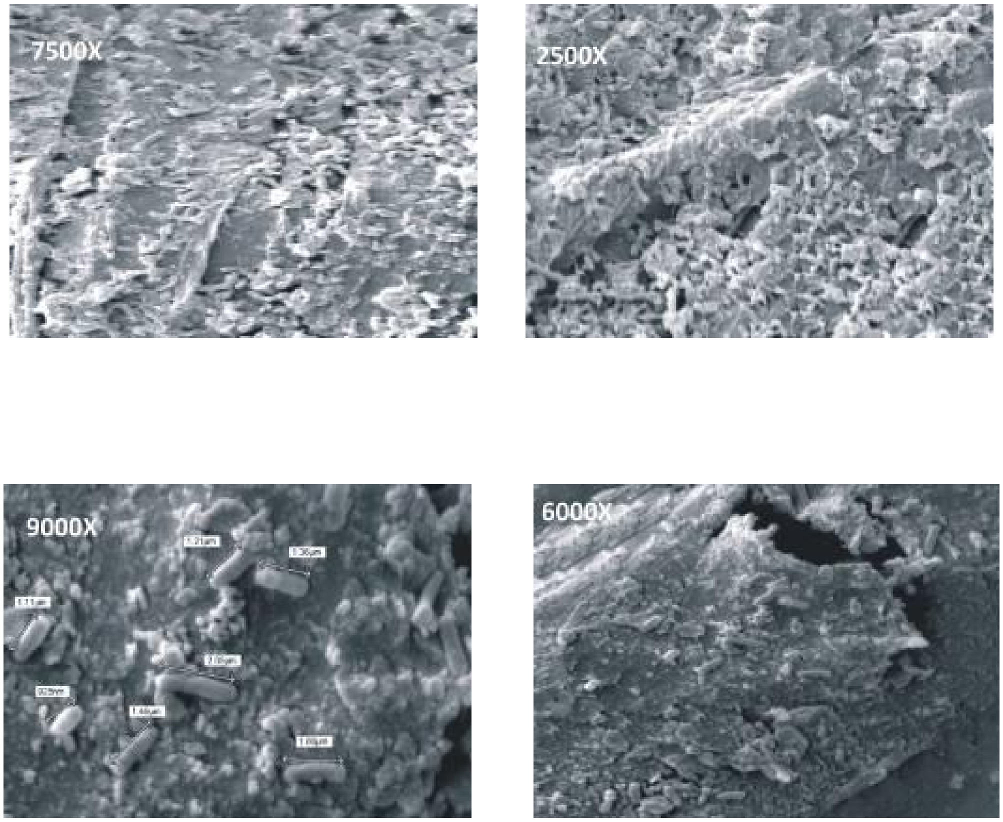
Fig. 12: Effect of the selected rhizobacteria inoculation and chitosan on chlorophyll content in tomato plants

Table 19: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants

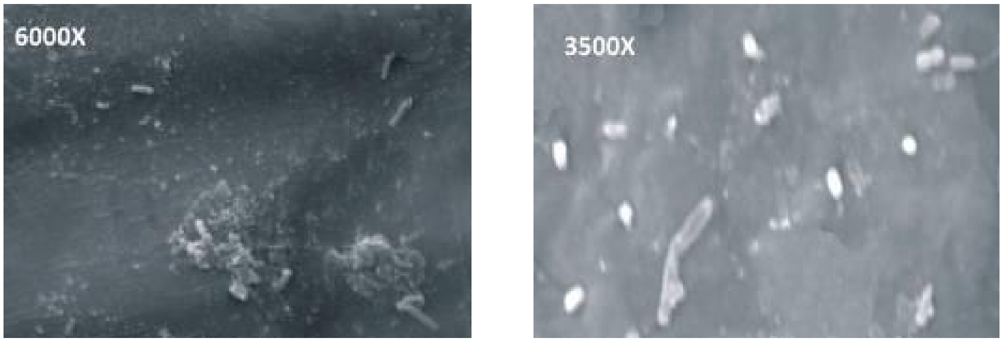
Sl. No.	Treatments	Total biomass (g/plant)		
		45 DAS	75 DAS	At Harvest
1	*Pseudomonas* B-15 +ToLCV	27.19	29.01	31.02
2	*Pseudomonas* JK-16 +ToLCV	26.86	28.74	30.52
3	*Pseudomonas* 206(4) +ToLCV	27.60	29.90	31.50
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	28.78	30.32	32.62
5	*Pseudomonas* B-15+ Chitosan +ToLCV	29.40	30.52	32.92
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	28.60	30.22	32.36
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	29.48	31.98	34.04
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	30.16	33.62	35.06
9	Chitosan +ToLCV	26.88	27.84	28.96
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	27.78	29.82	31.82
11	Chemical control +ToLCV	24.76	26.40	27.80
12	Diseased control (only ToLCV)	20.48	22.14	23.82
	SEm±	0.32	0.24	0.25
	CD @1%	1.21	0.92	0.96

Table 20: Effect of the selected rhizobacterial inoculation and chitosan on chlorophyll content in tomato plants

Sl. No	Treatments	Chlorophyll content (SPAD value)			
		30 DAS	45 DAS	75 DAS	At harvest
1	*Pseudomonas* B-15 +ToLCV	22.1	27.7	18.7	17.6
2	*Pseudomonas* JK-16 +ToLCV	21.9	25.9	18.2	16.8
3	*Pseudomonas* 206(4) +ToLCV	23.3	27.8	21.4	19.5
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	25.5	28.7	23.4	22.0
5	*Pseudomonas* B-15+ Chitosan +ToLCV	23.9	28.5	23.1	21.7
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	23.1	26.2	22.2	21.1
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	24.7	28.3	22.9	21.9
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	25.9	29.3	23.7	22.8
9	Chitosan +ToLCV	22.4	24.1	17.8	16.5
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.6	27.4	20.6	18.9
11	Chemical control +ToLCV	21.1	22.8	24.2	19.4
12	Diseased control (only ToLCV)	20.9	15.3	12.9	7.7
	SEm±	0.76	0.85	0.70	0.76
	CD @1%	2.86	3.24	2.67	2.89



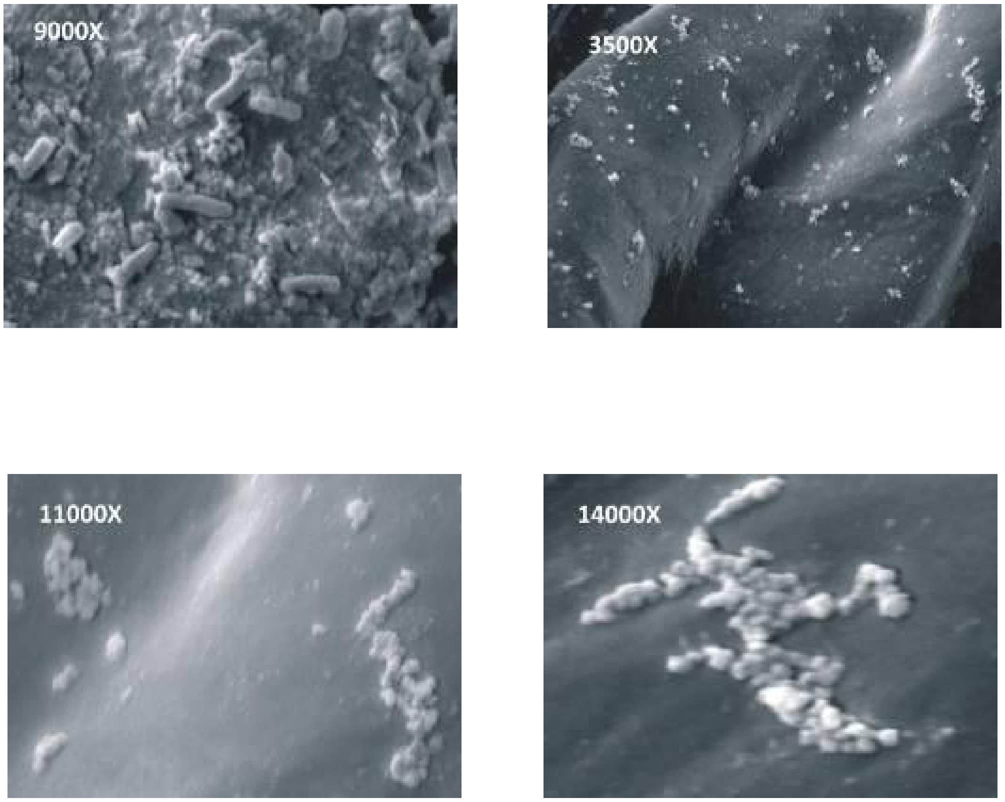
206(4) + Chitoson



206(4)

Plate 23 SEM pictures depicting higher colonization of *Pseudomonas* 206(4) to tomato roots due to chitosan treatment:

Plate 23 Contd…..



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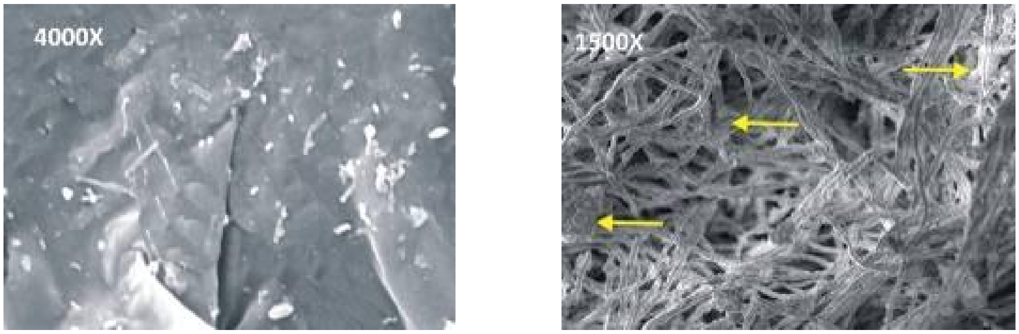


206(4)

*** - Arrow indicates cluster of cell entangled in root hair**

At harvest, chlorophyll content was decreased in all the treatments when compared to at 45 DAS. The maximum chlorophyll content was recorded in the rhizobacterial mixture + chitosan treated plants (22.80 SPAD value), which was followed by plants treated with rhizobacterial mixture treatment alone (22.00 SPAD value) the least chlorophyll was synthesized in the leaves of diseased control plants (7.70 SPAD value).

4.5.5.4 Fruit yield

Although plants inoculated with rhizobacterial strains with chitosan have shown good physiological attributes including early flowering compared to uninoculated control plants. There was no fruiting because all the flowers get withered due to high vector virus pressure which was maintained throughout the growth period under glass house condition.

4.5.6 Root colonization by *Pseudomonas* sp. 206(4) in presence and absence of chitosan

Colonization of rhizobacteria in tomato roots with and without chitosan was visualized through SEM pictures.Roots inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan showed higher level of bacterial populations. Here, the cells were observed encapsulated within a massive network of root derived material. In some places, cells were encapsulated and completely stuck to the root surface. (Plate 23)

Roots inoculated with *Pseudomonas* sp. 206(4) without chitosan exhibited lesser population of bacteria.However, no qualitative differences could be detected in the presence or absence of chitosan; i.e. cells exhibited similar size, same location and position towards the root surface.

4.6 Field evaluation of the combination of rhizobacterial isolates and chitosan for the biocontrol of ToLCV as well as growth promotion of Tomato under field condition during 2011

4.6.1 Biocontrol potential of the combination of selected rhizobacterial isolates and chitosan in field condition

All the rhizobacterial isolates were further tested for their ability to control ToLCV in tomato under field condition in combination with and without chitosan and the results are furnished in Tables 21, Fig.13. Application of this rhizobacterial isolates along with chitosan significantly reduced ToLCV severity both in glasshouse and field condition (Plate 24). At 45 DAI, all the isolates showed higher disease severity control ranging from 83.50 to 98.00 percent whereas at 75 DAI, the highest viral disease severity control (93.30%) was noticed in the plants inoculated with rhizobacterial mixture (206(4) +B-15+ JK-16) +Chitosan, which was followed by 206(4) +Chitosan treatment with 89.40 percent disease severity control. And, the least severity control was noticed when chitosan applied alone (73.40 percent control).

4.6.2 Plant growth promotional potential of the selected rhizobacterial strains in combination with chitosan

The effect of rhizobacterial combination with chitosan on growth and yield parameters of tomato was studied. The following parameters were recorded.

4.6.2.1 Plant height

The data pertaining to effect on plant height of Tomato plants at different intervals of time *viz.*, 30, 45, 60, 75 DAS and at harvest are given in Table 22, Fig.14, Plate 25. At 30 DAS, plant height was significantly improved due to various combination of chitosan and rhizobacterial inoculation. The highest length (23.5 cm / plant) was observed in the plants inoculated with rhizobacterial mixture + chitosan. The least height was observed in the diseased control treatment (15.80 cm / plant)

At 45 DAS, again rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (27.40 cm/plant). The next best treatment was 206(4)+chitosan which showed the plant height (26.2 cm / plant). The least plant height was in the disease control treatment (16.30 cm / plant).

Table 21: Disease severity of ToLCV as influenced by the selected rhizobacterial strains and chitosan in field conditions during 2011

SL. No.	Treatments	45 DAI		75 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
1	*Pseudomonas* 206(4) +ToLCV	12.5	87.50	13.3	86.70
2	*Pseudomonas* B-15 +ToLCV	12.5	87.50	16.7	83.30
3	*Pseudomonas* JK-16 +ToLCV	16.5	83.50	19.5	80.50
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	8.25	91.75	12.2	87.80
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	4.50	95.50	10.6	89.40
6	*Pseudomonas* B-15+ Chitosan +ToLCV	4.75	95.25	13.3	86.70
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	8.50	91.50	16.6	83.40
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	2.0	98.00	6.7	93.30
9	Chitosan +ToLCV	12.5	87.50	26.6	73.40
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	8.25	91.75	13.3	86.70
11	Chemical control +ToLCV	4.5	95.50	16.6	83.40
12	Diseased control (only ToLCV)	60.5	39.5	76.7	23.30

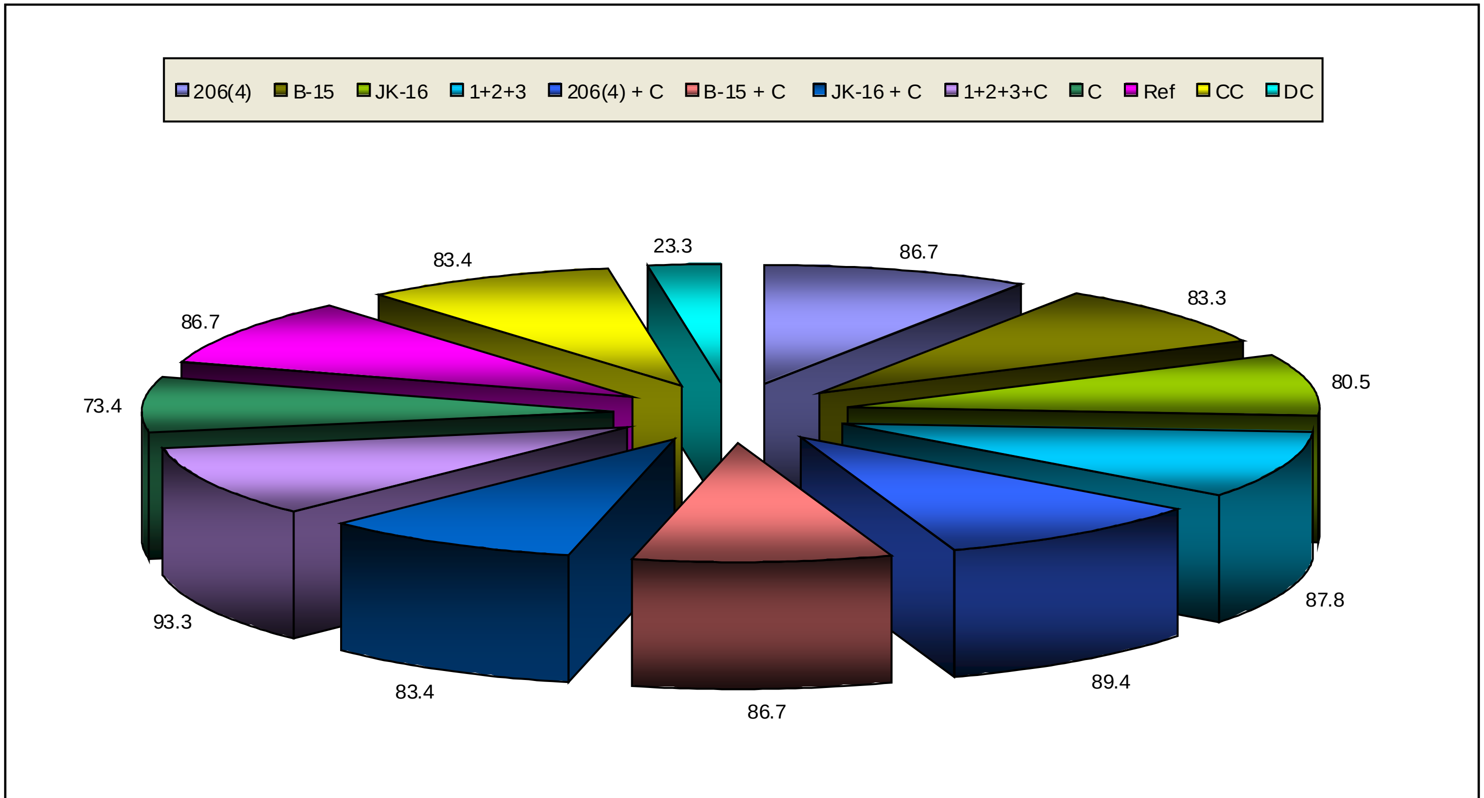


Fig. 13: Reduction in disease severity of ToLCV by the selected rhizobacterial strains and chitosan at 75 DAI in field condition during 2011

Fig. 13: Reduction in disease severity of ToLCV by the selected rhizobacterial strains and chitosan at 75 DAI in field condition during 2011

Plate 24 Contd……. …

Plate 24: Disease severity of ToLCV as influenced by inoculation with selected rhizobacteria during *kharif* 2011

Plate 25 Contd………

Plate : 25 Disease severity of ToLCV as influenced by inoculation with selected rhizobacteria and chitosan during *kharif* 2011

At 75 DAS also, rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (33.20 cm/plant). This was followed by 206(4) + chitosan (32.60 cm/plant) and rhizobacterial mixture alone with 31.70 cm/plant. The disease control treatment showed the least plant height (21.10cm / plant).

At harvest also, similar trend was observed. The highest plant height was observed in the treatment inoculated with rhizobacterial mixture + chitosan. It produced a height of 52.50 cm per plant. This was followed by the 206(4) + chitosan (48.20 cm / plant). The viral pathogen inoculated check exhibited the least plant height (28.70 cm/plant).

4.6.2.2 Plant biomass

In general, bacterized plants alone or in combination with chitosan produced significantly higher biomass than the uninoculated plants. And, rhizobacterial mixture + chitosan produced consistently the higher biomass throughout the growth period of tomato. At 45 DAS, the plants inoculated with rhizobacterial mixture + chitosan produced the highest biomass (31.70 g/plant) (Table 23, Fig.15 and Plate 26). This was followed by 206(4) + chitosan (29.70 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (16.20 g/plant).

At 75 DAS, significantly higher biomass was obtained in the treatment receiving rhizobacterial mixture + chitosan (38.30 g/plant). This was followed by the 206(4) + chitosan treatment (35.70 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (21.40 g/plant).

At the time of harvesting, the maximum biomass was recorded in rhizobacterial mixture + chitosan treatment (42.70 g/plant), which was followed by the 206(4) + chitosan treatment (41.80 g/plant). However, the least biomass was recorded in the viral pathogen inoculated treatment (25.10 g/plant).

4.6.2.3 Chlorophyll content

The chlorophyll content of tomato plants was significantly higher in rhizobacteria and chitosan treated plants. The data are presented in Table 24, Fig.16. Chlorophyll content was increased up to 45 DAS and declined thereafter.

At 30 DAS, rhizobacterial mixture + chitosan resulted in significantly higher chlorophyll content (24.00 SPAD value). This was followed by the rhizobacterial mixture treatment alone (22.30 SPAD value). The least chlorophyll content was recorded in the diseased control (18.60 SPAD value).

At 45 DAS, rhizobacterial mixture + chitosan treatment resulted in significantly higher chlorophyll content (45.30 SPAD value), which was followed by rhizobacterial mixture treatment alone (45.00 SPAD value). The least chlorophyll content was recorded in the diseased control (33.80 SPAD value).

At 75 DAS, the maximum chlorophyll content was recorded in the leaves of rhizobacterial mixture + chitosan treated plants plants (36.80 SPAD value) followed by rhizobacterial mixture alone treated leaves (36.00 SPAD value). The least chlorophyll content was recorded in the diseased control (26.90 SPAD value).

At harvest, the chlorophyll content was decreased in all the treatments when compared to at 45 DAS. The maximum chlorophyll content was recorded in the rhizobacterial mixture + chitosan treated plants (35.20 SPAD value), which was followed by plants treated with rhizobacterial mixture treatment alone (34.70 SPAD value). The least chlorophyll was synthesized in the leaves of diseased control plants (21.30 SPAD value).

4.6.2.4 Fruit yield

In general, rhizobacterial inoculation to tomato resulted in significantly higher fruit yield (Table 25, Fig.17).

The plants inoculated with rhizobacterial mixture + chitosan produced significantly higher no. of fruits per plant (26.60 / plant).

Table 22: Effect of the selected rhizobacteria and chitosan on plant height of tomato in field conditions during 2011

Sl. No	Treatments	Plant height (cm)			
		30 DAS	45 DAS	75 DAS	At Harvest
1	*Pseudomonas* 206(4) +ToLCV	22.4	25.9	31.2	43.7
2	*Pseudomonas* B-15 +ToLCV	21.8	24.4	30.5	41.6
3	*Pseudomonas* JK-16 +ToLCV	21.3	23.5	27.7	40.2
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	22.9	26.2	31.7	46.0
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	23.1	26.6	32.6	48.2
6	*Pseudomonas* B-15+ Chitosan +ToLCV	22.7	25.3	30.9	43.6
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	22.1	24.9	27.9	42.7
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	23.5	27.4	33.2	52.5
9	Chitosan +ToLCV	20.7	23.3	27.5	39.7
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	21.6	24.2	30.6	43.4
11	Chemical control +ToLCV	16.5	20.4	26.8	35.7
12	Diseased control (only ToLCV)	15.8	16.3	21.1	28.7
	SEm±	0.85	1.08	1.16	1.17
	CD @1%	2.43	3.07	3.31	3.33

LEGEND

206(4) = *Pseudomonas* 206(4) +ToLCV

B-15 = *Pseudomonas* B-15 +ToLCV

JK-16 = *Pseudomonas* JK-16 +ToLCV

1+2+3 = *Pseudomonas* (206(4) +B-15+JK-16) +ToLCV

206(4)+C = *Pseudomonas* 206(4) + Chitosan +ToLCV

B-15+C = *Pseudomonas* B-15+ Chitosan +ToLCV

JK-16+C = *Pseudomonas* JK-16 + Chitosan +ToLCV

1+2+3+C = *Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV

C = Chitosan +ToLCV

Ref = Reference strain (*P. fluorescens* NCIM 2099) +ToLCV

CC = Chemical control +ToLCV

DC = Diseased control (only ToLCV)

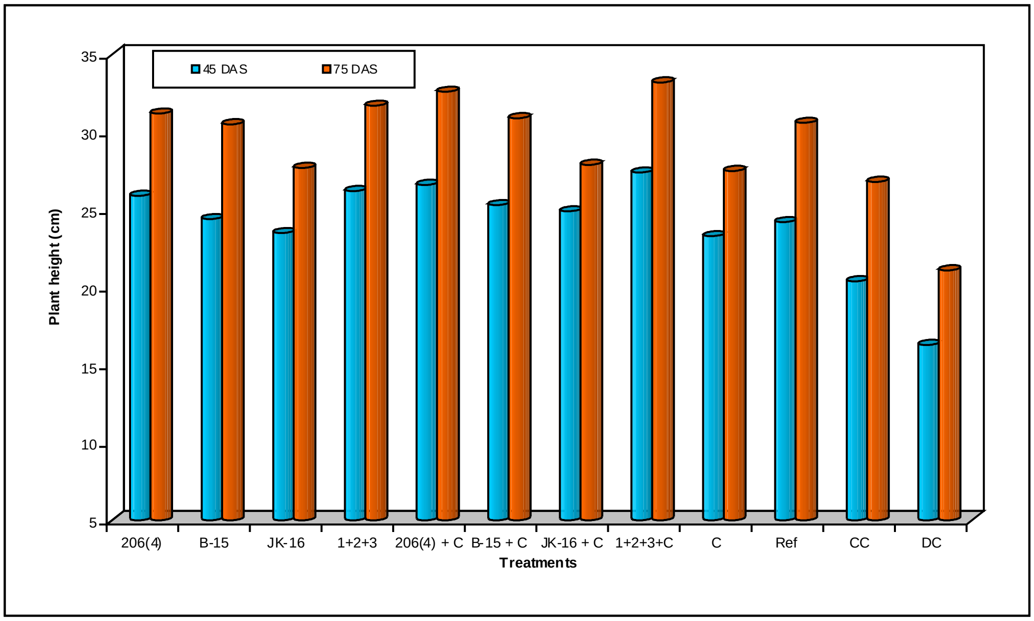


Fig. 14: Effect of the selected rhizobacteria and chitosan on plant height of tomato during 2011

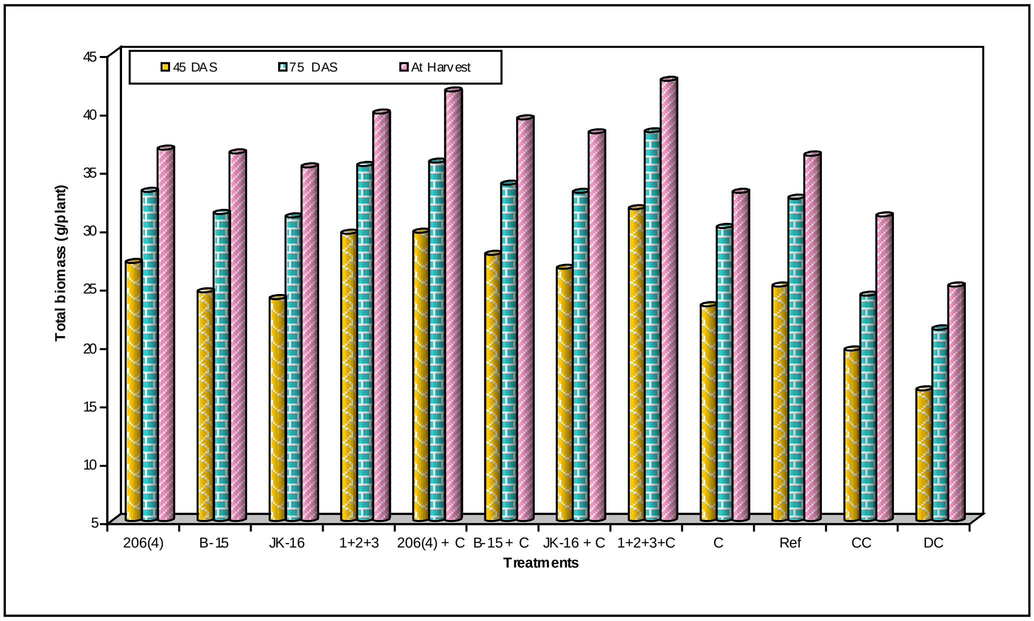


Fig. 15: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants during 2011

Plate 26: Plant growth promotion and disease severity control by selected rhizobacteria and chitosan

Table 23: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants in field conditions during 2011

Sl. No.	Treatments	Total biomass (g/plant)		
		45 DAS	75 DAS	At Harvest
1	*Pseudomonas* 206(4) +ToLCV	27.1	33.2	36.8
2	*Pseudomonas* B-15 +ToLCV	24.6	31.3	36.5
3	*Pseudomonas* JK-16 +ToLCV	24.0	31.0	35.3
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	29.6	35.4	39.9
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	29.7	35.7	41.8
6	*Pseudomonas* B-15+ Chitosan +ToLCV	27.8	33.8	39.4
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	26.6	33.1	38.2
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	31.7	38.3	42.7
9	Chitosan +ToLCV	23.4	30.1	33.1
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	25.1	32.6	36.3
11	Chemical control +ToLCV	19.6	24.3	31.1
12	Diseased control (only ToLCV)	16.2	21.4	25.1
	SEm±	0.36	0.36	0.43
	CD @5%	1.03	1.01	1.23

Table 24: Effect of the selected rhizobacterial inoculation and chitosan on chlorophyll content in tomato plants in field conditions during 2011

Sl. No	Treatments	Chlorophyll content (SPAD value)			
		30 DAS	45 DAS	75 DAS	At harvest
1	*Pseudomonas* 206(4) +ToLCV	21.1	44.0	35.8	32.7
2	*Pseudomonas* B-15 +ToLCV	19.8	42.6	33.8	31.9
3	*Pseudomonas* JK-16 +ToLCV	19.6	42.4	33.3	30.8
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	22.3	45.0	36.0	34.7
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	21.5	44.4	36.0	34.2
6	*Pseudomonas* B-15+ Chitosan +ToLCV	20.7	43.6	35.9	33.8
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	20.3	43.4	35.0	33.1
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	24.0	45.3	36.8	35.2
9	Chitosan +ToLCV	19.4	41.2	34.2	30.5
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	20.6	43.5	35.2	33.2
11	Chemical control +ToLCV	19.0	39.7	33.8	30.7
12	Diseased control (only ToLCV)	18.6	33.8	26.9	21.3
	SEm±	0.71	0.94	0.94	0.87
	CD @5%	2.01	2.67	2.69	2.49

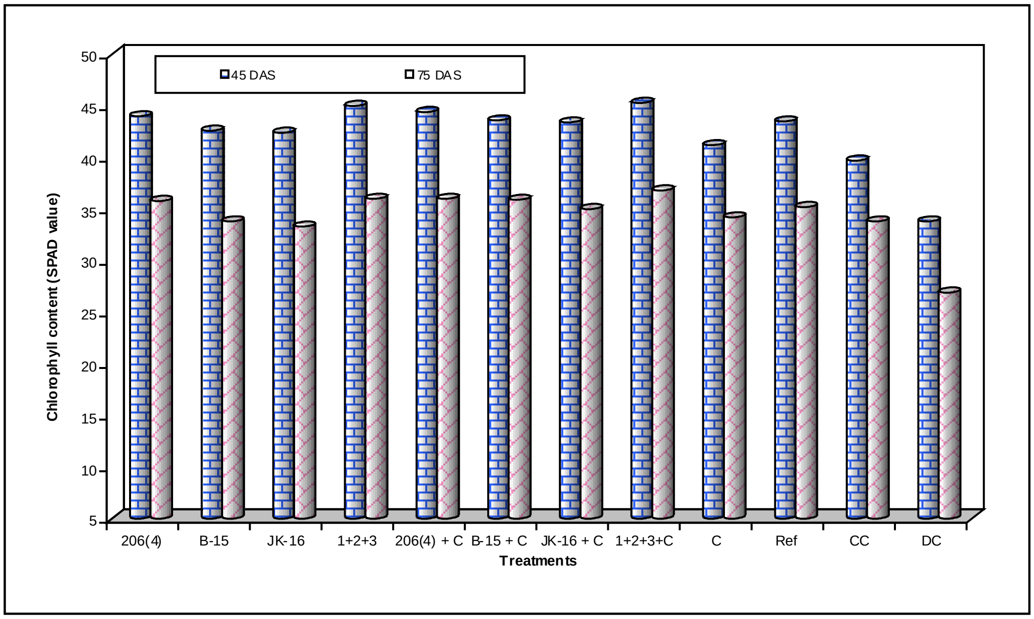


Fig. 16: Effect of the selected rhizobacterial inoculation and chitosan on chlorophyll content in tomato plants during 2011

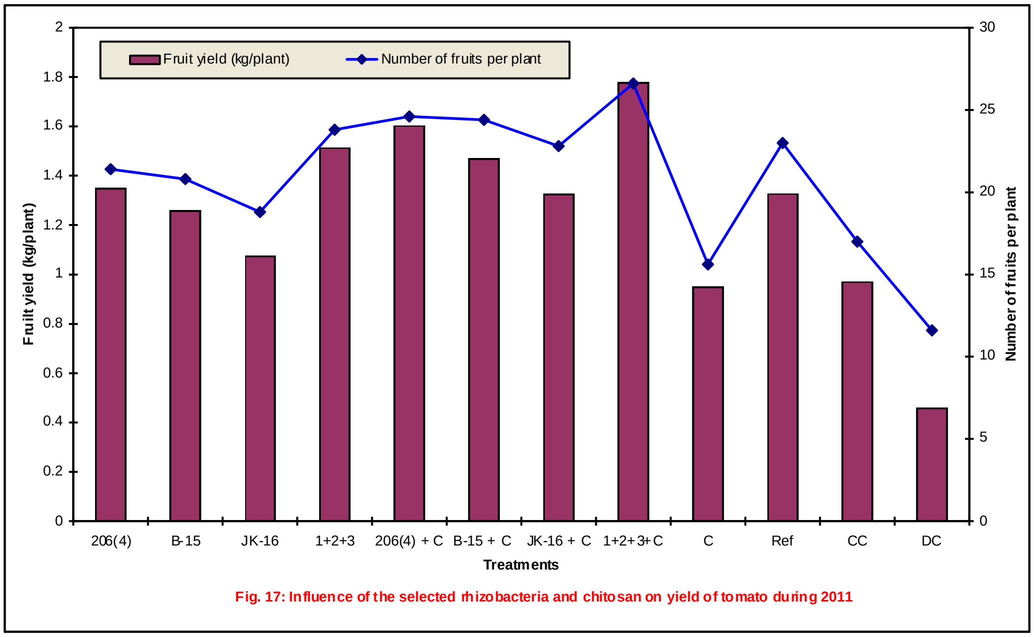


Fig. 17: Influence of the selected rhizobacteria and chitosan on yield of tomato during 2011

Table 25: Influence of the selected rhizobacteria and chitosan on yield of tomato in field conditions during 2011

Sl. No.	Treatments	Number of fruits per plant	Fruit yield (kg/plant)	Yield (ton/ha)	Shelf Life (days)
1	*Pseudomonas* 206(4) +ToLCV	21.4	1.348	40.43	10.6
2	*Pseudomonas* B-15 +ToLCV	20.8	1.257	37.70	10.2
3	*Pseudomonas* JK-16 +ToLCV	18.8	1.074	32.21	9.8
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	23.8	1.513	45.38	11.8
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	24.6	1.603	48.10	11.2
6	*Pseudomonas* B-15+ Chitosan +ToLCV	24.4	1.468	44.03	10.6
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	22.8	1.325	39.75	10.0
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	26.6	1.777	53.30	12.1
9	Chitosan +ToLCV	15.6	0.948	28.43	9.6
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.0	1.328	39.83	10.2
11	Chemical control +ToLCV	17.0	0.970	29.10	7.4
12	Diseased control (only ToLCV)	11.6	0.458	13.75	6.4
	SEm±	0.73	0.06	1.09	0.32
	CD @5%	2.06	0.17	3.10	0.91

This was followed by the treatment which received 206(4) + chitosan (24.60 / plant) and B-15 +chitosan (24.40 / plant). The pathogen inoculated treatment resulted in the least number of fruits (11.60/plant).

The plants inoculated with rhizobacterial mixture + chitosan recorded the highest fruit yield (1.77 Kg/plant). This was followed by the 206(4)+chitosan (1.603 Kg/plant). The least yield was recorded in the pathogen inoculated treatment (0.458 kg/plant).

4.6.2.5 Shelf life

The fruits produced by the rhizobacterial mixture + chitosan treated plants exhibited longest shelf life (12.10 days). This was followed by the treatment which received rhizobacterial mixture alone (11.8 days). The pathogen inoculated treatment produced fruits with minimum shelf life period (6.4 days) Table 25.

4.6.3 Histopathology

The histopathological studies were undertaken to detect structural and histochemical changes that took place subsequent to viral infection in treated, untreated and healthy control plants.

Structural staining for anatomical changes

The cross section of healthy dorsoventral leaves showed intact nature of all the cells. The palisade cells were more cylindrical, uniform, columnar in shape and prominent. The spongy cells were round, big and uniformly distributed.

The cross section of leaves treated with *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp.B-15 +chitosan revealed more or less similar kind of cell arrangement as seen in healthy leaves.

Pseudomonas sp.206(4) and *Pseudomonas* sp.B-15 treated leaves showed palisade cells, which were markedly reduced in size, slightly distorted with slight reduction in their compact nature. The spongy cells lost their normal round shape showing disintegration. There were distinct intercellular spaces.

The cross section of diseased leaves revealed palisade cells, which were markedly reduced in size, highly distorted and lost their columnar shape and compact nature. The spongy cells lost their normal round shape showing complete disintegration. There were distinct intercellular spaces (Plate 27).

Histochemical studies

The histochemical studies were undertaken on localization of polysaccharides, nucleic acid and total protein in different regions of healthy treated and infected leaves, the results of which are presented in Table 26.

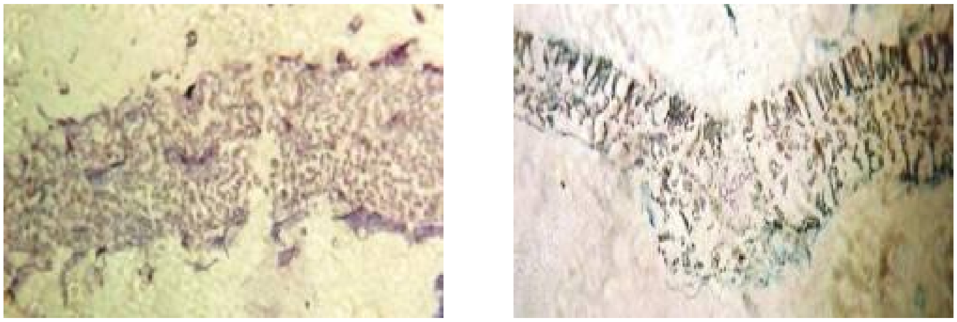
Polysaccharides

The leaves of infected plants showed low level of (+) polysaccharides in palisade and spongy parenchyma cells as compared to healthy plant, which showed rich (+++) polysaccharides in the cells indicating that virus infection reduced polysaccharides concentration.

The leaves treated with *Pseudomonas* sp.206(4) +chitosan and *Pseudomonas* sp.B-15 +chitosan also showed rich (+++) polysaccharides in leaves whereas, *Pseudomonas* sp.206(4) alone treated leaves showed medium (+ +) and *Pseudomonas* sp.B-15 showed low(+) level of polysaccharides in leaves (Plate 28).

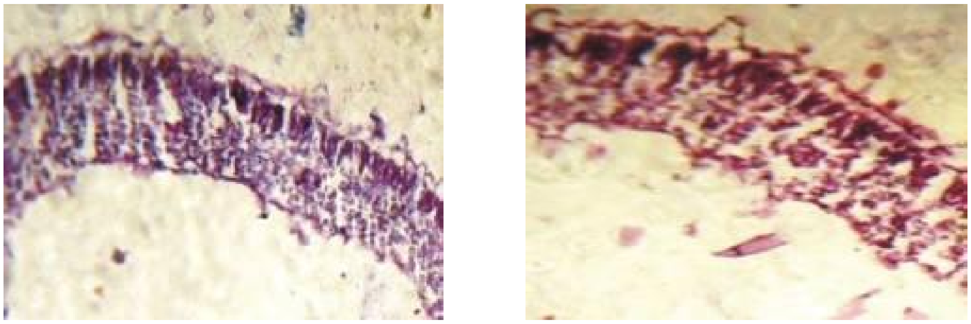
Nucleic acid

The staining for nucleic acid revealed moderately rich (+ +) concentration of nucleic acid in palisade and spongy parenchyma cells of healthy leaves, but there was a rise in nucleic acid concentration in diseased leaves, which showed very rich (++++) nucleic acid concentration in palisade and spongy cells.



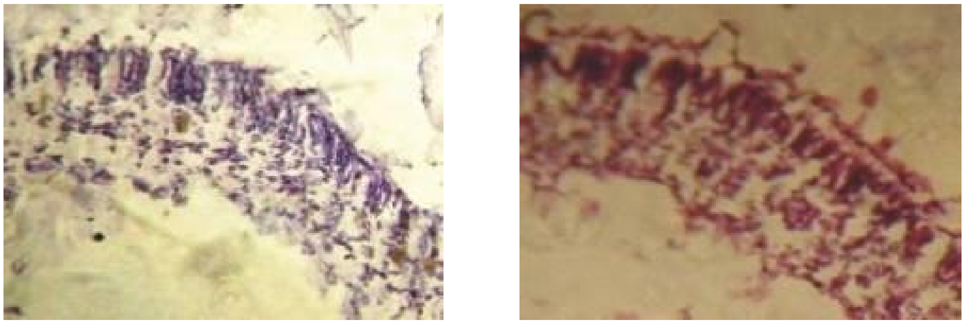
Disease Control

Healthy control



206(4)

206(4) + Chitoson



B – 15

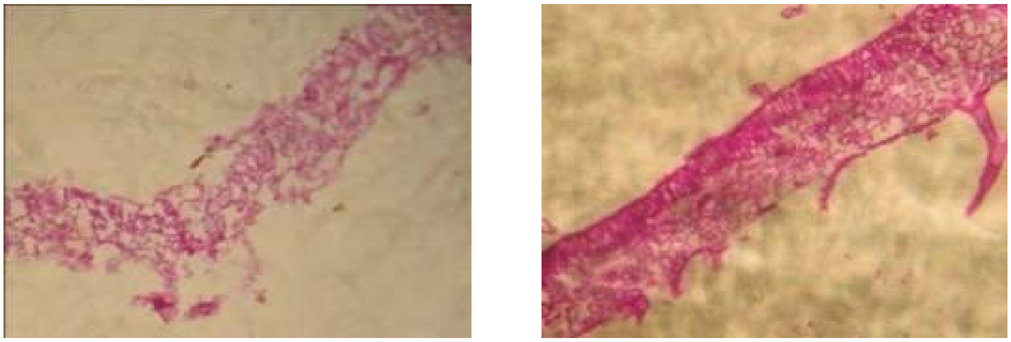
B – 15 + Chitoson

Plate 27. Structural changes in tomato leaves

Table 26: Histochemical changes in healthy, treated and ToLCV infected leaves of tomato plants

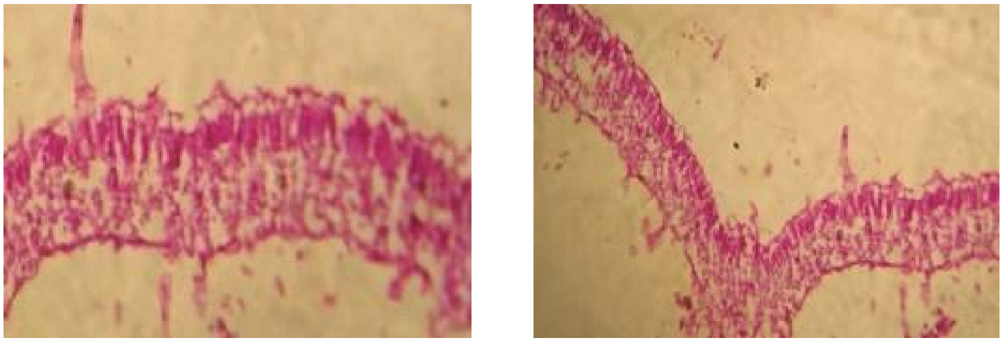
Status	Histochemical	Different regions of leaf		
		Epidermis	Palisade parenchyma	Spongy parenchyma
Healthy	Polysaccharides	+++	+++	+++
	Proteins	++++	++++	+++
	Nucleic acids	++	++	++
Infected	Polysaccharides	+	+	+
	Proteins	+	+	+
	Nucleic acids	++++	++++	++++
Pseudomonas sp.206(4)+Chitosan treatment	Polysaccharides	+++	+++	+++
	Proteins	+++	+++	++
	Nucleic acids	++	++	++
Pseudomonas sp.206(4) treatment	Polysaccharides	++	++	++
	Proteins	++	++	++
	Nucleic acids	+++	+++	+++
Pseudomonas sp.B-15+Chitosan treatment	Polysaccharides	+++	+++	+++
	Proteins	+++	+++	++
	Nucleic acids	++	++	++
Pseudomonas sp.B-15 treatment	Polysaccharides	+	+	+
	Proteins	++	++	++
	Nucleic acids	+++	+++	+++

Legend ++++ Very rich +++ Rich ++ Medium + Low



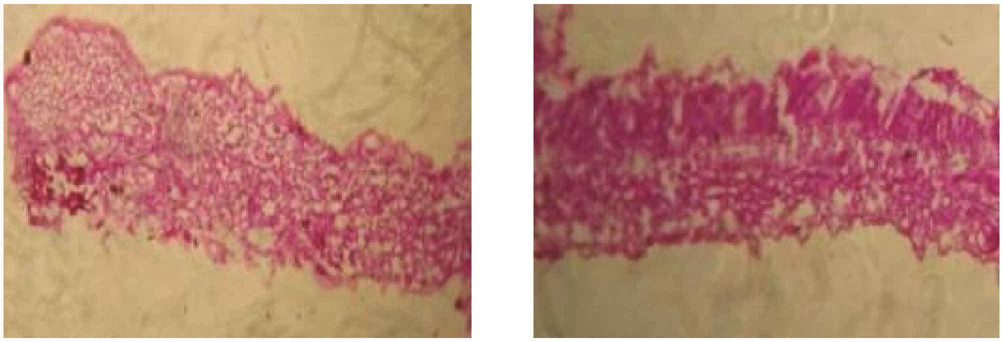
Disease control

Healthy control



206(4)

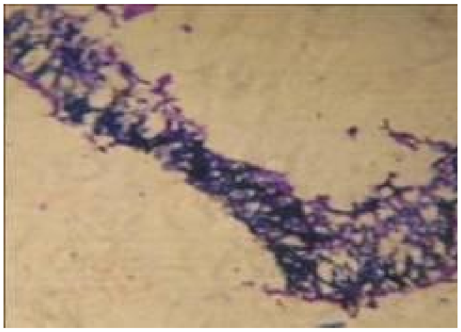
206(4) + Chitoson



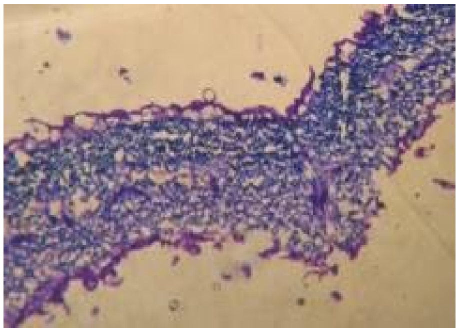
B – 15

B – 15 + Chitoson

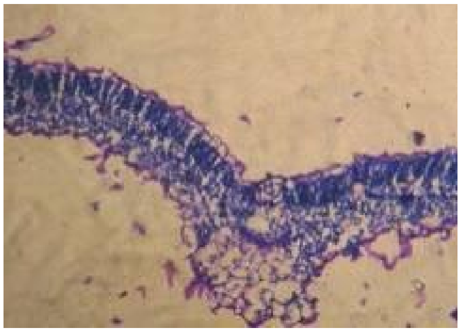
Plate 28: Histochemical changes (polysaccharides) in tomato leaves



Disease control

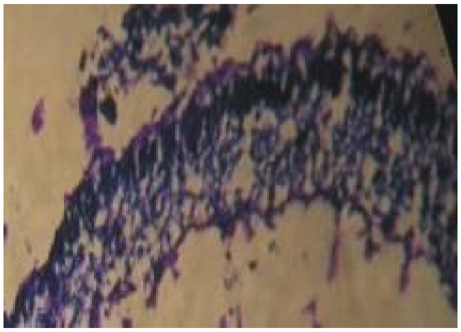


Healthy control

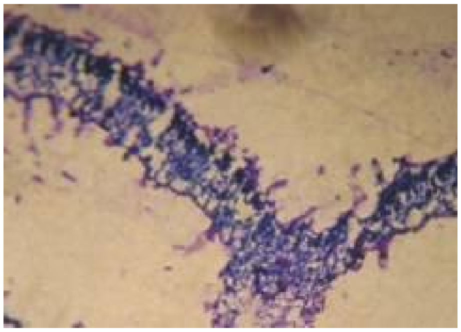


206(4)

206(4) + Chitoson

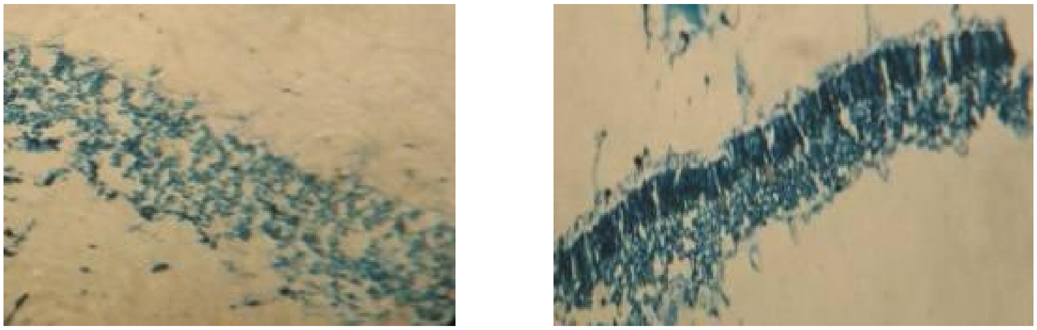


B – 15



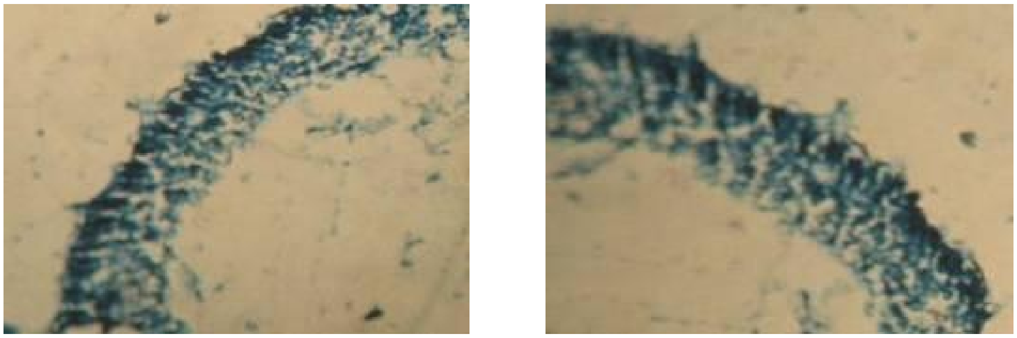
B – 15 + Chitoson

Plate 29. Histochemical changes (nucleic acid) in tomato leaves



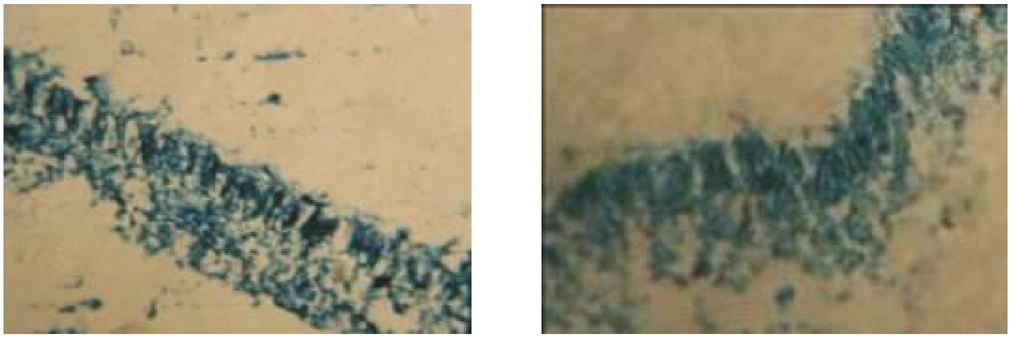
Disease control

Healthy control



206(4)

206(4) + Chitoson



B – 15

B – 15 + Chitoson

Histochemical changes (proteins) in tomato leaves

The leaves treated with *Pseudomonas* sp. 206(4) + chitosan and *Pseudomonas* sp.B-15 +chitosan also showed moderately rich (+ +) concentration of nucleic acid in palisade and spongy parenchyma cells of leaves, whereas, *Pseudomonas* sp. 206(4) alone treated leaves and *Pseudomonas* sp.B-15 showed rich (+++) concentration of nucleic acid in palisade and spongy cells (Plate 29).

Protein

The staining for protein revealed very rich (+ + + +) and rich (+ + +) concentration of proteins in palisade and spongy parenchyma cells respectively in healthy leaves, whereas it was lower (+) in palisade and spongy parenchyma cells of diseased leaves.

The leaves treated with *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp.B-15 +chitosan also showed rich (+++) and medium (+ +) concentration of proteins in palisade and spongy parenchyma cells of leaf respectively, whereas, *Pseudomonas* sp. 206(4) alone treated leaves and *Pseudomonas* sp.B-15 showed medium (++) concentration of proteins in palisade and spongy cells (Plate 30).

4.7 Field evaluation of the selected combination of rhizobacterial isolates and chitosan for the biocontrol of ToLCV as well as growth promotion of tomato under field condition during 2012

4.7.1 Biocontrol potential of the combination of the selected rhizobacterial isolates and chitosan under field condition

Based on the previous field experiment, three efficient treatments were further selected and tested for their ability to control ToLCV in tomato under field condition and the results are furnished in Tables 27, Fig.18 and Plate 31. Application of these rhizobacterial isolates along with chitosan significantly reduced ToLCV severity both in glass house and field conditions. At 45 DAI, all the isolates showed higher disease severity control ranging from 83.40 to 93.30 percent whereas at 75 DAI, the highest viral disease severity control (84.60%) was noticed in the plants inoculated with rhizobacterial mixture (206(4) +B-15+ JK-16) +Chitosan, which was followed by 206(4) +Chitosan treatment with 81.70 percent disease severity control. And, the least severity control was noticed when the reference strain was applied without chitosan (71.70 percent control). Chemical control treatment also exhibited 81.40 percent disease severity control.

4.7.2 Ability of the rhizobacteria and chitosan to induce systemic resistance against ToLCV in tomato plants

The ISR activity of the isolates along with chitosan was tested by estimating defense enzymes such as peroxidase, chitinase, polyphenoloxidase, phenylalanine ammonia lyase (PALase) and phenol content in plants at different intervals of time.

4.7.2.1 Phenol content

The phenol content of leaves, at different intervals *viz.*, 45 and 75 DAS, as influenced by inoculation with rhizobacteria are presented in Table 28 a, Fig.19a.

At 45 DAS, the treatment receiving rhizobacterial mixture (206(4) +B-15+ JK-16) +Chitosan recorded the maximum phenol content (0.36mg/g), which was followed by the *Pseudomonas*206(4) +Chitosan treatment (0.31 mg/g). The least phenol content was recorded in the chemical control treatment (0.24mg/g).

At 75 DAS, phenol content was slightly decreased in all the treatments as compared to 45 DAS. The highest phenol content was recorded in the plants inoculated with rhizobacterial mixture with chitosan (0.30 mg/g). This was followed by *Pseudomonas* 206(4) +Chitosan treatment (0.28 mg/g). Chemical control recorded significantly lower amount of phenol content (0.21 mg/g).

4.7.2.2 Phenylalanine ammonia lyase (PALase) activity

The results on biosynthesis of PALase as influenced by rhizobacterial strains in combination with chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in Table 28b, Fig.19e.

Table 27: Biocontrol of ToLCV disease by the selected rhizobacterial strains and chitosan in field conditions during 2012

SL. No.	Treatments	45 DAI		75 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
1	*Pseudomonas* B-15+ Chitosan +ToLCV	13.3	86.70	21.6	78.40
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	10.6	89.40	18.3	81.70
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	6.7	93.30	15.4	84.60
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	16.6	83.40	28.3	71.70
5	Chemical control +ToLCV	13.3	86.70	18.6	81.40
6	Diseased control (only ToLCV)	56.7	43.30	86.6	13.40

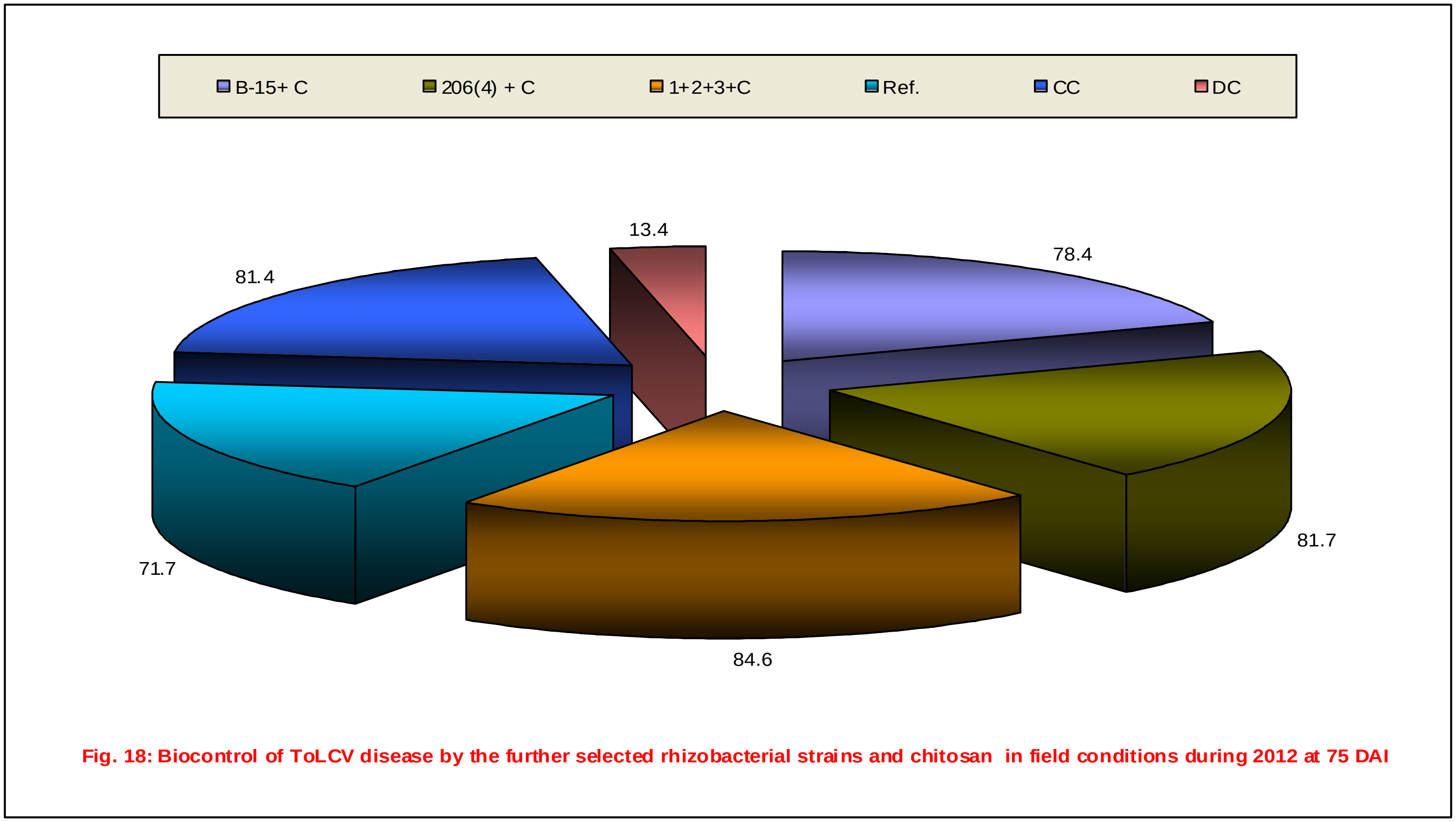


Fig. 18: Biocontrol of ToLCV disease by the further selected rhizobacterial strains and chitosan in field conditions during 2012 at 75 DAI

Plate 31. Disease incidence of ToLCV as influenced by inoculation with selected rhizobacteria and chitosan during summer 2012

Table 28a: Effect of the selected rhizobacteria strains and chitosan on defense molecules activity in field conditions during 2012

SL. No.	Treatments	Phenol(mg/g dry weight)		Peroxidase(∆OD/g protein/min)		Chitinase(µgGlc NAc/ µg protein/ min)	
		45 DAS	75 DAS	45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* B-15+ Chitosan +ToLCV	0.30	0.26	1.49	0.52	2.46	1.14
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	0.31	0.28	1.67	0.57	2.54	1.22
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	0.36	0.30	2.19	1.11	3.10	1.47
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	0.28	0.24	1.37	0.49	2.41	1.11
5	Chemical control +ToLCV	0.24	0.21	1.05	0.43	1.93	1.05
6	Diseased control (only ToLCV)	0.25	0.23	1.11	0.44	2.08	1.09
	SEm±	0.01	0.01	0.10	0.04	0.10	0.05
	CD @5%	0.02	0.02	0.31	0.12	0.29	0.15

LEGEND

B-15+ C = *Pseudomonas* B-15+ Chitosan +ToLCV

206(4)+C = *Pseudomonas* 206(4) + Chitosan +ToLCV

1+2+3+C = *Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV

Ref = Reference strain (*P. fluorescens* NCIM 2099) +ToLCV

CC = Chemical control +ToLCV

DC = Diseased control (only ToLCV)

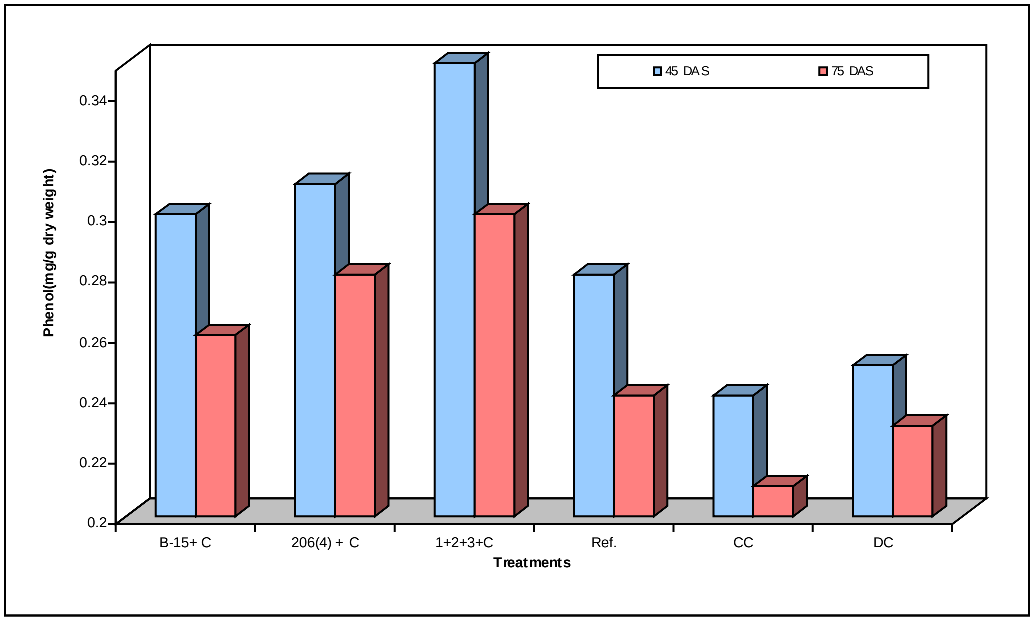


Fig. 19a: Effect of the further selected rhizobacterial strains and chitosan on defense molecules activity in field conditions during 2012

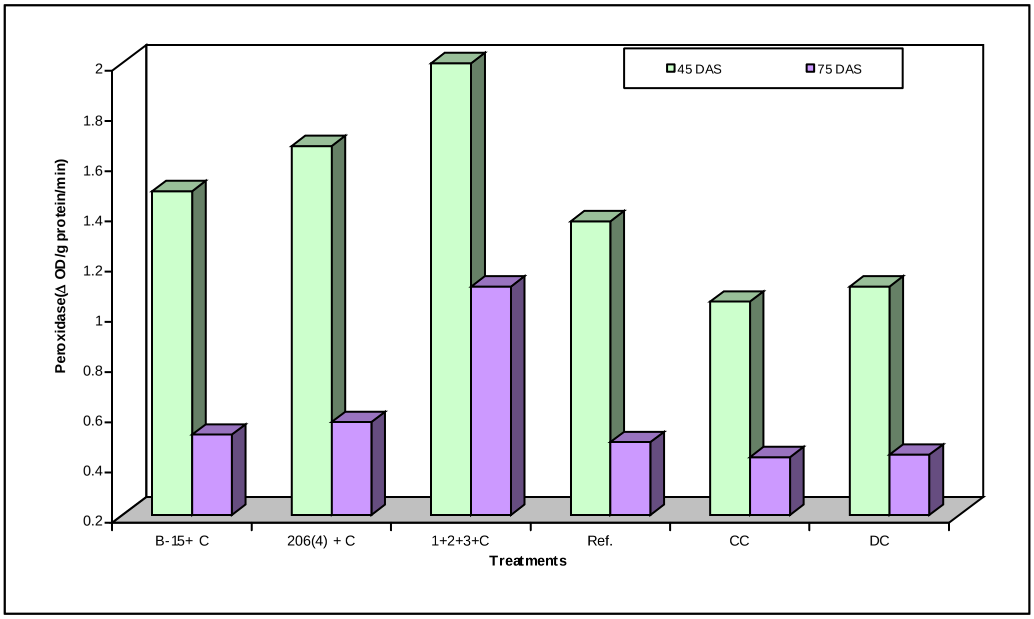


Fig. 19b: Effect of the further selected rhizobacterial strains and chitosan on defense molecules activity in field conditions during 2012

It was observed that PALase activity was significantly increased in all the rhizobacteria and chitosan inoculated treatments. At 45 DAS, the maximum PALase activity was recorded in the treatment receiving rhizobacterial mixture + chitosan (0.231 change in cinnamic acid/min/g) followed by the plants inoculated with *Pseudomonas* 206(4) + Chitosan treatment (0.210 change in Cinnamic acid/min/g). The least PALase activity was recorded in the chemical control (0.138 change in Cinnamic acid/min/g each).

At 75 DAS, PALase activity was found decreasing in all the treatments when compared to 45 DAS. The highest activity was seen in plants inoculated with rhizobacterial mixture + chitosan (0.190 change in cinnamic acid/min/g) followed by the plants inoculated with *Pseudomonas* 206(4) + Chitosan treatment (0.173 change in Cinnamic acid/min/g).The least was exhibited by the chemical control (0.123 change in Cinnamic acid/min/g).

4.7.2.3 Peroxidase activity

The data pertaining to peroxidase activity as influenced by rhizobacterial combination with chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in Table 28a, Fig.19b.

There was a tremendous increase in peroxidase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest peroxidase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (2.19 ∆OD/g protein/ min). *Pseudomonas* 206(4) + Chitosan was the next best treatment with 1.67 ∆OD/g protein/ min. The disease control treatment exhibited 1.11 ∆OD/g protein/ min. And, the least peroxidase activity was recorded in the chemical control (1.05 ∆OD/g protein/ min).

At 75 DAS, the peroxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest peroxidase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (1.11 ∆OD/g protein/ min), followed by *Pseudomonas* 206(4) +Chitosan (0.57 ∆OD/g protein/ min). The least peroxidase activity was recorded in the chemical control (0.43 ∆OD/g protein/ min). The disease control plants showed an activity of 0.44 ∆OD/g protein/ min.

4.7.2.4 Chitinase activity

The results on biosynthesis of chitinase as influenced by rhizobacterial strains with and without chitosan in tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table 28a, Fig.19c.

At 45 DAS, the highest chitinase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (3.10 µgGlc NAc/ µg protein/ min). *Pseudomonas* 206(4) +Chitosan was the next best treatment with 2.54 µgGlc NAc/ µg protein/ min.The disease control treatment exhibited 2.08 µgGlc NAc/ µg protein/ min. And, the least chitinase activity was recorded in the chemical control (1.93 µgGlc NAc/ µg protein/ min).

At 75 DAS, the chitinase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest chitinase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (1.47 µgGlc NAc/ µg protein/ min), followed by *Pseudomonas* 206(4) +Chitosan (1.22 µgGlc NAc/ µg protein/ min). The least chitinase activity was recorded in the chemical control (1.05 µgGlc NAc/ µg protein/ min). The disease control plants showed an activity of 1.09 µgGlc NAc/ µg protein/ min.

4.7.2.5 Polyphenol oxidase activity

The polyphenol oxidase activity as influenced by rhizobacterial strains and chitosan treatment in tomato leaves at different intervals of time *viz*., 45 and 75 DAS are furnished in Table 28b, Fig.19d.

In general, there was a tremendous increase in polyphenol oxidase activity in both the stages due to rhizobacterial and chitosan treatment. At 45 DAS, the highest polyphenol oxidase activity was recorded in the plant leaves receiving rhizobacterial mixture + chitosan (1.83 ∆OD/g protein/ min). *Pseudomonas* 206(4) +Chitosan was the next best treatment with 1.64 ∆OD/g protein/ min.The disease control treatment exhibited 1.12 ∆OD/g protein/ min. And, the least polyphenol oxidase activity was recorded in the chemical control (1.08 ∆OD/g protein/ min).

Plate 32. Disease severity of ToLCV as influenced by inoculation with selected rhizobacteria and chitosan during summer 2012

Table 28b: Effect of the selected rhizobacteria and chitosan on defense molecules activity in field conditions during 2012

SL. No.	Treatments	Polyphenol oxidase (∆OD/g protein/min)		Phenylammonia lyase(changes in cinnamic acid/min/g)	
		45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* B-15+ Chitosan +ToLCV	1.43	0.87	0.187	0.167
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	1.64	0.90	0.210	0.173
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	1.83	0.98	0.231	0.190
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	1.28	0.69	0.178	0.153
5	Chemical control +ToLCV	1.08	0.48	0.138	0.123
6	Diseased control (only ToLCV)	1.12	0.51	0.145	0.137
	SEm±	.06	.03	.02	.01
	CD @5%	.17	.10	.05	.03

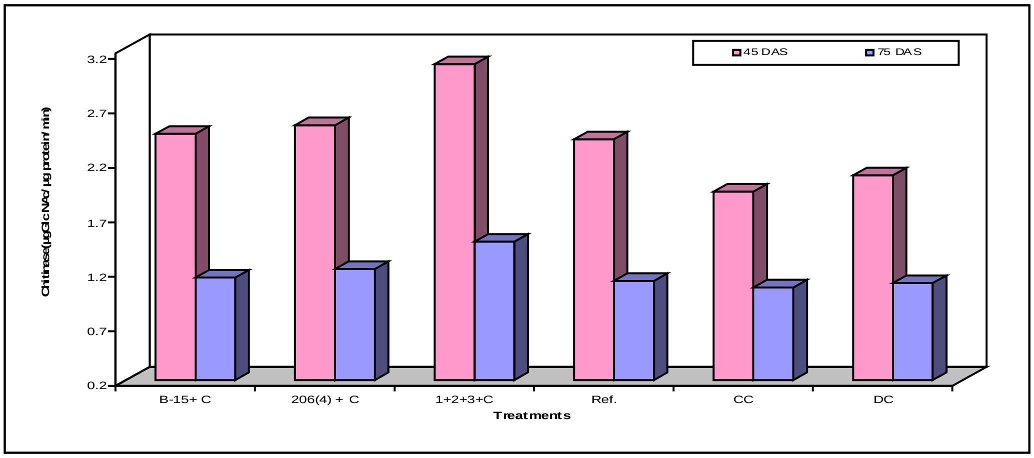


Fig. 19c: Effect of the further selected rhizobacterial strains and chitosan on defense molecules activity in field conditions during 2012

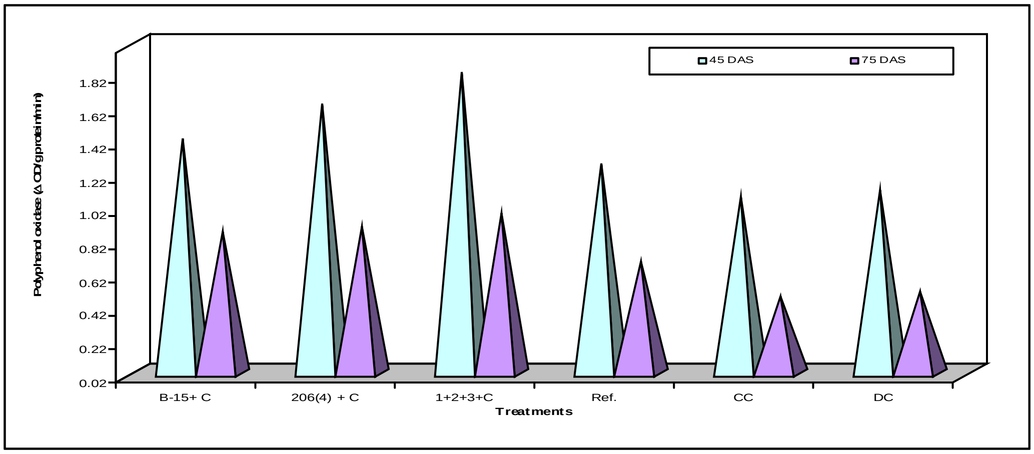


Fig. 19d: Effect of the further selected rhizobacterial strains and chitosan on defense molecules activity in field conditions during 2012

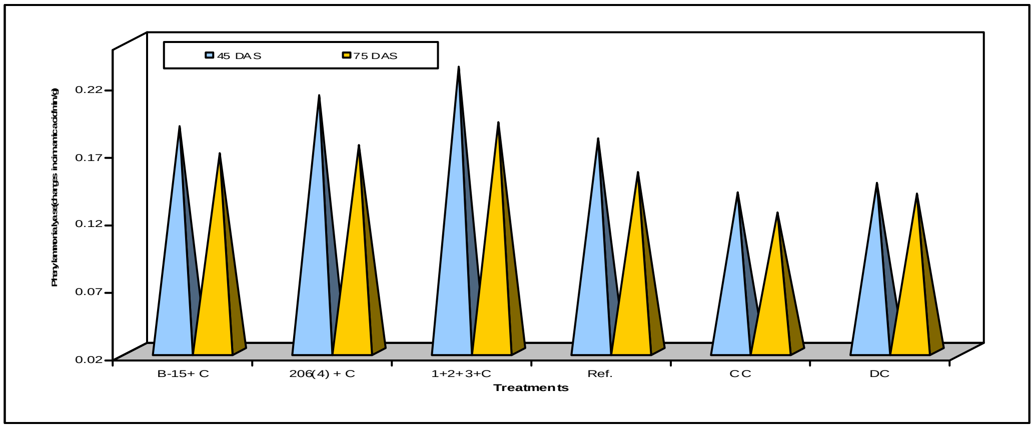


Fig. 19e: Effect of the further selected rhizobacterial strains and chitosan on defense molecules activity in field conditions during 2012

Table 29a: Quantification of viral load in the rhizobacteria and chitosan treated tomato plants at 45 DAS in field conditions during 2012

Sl. No.	Treatments	AT 45 DAS	
		Threshold cycle	Viral load
1	*Pseudomonas* B-15+ Chitosan +ToLCV	20	++
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	20	++
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	20	+
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	20	++
5	Chemical control +ToLCV	20	+
6	Diseased control (only ToLCV)	20	+++

+ - indicates visible band appeared at 20th cycle; low amount of viral load
++ - indicates visible band appeared at 20th cycle; higher amount of viral load
+++ - indicates visible band appeared at 20th cycle; the highest amount of viral load

Table 29b: Quantification of viral load in the rhizobacteria and chitosan treated tomato plants at 75 DAS in field conditions during 2012

Sl. No.	Treatments	AT 75 DAS	
		Threshold cycle	Viral load
1	*Pseudomonas* B-15+ Chitosan +ToLCV	20	+
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	20	+
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	20	+
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	20	++
5	Chemical control +ToLCV	20	+
6	Diseased control (only ToLCV)	10	+++

+ - indicates visible band appeared at 20th cycle; low amount of viral load
++ - indicates visible band appeared at 20th cycle; higher amount of viral load
+++ - indicates visible band appeared at 10th cycle; the highest amount of viral load

LEGEND

M Double digest marker(ECoRI+Hind III)

Lane 1: ToLCV amplicon after 10,20 and 30 cycles(Positive control)

Lane 2-12: PGPR treated plants after 10,20 and 30 cycles

1 – Disese control

2- Enterobacter E-21

3- *Pseudomonas* 207(1)

4- *Pseudomonas* 212(1)

5- *Pseudomonas* JK-33

6- *Pseudomonas* B-25

7- *Pseudomonas* JK-5

8 - *Pseudomonas* JK-16

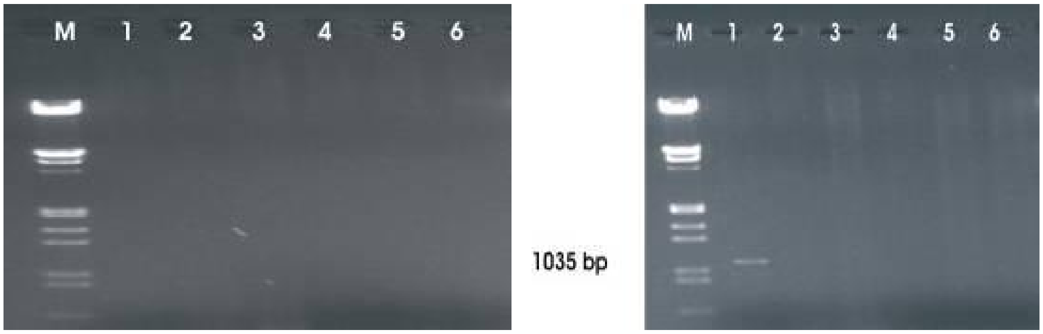
9 - *Pseudomonas* B-15

10 - *Pseudomonas* 206(4)

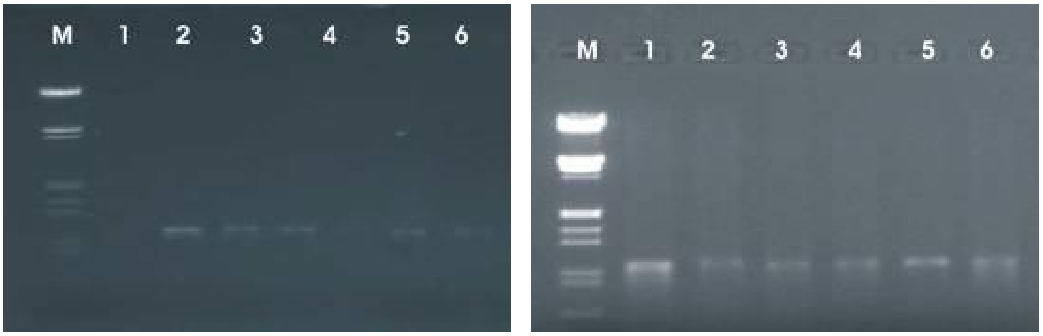
11 - *Pseudomonas* 218(1)

12 - Reference strain (*P. fluorescens* NCIM 2099)

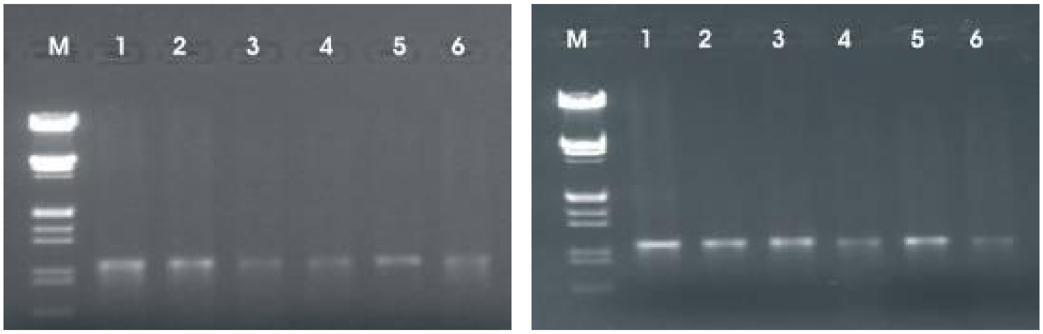
45DAS **75DAS**



10 Cycles



20 Cycles



30 Cycles

Plate 33. Quantification of viral load in the rhizobacteria and chitosan treated tomato plants

At 75 DAS, the polyphenol oxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest polyphenol oxidase activity was seen in leaves inoculated with rhizobacterial mixture + chitosan (0.98 ∆OD/g protein/ min) followed by *Pseudomonas* 206(4) + chitosan (0.90 ∆OD/g protein/ min).The least polyphenol oxidase activity was recorded in the chemical control (0.48 ∆OD/g protein/ min). The disease control plants showed an activity of 0.51 ∆OD/g protein/ min.

4.7.3 Detection of ToLCV in leaves

In order to quantify the viral inoculum in tomato plants treated with rhizobacterial isolates, a semi quantitative PCR analysis was carried out. This analysis detects and estimates the viral DNA accumulation in the challenge inoculated plants.

All the rhizobacterial and chitosan treated plants and the disease control plants were analyzed by means of semi quantitative PCR.

Equal amount of total DNA (100ng) isolated from rhizobacterial+chitosan treated plants and from the check were used. PCR reaction using coat protein gene specific primers were performed for different number of cycles *viz*., 10, 20 and 30. PCR products were detected on agarose gel electrophoresis, and the threshold cycle was determined for each treatment. In general, a slight reduction in the viral inoculum was observed in most of the treated plants. Almost all the plants showed the viral inoculum with less amount of viral load except the diseased control plant which showed the highest amount of viral load. In all the treatments, plants inoculated with rhizobacterial mixture + chitosan and chemical control plants showed the least amount of viral load after 20th cycle of the PCR at 45 DAS (Table 29a, Plate 33).

At 75 DAS, almost all the treated plants showed the viral inoculum after 20th cycle of the PCR, except in the positive control where DNA showed viral inoculum after 10th cycle.All the treatments, showed the viral inoculum with low amount of viral load and the reference strain without chitosan showed higher amount of viral load but the diseased control plant showed the highest amount of viral load after 20th cycle of the PCR (Table 29 b, Plate 33).

4.7.4 Plant growth promotional potential of the selected rhizobacterial strains in combination with chitosan

The effect of rhizobacterial combination with chitosan on growth and yield parameters of tomato was studied. The following parameters were recorded.

4.7.4.1 Plant height

The data pertaining to effect on plant height of Tomato plants at different intervals of time *viz.*, 30, 45, 60, 75 DAS and at harvest are given in Table 30, Fig.20 Plate 32.

At 30 DAS, plant height was significantly improved due to various combination of chitosan and rhizobacterial inoculation. The highest length (20.3 cm / plant) was observed in the plants inoculated with rhizobacterial mixture + chitosan. The least height was observed in the diseased control treatment (13.90 cm / plant)

At 45 DAS, again rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (23.70 cm/plant). The next best treatment was 206(4)+chitosan which showed the plant height (22.90 cm / plant). The least plant height was in the disease control treatment (16.40 cm / plant).

At 75 DAS also, rhizobacterial mixture + chitosan inoculated plants resulted in the highest plant height (27.40 cm/plant). This was followed by the 206(4) + chitosan (26.70 cm / plant). The disease control treatment shown least plant height (19.70 cm / plant).

At harvest, a similar trend was observed. The highest plant height was observed in the treatment inoculated with rhizobacterial mixture + chitosan.It produced a height of 38.10 cm per plant. This was followed by the 206(4) + chitosan (35.90 cm / plant). The viral pathogen inoculated check exhibited the least plant height (26.00 cm/plant).

Table 30: Effect of the selected rhizobacteria and chitosan on plant height of tomato in field conditions during 2012

Sl. No.	Treatments	Plant height (cm)			
		30 DAS	45 DAS	75 DAS	At Harvest
1	*Pseudomonas* B-15+ Chitosan +ToLCV	19.4	22.4	25.9	35.0
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	19.7	22.9	26.7	35.9
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	20.3	23.7	27.4	38.1
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	19.3	22.1	25.2	34.7
5	Chemical control +ToLCV	15.0	18.2	20.4	30.2
6	Diseased control (only ToLCV)	13.9	16.4	19.7	26.0
	SEm±	.72	.64	.95	1.07
	CD @1%	2.13	1.88	2.81	3.17

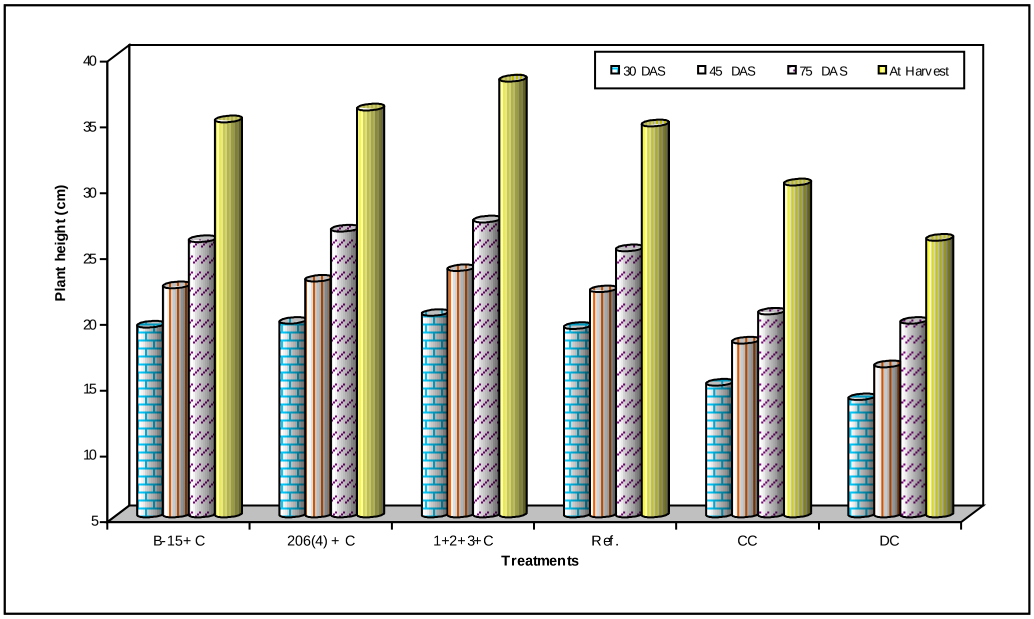


Fig. 20: Effect of the further selected rhizobacterial strains and chitosan on plant height of tomato during 2012

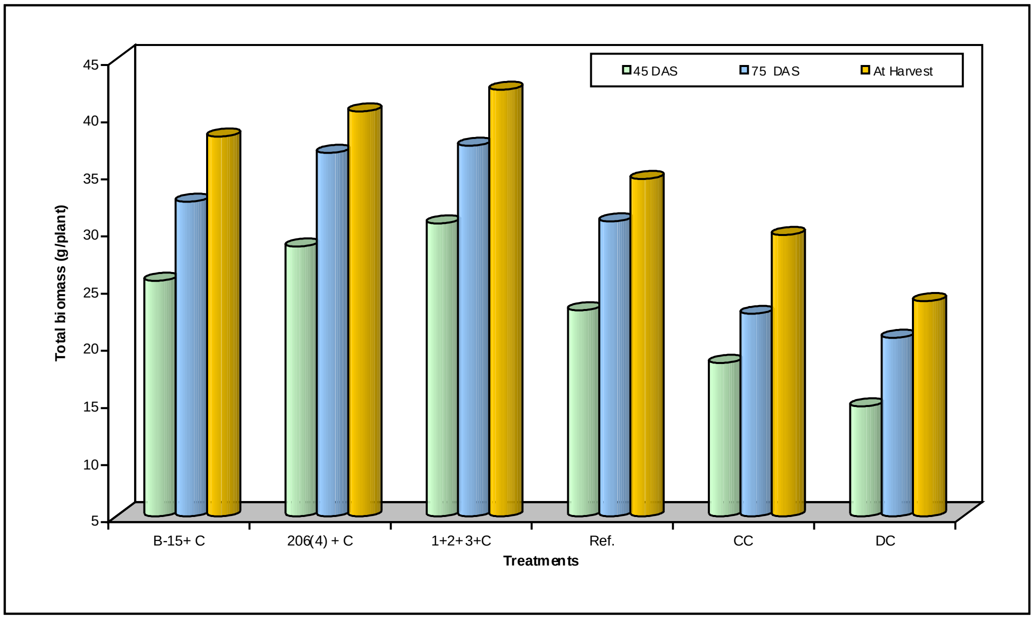


Fig. 21: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants during 2012 in field conditions

4.7.4.2 Plant biomass

In general, bacterized plants alone or in combination with chitosan produced significantly higher biomass than the uninoculated plants. And, rhizobacterial mixture + chitosan produced consistently higher biomass throughout the growth period of tomato. At 45 DAS, the plants inoculated with rhizobacterial mixture + chitosan produced the highest biomass (30.60 g/plant) (Table 31, Fig.21).

Table 34). This was followed by 206(4) + chitosan (28.60 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (14.60 g/plant).

At 75 DAS, significantly higher biomass was obtained in the treatment receiving rhizobacterial mixture + chitosan (37.40 g/plant). This was followed by 206(4)+chitosan treatment (36.80 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (20.60 g/plant).

At the time of harvesting, the maximum biomass was recorded in rhizobacterial mixture + chitosan treatment (42.30 g/plant), which was followed by 206(4)+chitosan treatment (40.40 g/plant). However, the least biomass was recorded in the viral pathogen inoculated treatment (23.80 g/plant).

4.7.4.3 Chlorophyll content

The chlorophyll content of tomato plants was significantly higher in rhizobacteria and chitosan treated plants. The data are presented in Table 32 and Fig.22. Chlorophyll content was increased up to 45 DAS and declined thereafter.

At 30 DAS, rhizobacterial mixture + chitosan resulted in significantly higher chlorophyll content (25.90 SPAD value). This was followed by 206(4)+chitosan treatment (24.20 SPAD value). The least chlorophyll content was recorded in the diseased control (22.20 SPAD value).

At 45 DAS, rhizobacterial mixture + chitosan treatment resulted in significantly higher chlorophyll content (49.90 SPAD value), which was followed by 206(4)+ chitosan treatment (49.40 SPAD value). The least chlorophyll content was recorded in the diseased control (34.30 SPAD value).

At 75 DAS, the maximum chlorophyll content was recorded in the leaves of rhizobacterial mixture + chitosan treated plants (43.50 SPAD value) followed by 206(4) + chitosan treated leaves (40.80 SPAD value). The least chlorophyll content was recorded in the diseased control (31.00 SPAD value).

At harvest, the chlorophyll content was decreased in all the treatments when compared to at 45 DAS. The maximum chlorophyll content was recorded in the rhizobacterial mixture + chitosan treated plants (35.60 SPAD value), which was followed by plants treated with 206(4)+chitosan (32.80 SPAD value). The least chlorophyll was synthesized in the leaves of diseased control plants (18.40 SPAD value).

4.7.4.4 Fruit yield

In general, rhizobacterial inoculation to tomato resulted in significantly higher fruit yields (Table 33, Fig. 23 and Plate 34).

The plants inoculated with rhizobacterial mixture + chitosan produced significantly higher no. of fruits per plant (26.80 / plant). This was followed by the treatment which received 206(4) + chitosan (22.40 / plant). The pathogen inoculated treatment resulted in the least number of fruits (9.40/plant).

The plants inoculated with rhizobacterial mixture + chitosan recorded the highest fruit yield (1.011Kg/plant). This was followed by the 206(4)+chitosan (0.839 Kg/plant). The least yield was recorded in the pathogen inoculated treatment (0.310 kg/plant).

4.7.4.5 Shelf life

The fruits produced by the rhizobacterial mixture + chitosan treated plants exhibited longest shelf life (8.6 days). This was followed by the treatment which received 206(4)+chitosan (8.2 days). The pathogen inoculated treatment produced fruits with minimum shelf life period (5.1 days) Table 33

Table 31: Influence of inoculation of the selected rhizobacteria and chitosan on total biomass of tomato plants in field conditions during 2012

Sl. No.	Treatments	Total biomass (g/plant)		
		45 DAS	75 DAS	At Harvest
1	*Pseudomonas* B-15+ Chitosan +ToLCV	25.6	32.5	38.2
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	28.6	36.8	40.4
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	30.6	37.4	42.3
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.0	30.8	34.5
5	Chemical control +ToLCV	18.4	22.7	29.6
6	Diseased control (only ToLCV)	14.6	20.6	23.8
	SEm±	.40	.58	.51
	CD @5%	1.17	1.72	1.52

Table 32: Effect of the selected rhizobacterial inoculation and chitosan on chlorophyll content in tomato plants in field conditions during 2012

Sl. No	Treatments	Chlorophyll content (SPAD value)			
		30 DAS	45 DAS	75 DAS	At harvest
1	*Pseudomonas* B-15+ Chitosan +ToLCV	23.5	46.2	37.3	30.8
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	24.2	49.4	40.8	32.8
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	25.9	49.9	43.5	35.6
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	23.1	45.5	36.5	27.7
5	Chemical control +ToLCV	22.7	42.8	35.8	26.0
6	Diseased control (only ToLCV)	22.2	34.3	31.0	18.4
	SEm±	.59	1.07	.85	.98
	CD @5%	1.73	3.15	2.50	2.90

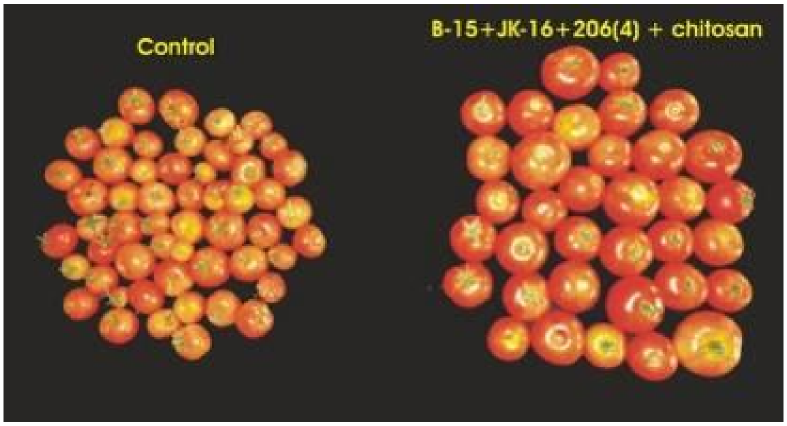


Plate 34. Effect of inoculation of rhizobacterial mixture and chitosan on fruit quality of tomato

4.7.5 Subcellular changes due to ToLCV and rhizobacterial inoculation

The cellular structures of the of the healthy, treated (*Pseudomonas* sp. 206(4) +chitosan) and diseasd plants were studied by transmission electron microscopy (TEM) which showed the subcellular changes which occurred inside the cells due to ToLCV and rhizobacterial inoculation. Incase of diseased cells, there is complete loss of subcellular architecture. Cell boundaries are lost.Cells got completely shrunken with moon shaped chloroplast whereas at some part of the cells, there was complete loss of chloroplast observed. No vacuolation was observed. But, incase of treated leaves, cells appeared somewhat close to normal cells. Some extent of distortion was seen in chloroplast whereas mitochondria, endoplasmic reticulum and other organells appeared to be normal. Shrikage of protoplast was observed.cell membrane was intact. At intercellular junction, electron densed materials were seen.Vacuolation was observed inside the chloroplast. Healthy plant cells apperas normal with intact histological structure (Plate 35).

4.8 Field evaluation of the best rhizobacterial isolate and chitosan treatment for the biocontrol of ToLCV as well as growth promotion of tomato

4.8.1 Biocontrol potential of the combination of the selected rhizobacterial isolate and chitosan under field condition

Based on the previous field experiment, the most promising strain 206(4) was selected and tested for its ability to control ToLCV in tomato with chitosan combination under field condition and the results are furnished in Table 34, Fig.24, Plate 36). At 45 DAI, 206(4)+chitosan treatment showed 48 percent disease severity control whereas 36 percent disease severity control was noticed in the chemical control treated plants. At 75 DAI, the highest viral disease severity control (24%) was observed in 206(4) +Chitosan treatment which was followed by chemical control treatment with 18 percent disease severity control.

4.8.2 Ability of the *Pseudomonas* sp. 206(4) and chitosan to induce systemic resistance against ToLCV in tomato plants

The ISR activity of the best isolate along with chitosan was tested by estimating defense enzymes such as peroxidase, chitinase, polyphenoloxidase, phenylalanine ammonia lyase (PALase) and phenol content in plants at different intervals of time.

4.8.2.1 Phenol content

The phenol content of leaves, at different intervals *viz.*, 45 and 75 DAS, as influenced by inoculation with rhizobacteria are presented in Table 35a, Fig.25a.

Table 33: Influence of the selected rhizobacteria and chitosan on yield of tomato in field conditions during 2012

Sl. No.	Treatments	Number of fruits per plant	Fruit yield (kg/plant)	Yield (ton/ha)	Shelf Life (days)
1	*Pseudomonas* B-15+ Chitosan +ToLCV	20.2	0.721	16.22	7.4
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	22.4	0.839	18.87	8.2
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	26.8	1.011	22.75	8.6
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	19.6	0.704	15.84	7.3
5	Chemical control +ToLCV	17.4	0.568	12.78	5.6
6	Diseased control (only ToLCV)	9.4	0.310	6.98	5.1
	SEm±	0.72	0.02	0.48	0.28
	CD @5%	2.10	0.06	1.43	0.83

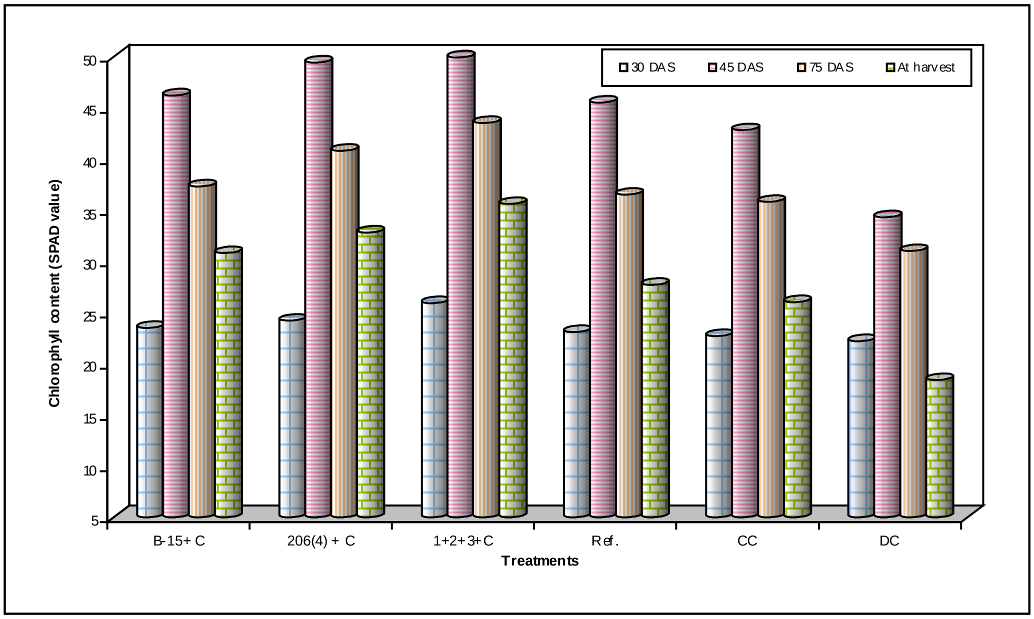


Fig. 22: Effect of the further selected rhizobacterial strains and chitosan on chlorophyll content in tomato plants during 2012 in field conditions

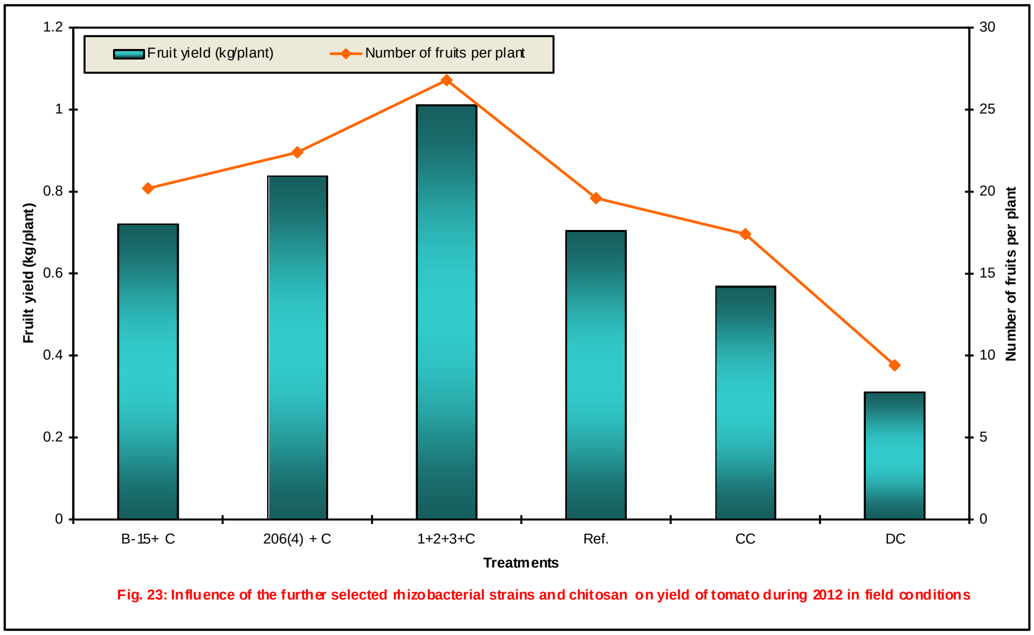
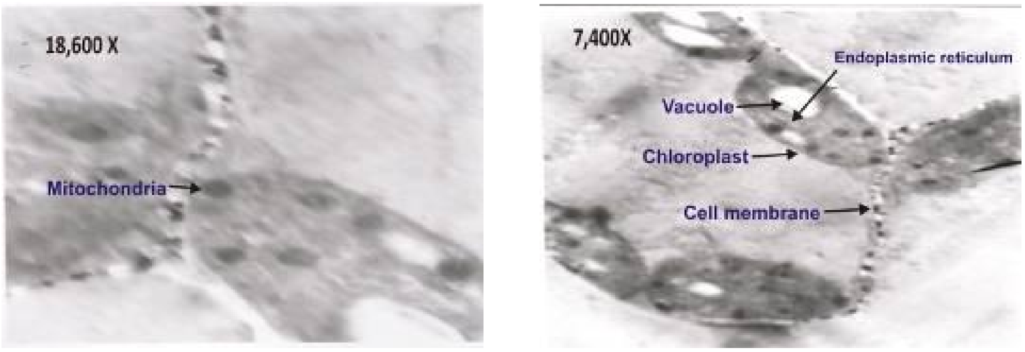
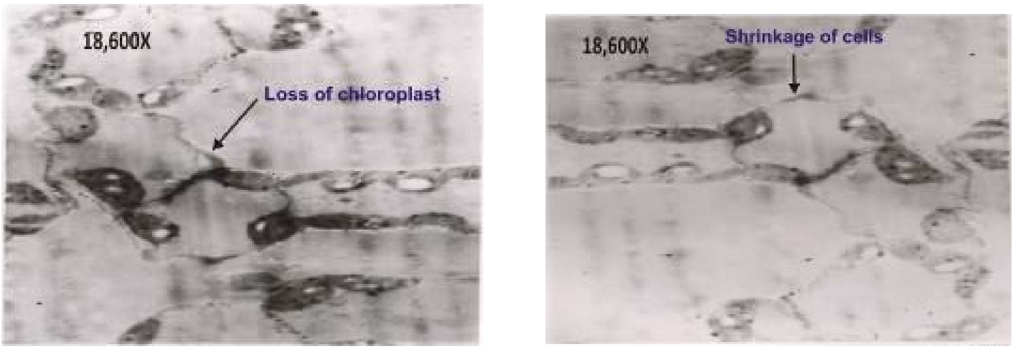


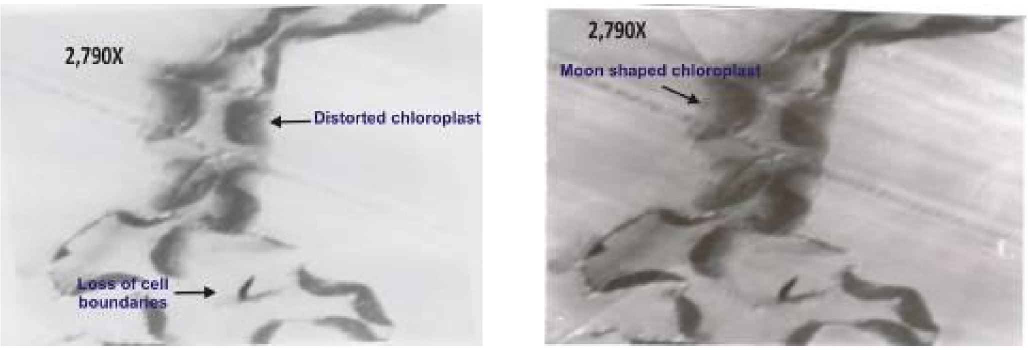
Fig. 23: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions



Healthy leaves



Virus infected leaves, but inoculated with Pseudomonas 206(4)



Virus infected leaves

Plate 35. TEM pictures depicting subcellular changes due to ToLCV and rhizobacterial inoculation

Table 34: Disease severity of ToLCV as influenced by the best selected rhizobacterial strain and chitosan at high disease pressure in field conditions

SL. No.	Treatments	45 DAI		75 DAI	
		Per cent disease severity	Per cent disease severity control	Per cent disease severity	Per cent disease severity control
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	52	48	76	24
2	Chemical control +ToLCV	64	36	82	18
3	Diseased control (only ToLCV)	100	0	100	0

LEGEND

206(4)+C = *Pseudomonas* 206(4) + Chitosan +ToLCV

CC = Chemical control +ToLCV

DC = Diseased control (only ToLCV)

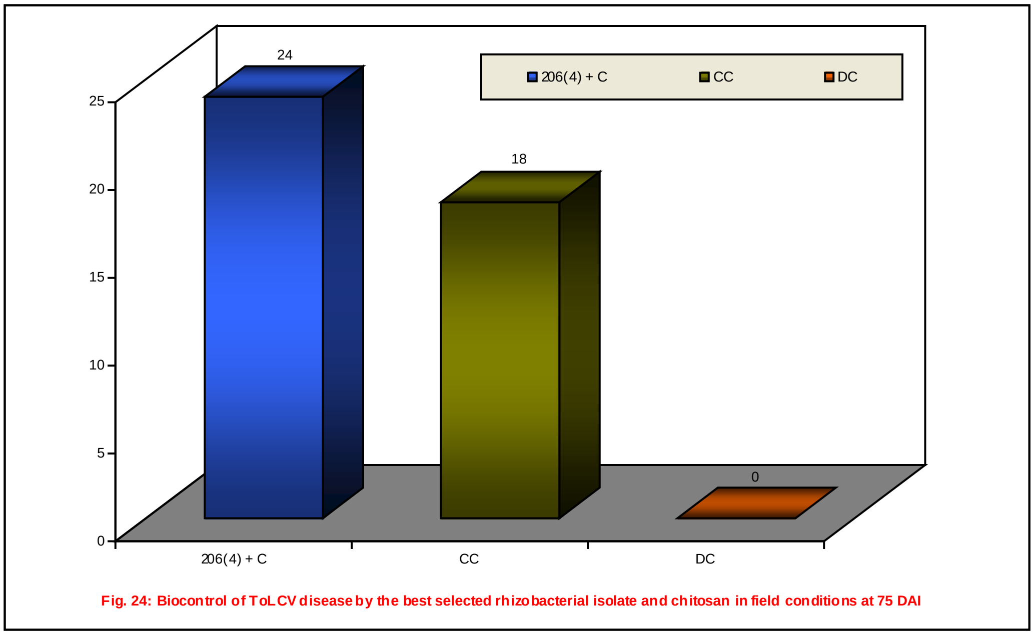


Fig. 24: Biocontrol of ToLCV disease by the best selected rhizobacterial isolate and chitosan in field conditions at 75 DAI

Disease control

Chemical control

206(4) + Chitoson

Plate 36. Disease severity of ToLCV as influenced by inoculation with best rhizobacterial isolate and chitosan during summer 2012

At 45 DAS, the treatment receiving *Pseudomonas* sp. 206(4) + chitosan recorded the maximum phenol content (0.26mg/g), which was followed by the disease control treatment (0.23 mg/g). The least phenol content was recorded in the chemical control treatment (0.21mg/g).

At 75 DAS, phenol content was slightly decreased in all the treatments as compared to 45 DAS. The highest phenol content was recorded in the plants inoculated with *Pseudomonas* sp. 206(4) + chitosan (0.23 mg/g). This was followed by the disease control (0.21 mg/g). Chemical control recorded significantly lower amount of phenol content (0.20 mg/g).

4.8.2.2 Phenylalanine ammonia lyase (PALase) activity

The results on biosynthesis of PALase activity as influenced by rhizobacterial strains in combination with chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in 35b, Fig.25e.

At 45 DAS, the maximum PALase activity was recorded in the treatment receiving *Pseudomonas* sp. 206(4) + chitosan (0.184 change in cinnamic acid/min/g) followed by the disease control (0.164 change in Cinnamic acid/min/g). The least PALase activity was recorded in the chemical control (0.148 change in Cinnamic acid/min/g each).

At 75 DAS, PALase activity was found decreasing in all the treatments when compared to 45 DAS. The highest activity was seen in plants inoculated with *Pseudomonas* sp. 206(4) + chitosan (0.176 change in cinnamic acid/min/g) followed by disease control (0.148 change in Cinnamic acid/min/g).The least was exhibited by the chemical control (0.128 change in Cinnamic acid/min/g).

4.8.2.3 Peroxidase activity

The data pertaining to peroxidase activity as influenced by rhizobacterial combination with chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in 35a, Fig.25b.

There was a tremendous increase in peroxidase activity in both the stages due to rhizobacterial inoculation. At 45 DAS, the highest peroxidase activity was recorded in the plant leaves receiving *Pseudomonas* sp. 206(4) + chitosan (1.51 ∆OD/g protein/ min). The disease control treatment exhibited 1.26 ∆OD/g protein/ min. And, the least peroxidase activity was recorded in the chemical control (1.14 ∆OD/g protein/ min).

At 75 DAS, the peroxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest peroxidase activity was seen in leaves inoculated with *Pseudomonas* sp. 206(4) + chitosan (0.55 ∆OD/g protein/ min). The least peroxidase activity was recorded in the chemical control (0.41 ∆OD/g protein/ min). The disease control plants showed an activity of 0.43 ∆OD/g protein/ min.

4.8.2.4 Chitinase activity

The results on biosynthesis of chitinase as influenced by rhizobacterial strains with and without chitosan in tomato leaves at different intervals of time viz., 45 and 75 DAS are furnished in 35a, Fig.25c.

At 45 DAS, the highest chitinase activity was recorded in the plant leaves receiving *Pseudomonas* sp. 206(4) + chitosan (2.65 µgGlc NAc/ µg protein/ min).The disease control treatment exhibited 1.96 µgGlc NAc/ µg protein/ min. And, the least chitinase activity was recorded in the chemical control (1.74 µgGlc NAc/ µg protein/ min).

At 75 DAS, the chitinase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest chitinase activity was seen in leaves inoculated with *Pseudomonas* sp. 206(4) + chitosan (1.63 µgGlc NAc/ µg protein/ min). The least chitinase activity was recorded in the chemical control (1.12 µgGlc NAc/ µg protein/ min). The disease control plants showed an activity of 1.25 µgGlc NAc/ µg protein/ min.

Table 35a: Effect of the best selected rhizobacteria and chitosan on defense molecules activity

SL. No.	Treatments	Phenol(mg/g dry weight)		Peroxidase(ΔOD/g protein/min)		Chitinase(µgGlc NAc/ µg protein/ min)	
		45 DAS	**75 DAS**	**45 DAS**	**45 DAS**	**75 DAS**	**45 DAS**
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	0.26	0.23	1.51	0.55	2.65	1.63
2	Chemical control +ToLCV	0.21	0.20	1.14	0.41	1.74	1.12
3	Diseased control (only ToLCV)	0.23	0.21	1.26	0.43	1.96	1.25
	SEm ±	0.01	0.01	0.07	0.01	0.09	0.07
	CD @5%	0.02	0.03	0.21	**0.03**	0.26	0.22

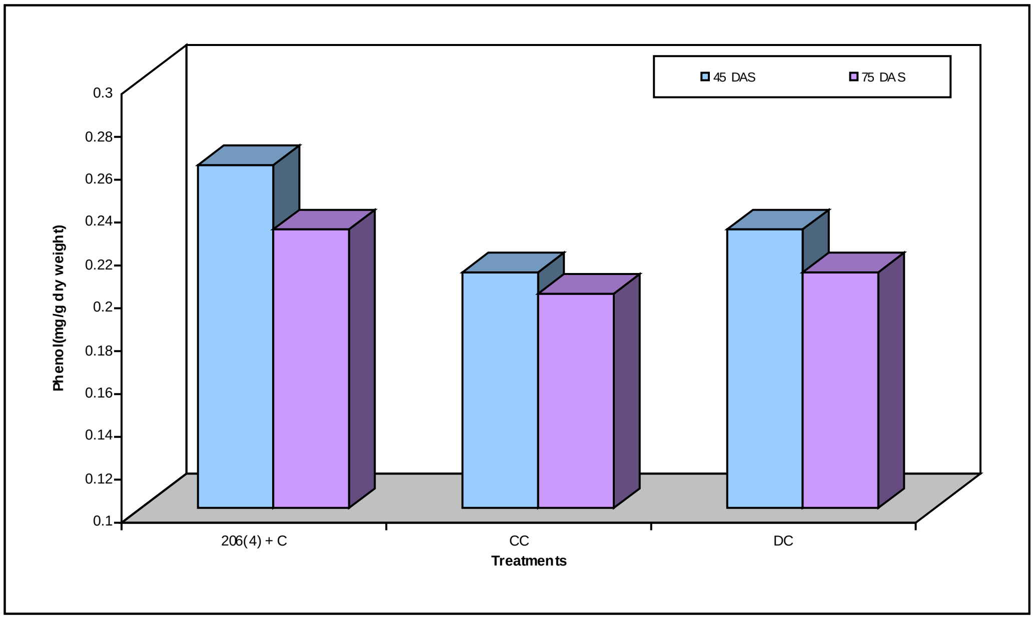


Fig. 25a: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions

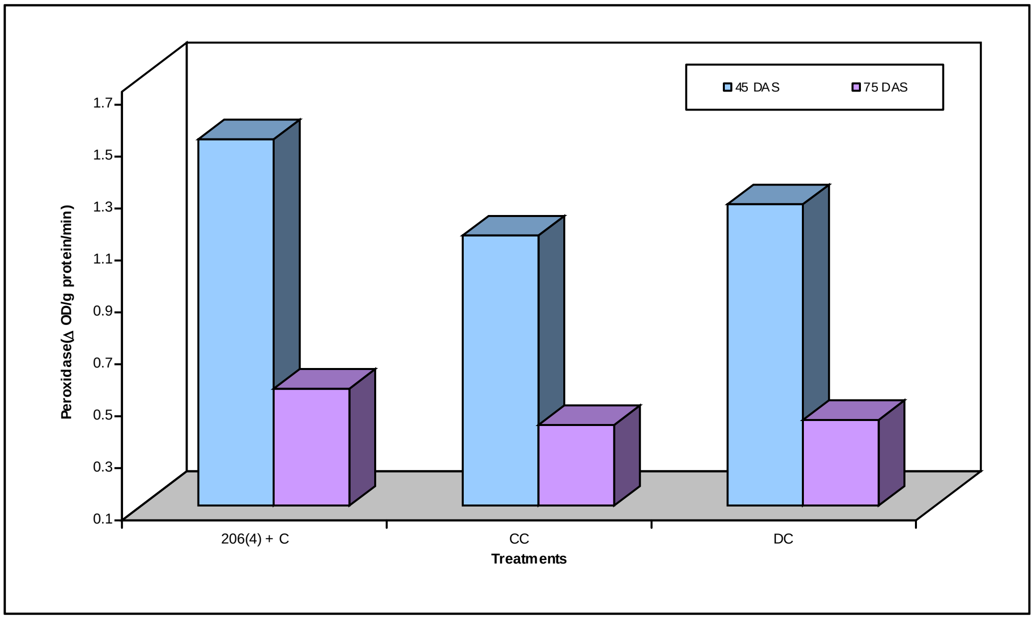


Fig. 25b: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions

Table 35b: Effect of the best selected rhizobacteria and chitosan on defense molecules activity

SL. No.	Treatments	Polyphenol oxidase (ΔOD/g protein/min)		Phenylammonia lyase(changes in cinnamic acid/min/g)	
		45 DAS	75 DAS	45 DAS	75 DAS
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	1.37	0.87	0.184	0.176
2	Chemical control +ToLCV	0.93	0.48	0.148	0.128
3	Diseased control (only ToLCV)	1.02	0.56	0.164	0.148
	SEm±	0.04	0.01	0.02	0.01
	CD at 5%	0.12	0.04	0.05	0.03

Table 36: Effect of the best selected rhizobacteria and chitosan on plant height of tomato

Sl. No.	Treatments	Plant height (cm)			
		30 DAS	45 DAS	75 DAS	At Harvest
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	18.5	20.5	25.3	31.1
2	Chemical control +ToLCV	15.4	17.4	20.1	26.9
3	Diseased control (only ToLCV)	14.4	15.7	17.5	20.3
	SEm±	0.51	0.51	0.92	0.59
	CD @1%	1.59	1.57	2.84	1.81

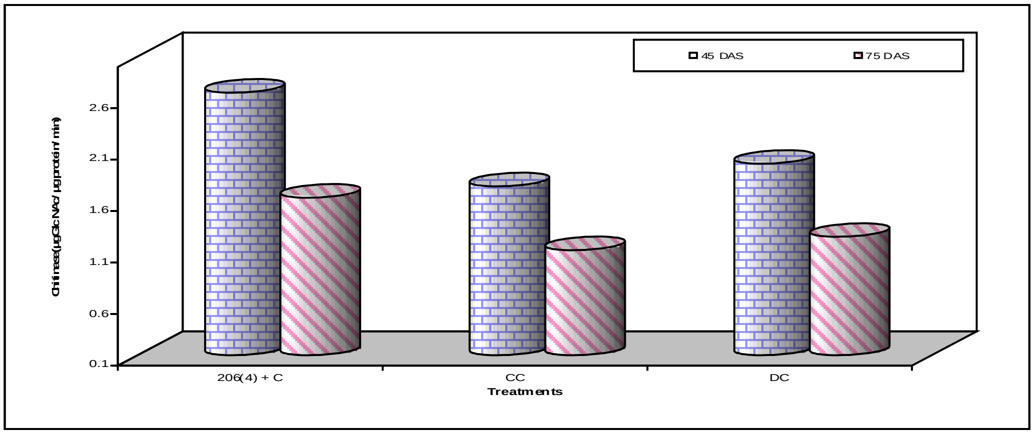


Fig. 25c: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions

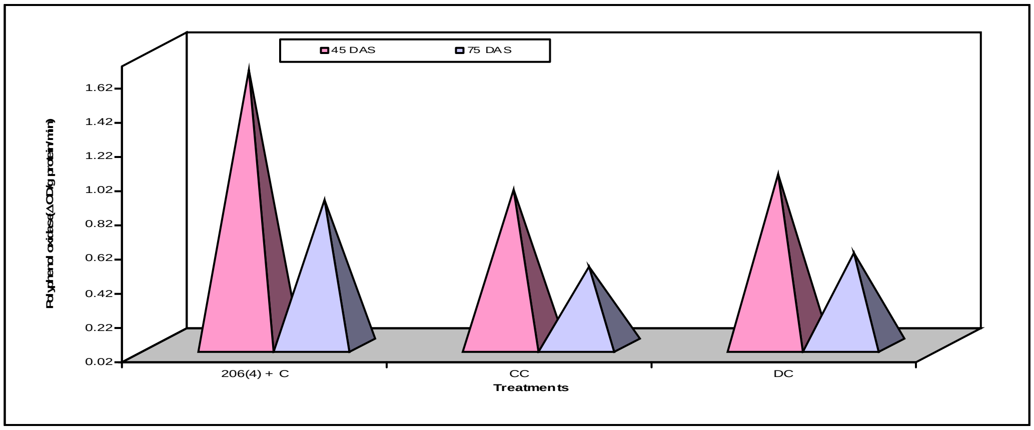


Fig. 25d: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions

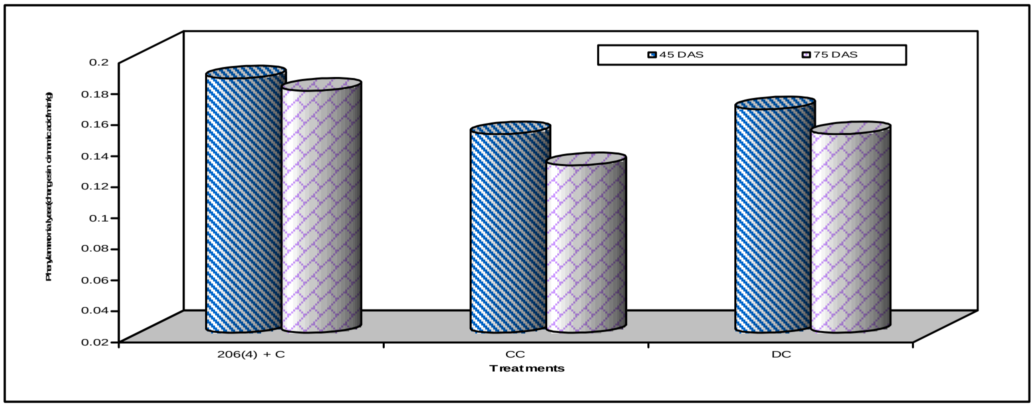


Fig. 25e: Influence of the further selected rhizobacterial strains and chitosan on yield of tomato during 2012 in field conditions

4.8.2.5 Polyphenol oxidase activity

The polyphenol oxidase activity as influenced by rhizobacterial strains and chitosan treatment in tomato leaves at different intervals of time *viz.*, 45 and 75 DAS are furnished in 35b, Fig.25d.

In general, there was a substantial increase in polyphenol oxidase activity in both the stages due to rhizobacterial and chitosan treatment. At 45 DAS, the highest polyphenol oxidase activity was recorded in the plant leaves receiving *Pseudomonas* sp. 206(4) + chitosan (1.37 ΔOD/g protein/ min). The disease control treatment exhibited 1.02 ΔOD/g protein/ min. And, the least polyphenol oxidase activity was recorded in the chemical control (0.93 ΔOD/g protein/min).

At 75 DAS, the polyphenol oxidase activity was slightly decreased in all the treatments when compared to 45 DAS. The highest polyphenol oxidase activity was seen in leaves inoculated with *Pseudomonas* sp. 206(4) + chitosan (0.87 ΔOD/g protein/ min). The least polyphenol oxidase activity was recorded in the chemical control (0.48 ΔOD/g protein/ min). The disease control plants showed an activity of 0.56 ΔOD/g protein/ min.

4.8.3 Plant growth promotional potential of the *Pseudomonas* sp. 206(4) in combination with chitosan

The effect of *Pseudomonas* sp. 206(4) in combination with chitosan on growth and yield parameters of tomato was studied. The following parameters were recorded.

4.8.3.1 Plant height

The data pertaining to effect on plant height of Tomato plants at different intervals of time *viz.*, 30, 45, 60, 75 DAS and at harvest are given in Table 36, Fig. 26.

At 30 DAS, plant height was significantly improved due to the combination of chitosan and rhizobacterial inoculation. The maximum height (18.5 cm / plant) was observed in the plants inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan. The least height was observed in the diseased control treatment (14.40 cm / plant)

At 45 DAS, again *Pseudomonas* sp. 206(4) + chitosan plants resulted in the highest plant height (20.5 cm/plant). The least plant height was in the disease control treatment (15.70 cm / plant).

At 75 DAS also, *Pseudomonas* sp. 206(4) + chitosan inoculated plants resulted in the highest plant height (25.30 cm/plant). This was followed by the chemical control treatment with 20.10 cm/plant. The disease control treatment shown least plant height (17.50 cm / plant).

At harvest, similar trend was observed. The highest plant height was observed in the treatment inoculated with *Pseudomonas* sp. 206(4) + chitosan. It produced a height of 31.10 cm per plant. This was followed by the chemical control plant (26.90 cm / plant). The viral pathogen inoculated check exhibited the least plant height (20.30 cm/plant).

4.8.3.2 Plant biomass

In general, bacterized plants in combination with chitosan produced significantly higher biomass than the uninoculated plants. And, *Pseudomonas* sp. 206(4) + chitosan produced consistently higher biomass throughout the growth period of tomato. At 45 DAS, the plants inoculated with *Pseudomonas* sp. 206(4) + chitosan produced the highest biomass (21.30 g/plant) (Table 37 and Fig.27). This was followed by the chemical control treatment (15.40 g/plant).The least biomass was recorded in the viral pathogen inoculated treatment (10.20 g/plant).

At 75 DAS, significantly higher biomass was obtained in the treatment receiving *Pseudomonas* sp. 206(4) + chitosan (24.70 g/plant). This was followed by the chemical control treatment (20.30 g/plant). The least biomass was recorded in the viral pathogen inoculated treatment (11.70 g/plant).

Table 37: Influence of inoculation of the best selected rhizobacteria and chitosan on total biomass of tomato plants

Sl. No.	Treatments	Total biomass (g/plant)		
		45 DAS	75 DAS	At Harvest
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	21.3	24.7	30.9
2	Chemical control +ToLCV	15.4	20.3	23.5
3	Diseased control (only ToLCV)	10.2	11.7	13.6
	SEm±	0.44	0.64	0.57
	CD @5%	1.37	1.99	1.75

Table 38: Effect of the best selected rhizobacterial inoculation and chitosan on chlorophyll content in tomato plants

Sl. No	Treatments	Chlorophyll content (SPAD value)			
		30 DAS	45 DAS	75 DAS	At harvest
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	24.0	45.8	30.7	20.7
2	Chemical control +ToLCV	20.1	41.8	28.2	17.7
3	Diseased control (only ToLCV)	19.2	32.6	17.4	10.7
	SEm±	.65	.94	1.42	1.21
	CD @5%	2.01	2.90	4.37	3.73

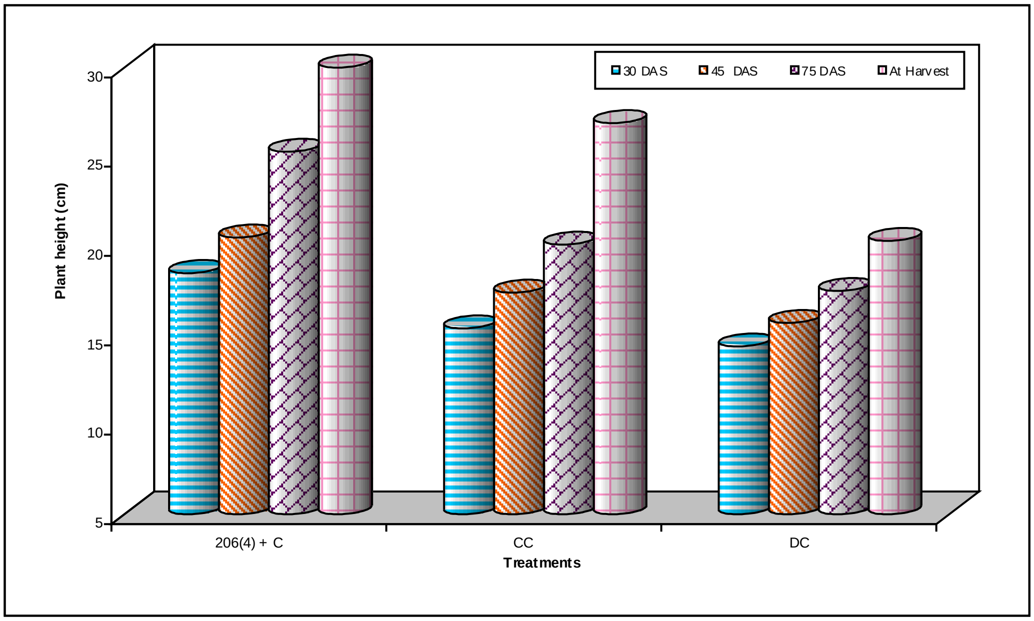


Fig. 26: Effect of the selected rhizobacteria on plant height of tomato

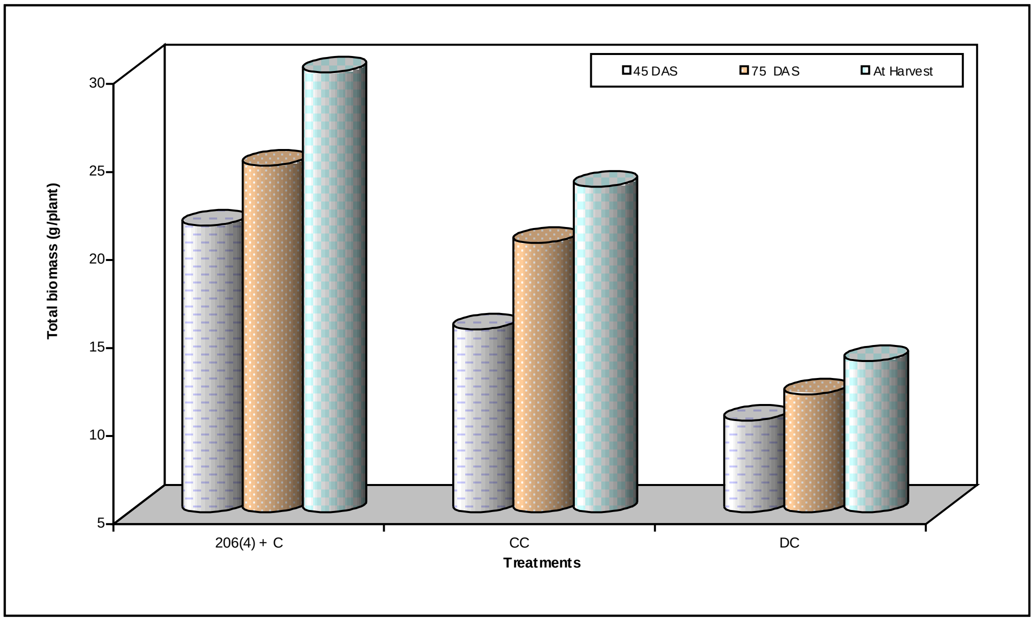


Fig. 27: Influence of inoculation of the best selected rhizobacterial isolate and chitosan on total biomass of tomato plants

At the time of harvesting, the maximum biomass was recorded in *Pseudomonas* sp. 206(4) + chitosan treatment (30.90 g/plant), which was followed by the chemical control treatment (23.50 g/plant). However, the least biomass was recorded in the viral pathogen inoculated treatment (13.60 g/plant).

4.8.3.3 Chlorophyll content

The chlorophyll content of tomato plants was significantly higher in rhizobacteria and chitosan treated plants. The data are presented in Table 38, Fig.28. Chlorophyll content was increased up to 45 DAS and declined thereafter.

At 30 DAS, *Pseudomonas* sp. 206(4) + chitosan resulted in significantly higher chlorophyll content (24.00 SPAD value). This was followed by the chemical control treatment (20.10 SPAD value). The least chlorophyll content was recorded in the diseased control (19.20 SPAD value).

At 45 DAS, *Pseudomonas* sp. 206(4) + chitosan treatment resulted in significantly higher chlorophyll content (45.80 SPAD value), which was followed by chemical control treatment (41.80 SPAD value). The least chlorophyll content was recorded in the diseased control (32.60 SPAD value).

At 75 DAS, the maximum chlorophyll content was recorded in the leaves of *Pseudomonas* sp. 206(4) + chitosan treated plants (30.70 SPAD value) followed by chemical control treated leaves (28.20 SPAD value). The least chlorophyll content was recorded in the diseased control (17.40 SPAD value).

At harvest, chlorophyll content was decreased in all the treatments when compared to at 45 DAS. The maximum chlorophyll content was recorded in *Pseudomonas* sp. 206(4) + chitosan treated plants (20.70 SPAD value), which was followed by chemical control treated plants (17.70 SPAD value). The least chlorophyll was synthesized in the leaves of diseased control plants (10.70 SPAD value).

4.8.3.4 Fruit yield

In general, rhizobacterial inoculation to tomato resulted in significantly higher fruit yields (Table 39, Fig.29). The plants inoculated with *Pseudomonas* sp. 206(4) + chitosan produced significantly higher no. of fruits per plant (12.20/plant). This was followed by the chemical control treatment (6.40/plant).The pathogen inoculated treatment resulted in the least number of fruits (2.60/plant).

The plants inoculated with *Pseudomonas* sp. 206(4) + chitosan recorded the highest fruit yield (0.207 kg/plant). This was followed by the chemical control (0.092 Kg/plant). The least yield was recorded in the pathogen inoculated treatment (0.058 kg/plant).

4.8.3.5 Shelf life

The fruits produced by the *Pseudomonas* sp. 206(4) + chitosan treated plants exhibited the longest shelf life (5.4 days). This was followed by the chemical control (4.7 days). The pathogen inoculated treatment produced fruits with the least shelf life period (4.4 days) (Table 39).

Table 39: Influence of the best selected rhizobacteria and chitosan on yield of tomato

Sl. No.	Treatments	Number of fruits per plant	Fruit yield (kg/plant)	Yield (ton/ha)	Shelf Life (days)
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	12.2	0.207	5.73	5.4
2	Chemical control +ToLCV	6.4	0.092	2.55	4.7
3	Diseased control (only ToLCV)	2.6	0.058	1.62	4.4
	SEm±	0.47	0.01	0.28	0.24
	CD @5%	1.45	0.03	0.87	0.75

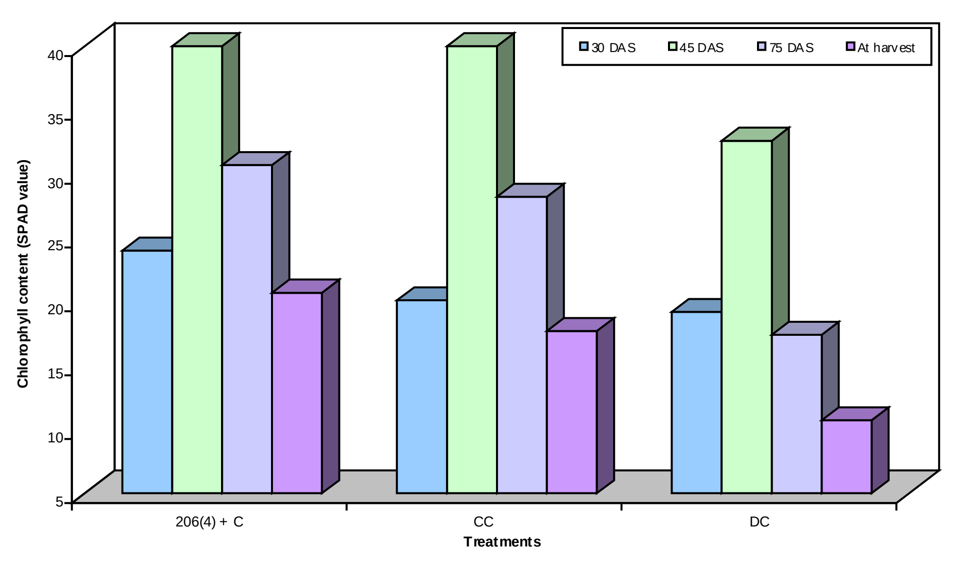


Fig. 28: Effect of the best selected rhizobacterial isolate and chitosan on chlorophyll content in tomato plants

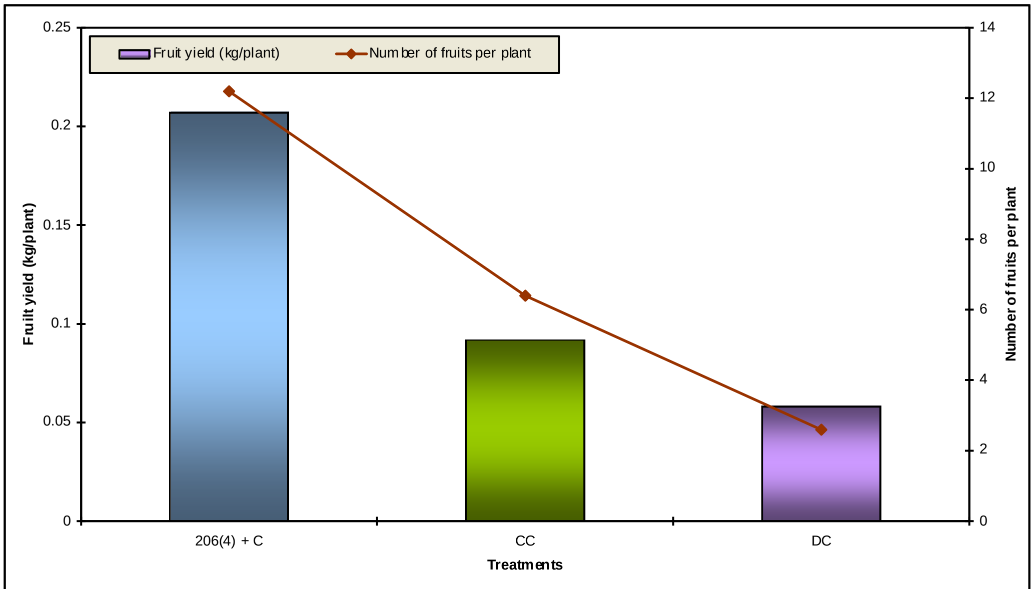


Fig. 29: Influence of the best selected rhizobacterial isolate and chitosan on yield of tomato

Fig. 29: Influence of the best selected rhizobacterial isolate and chitosan on yield of tomato

5. DISCUSSION

The plant diseases caused by insect transmitted viruses are among the most serious production problems encountered by vegetable growers. Effective control of insect borne virus diseases is problematic since most viruses are transmitted by highly mobile insects and may colonize fields rapidly, before their presence is felt.

Tomato (*Lycopersicon esculentum* Mill.) is the world's largest vegetable crop and is known as a protective food because of its special nutritive value. It is a major source of minerals and vitamins A, B and C and also regarded as an anticancer food. It is rich in lycopene which is known to help prevent cancer (Anon., 2003).

Of all the diseases reported in tomato, the tomato leaf curl virus (ToLCV) disease is cited to be most devastating, as quantitative and qualitative yield losses often reach cent per cent in summer month in Karnataka (Devaraja *et al.,* 2005). Control of the vector whitefly has not been effective in preventing epidemics and yield losses due to these viruses. So far, no economically viable management practices are available to manage ToLCV in tomato crop. However, they are slowly being replaced by naturally occurring and introduced biocontrol agents. Strains of *Pseudomonas fluorescens* have been used to manage soil-borne diseases and success has been achieved in the management of virus diseases also (Maurhofer *et al*., 1994; Raupach *et al.,* 1996). During previous investigation, as many as 50 rhizosphere isolates were screened against TMV in tomato (Kirankumar, 2007) and BYVMV in bhendi (Patil, 2010) and good control of the viral diseases observed. Most of these isolates showed induced systemic resistance against the viral disease, as evidenced through increased synthesis of phenols, phenylalanine ammonia lyase, peroxidase and chitinase.

Before start of the investigation, the disease under experimentation was confirmed as ToLCV through Transmission Electron Microscopy studies. The TEM examination of the partially purified preparation of ToLCV revealed the presence of isometric and pentagonal with single and paired Gemini virus particles, with a dimension of 20 × 30 nm to 24 × 30 nm, when negatively stained with 2 per cent Uranyl acetate pH 7.0. The particles exhibited the typical characteristic shape of geminivirus. Similar kind of observation was also made by Muniyappa *et al.* (1991), Abd El-Monem *et al*. (2011), Abdel-Salam (1991), Lazarwaitz (1992), Argüello-Astorga *et al*. (1994), El-Dougdoug *et al.* (1996), Harrison and Robinson (1999), Varma and Malathi (2003) and Ajlan *et al.* (2006).

The presence of ToLCV was further confirmed through sequencing studies. Early and accurate diagnosis of plant viruses is a key component of any crop disease management system. Since the biological methods of viral detection are too slow and not amenable to large scale application, molecular biology tools are being applied for rapid, specific and sensitive detection of viral pathogens (Miller and Martin, 1988). The increasingly popular use of PCR and RT-PCR for the diagnosis of plant viruses is appreciated due to highly sensitive and reliable methods for viral nucleic acid detection (Thomson and Dietzgen, 1995).

In order to rule out the possibility of amplification of off-targets, the PCR product of ToLCV were cloned in PTZ57R/T cloning vector and sequenced. The resulted sequence showed 98 per cent homology with Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment DNA A , confirming the ToLCV disease of tomato. These sequences have already been submitted in NCBI database (AY428770).

Hence, the present investigation was carried out to evaluate these potent rhizobacterial isolates alone and along with elicitor molecules such as chitosan, for their ability to control ToLCV. All these isolates were industrially formulated (lignite based formulation), bringing this approach closer to commercial implementation, and seed treated. The formulation was also applied as a foliar spray. Initial screening of these 50 isolates under pot cultures yielded ten efficient isolates controlling the disease completely at 30 DAI.

Various biochemical tests were employed for characterizing these isolates. Based on morphological and biochemical characteristics and by referring to the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994), the strains were tentatively identified up to generic level. It was interesting to note that out of ten, nine isolates belonged to the genus, *Pseudomonas* and the remaining one belonged to *Enterobacter.*

Out of nine isolates, three isolates (218(1), 207(1) and JK-33) were fluorescent isolates and the rest were non- fluorescent isolates. Many scientists have also reported the efficacy of fluorescent *Pseudomonas* in controlling wide spectrum of plant pathogens such as *Sclerotium rolfsii* (Bhatia *et al.,* 2005), *Alternaria helianthi* (Prasad *et al.,* 2003), *Pythium aphanidermatum* (Ramesh and Korikanthimath, 2003), *Rhizoctonia solani* (Nandakumar *et al.,* 2001a) and *Collecotrichum gleosporiodes* (Vivekananthan *et al*., 2004). PGPR belonging to non-fluorescent *Pseudomonas* antagonistic to a number of phytopathogens have also been reported (Rangeswaran and Prasad, 1998).

The biocontrol ability of ten isolates was evaluated under high virus - vector pressure conditions. All the isolates significantly reduced the ToLCV severity both in glass house and field conditions. Three isolates exhibited more than 70 per cent disease severity control. *Pseudomonas* 206(4) controlled the disease severity to the maximum extent of 85.72 per cent which was on par with the reference strain (*P. fluorescens* NCIM 2099).

Many reports on the potential of *Pseudomonas fluorescens* in controlling viral diseases are available in the literature. Maurhofer *et al.* (1994) reported the use of *P. fluorescens* in the management of the tobacco necrosis virus (TNV) in tobacco under glasshouse conditions. Raupach *et al.* (1996) demonstrated induced resistance by *P. fluorescens* against cucumber mosaic virus (CMV) in cucumber and tomato. Zhender *et al*. (1999) and Murphy *et al.* (2000) showed the biocontrol efficacy of *P. fluorescens* against CMV and tomato mottle virus respectively in tomato under field conditions. Kandan *et al.* (2003) also showed the biocontrol efficacy of *P. fluorescens* strains against tomato spotted wilt virus (TSWV) in tomato under field conditions. Kavino *et al* (2007) demonstrated the biocontrol potential of *P. fluorescens* strains Pf1 and CHAO against banana bunchy top virus. All these findings support the potential role of *Pseudomonas* isolates in managing viral diseases in crop plants.

Utilization of plant's defense mechanisms is of current interest in the management of plant diseases. Induced protection in plants against various pathogens, by biotic and abiotic inducers has been reported in many crops (Baker *et al*., 1997). The rhizobacteria are well known inducers of defense mechanisms and their application has often resulted in increased rates of plant growth and reduced disease incidence in many crops (Leeman *et al.,* 1995; Liu *et al.,* 1995; Nandakumar *et al*., 2001b; Viswanathan and Samiyappan, 2002).

Recent investigations on the mechanisms of biocontrol by *P. fluorescens* have revealed that several strains protect plants from various pathogens including viruses by activating defense molecules. All the ten promising rhizobacterial strains were tested for their ability to induce peroxidase, polyphenol oxidase, PALase, chitinase and production of phenol in tomato plants. It was interesting to note that all the strains triggered the defence related enzymes in plants at varied levels.

One of the major biological properties of phenolic compounds is their antimicrobial activity (Saini *et al*., 1988) and it is, often, assumed that their main role in plants is to act as protective compounds against disease causing agents such as fungi, bacteria and viruses. Phenolic compounds are known to enhance the mechanical strength of the host cell wall and also to inhibit the invading pathogens. Phenolic compounds are known to be formed in plants through phenylpropanoid metabolism and are associated with disease resistance (Vidhyasekaran *et al.*, 1997b).

In the present study, higher level of accumulation of phenolics was observed in both *Pseudomonas* 206(4) and B-15 strain treated tomato plants. These strains recorded 37.83 and 32.43 per cent higher phenol content respectively than the diseased control. The present findings are in agreement with Kandan *et al.* (2003), who also reported increased production of phenolic compounds in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus. Seed treatment with *P. fluorescens* 63 induced the accumulation of phenolics in tomato root tissue (M'piga *et al*., 1997). Kirankumar (2007) also reported that all the six PGPR strains tested resulted in increased phenol content in tomato leaves when compared to healthy control, thus protecting plants from TMV.

Tomato plants treated with *Pseudomonas* sp. 206(4) showed the highest induction of peroxidase (84.82% higher than the diseased control). Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989).

Table 40: Per cent increase in ISR molecules as influenced by rhizobacterial isolates at 45 DAS (greenhouse experiment no.2)

Sl. No.	Treatments	Per cent increase over uninoculated control (diseased control)				
		Phenol content	Peroxidase activity	PALase activity	Polyphenol oxidase activity	Chitinase activity
1	*Pseudomonas* JK-33 +ToLCV	18.90	26.20	17.39	7.01	29.01
2	*Pseudomonas* B-25 +ToLCV	24.32	21.37	18.63	10.52	20.20
3	*Pseudomonas* 207(1) +ToLCV	10.81	20.68	7.45	5.26	7.77
4	*Pseudomonas* 218 +ToLCV	13.50	11.03	18.01	24.56	13.47
5	*Pseudomonas* JK-5 +ToLCV	16.20	16.55	14.28	17.54	19.68
6	*Pseudomonas* 206(4) +ToLCV	37.83	84.82	63.97	34.21	41.45
7	*Pseudomonas* JK-16 +ToLCV	29.72	71.72	49.68	24.56	35.75
8	*Pseudomonas* 212(1) +ToLCV	29.72	66.20	14.28	14.91	8.29
9	*Pseudomonas* B-15 +ToLCV	32.43	77.93	58.38	33.33	32.12
10	*Enterobacter* E-21 +ToLCV	10.81	32.41	43.47	15.78	36.26
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	29.72	76.55	55.90	64.03	46.63
12	Healthy control (No rhizobacteria , no ToLCV)	_	_	_	_	_
13	Diseased control (only ToLCV)	_	_	_	_	_

Table 41: Per cent increase in plant growth parameters as influenced by rhizobacterial isolates in tomato at 45 DAS (greenhouse experiment no. 2)

Sl. No.	Treatments	Per cent increase over inoculated control		
		Plant height	Biomass	Chlorophyll content
1	*Pseudomonas* JK-33 +ToLCV	18.61	24.13	23.01
2	*Pseudomonas* B-25 +ToLCV	19.23	24.83	10.94
3	*Pseudomonas* 207(1) +ToLCV	24.37	21.61	20.01
4	*Pseudomonas* 218 +ToLCV	19.38	26.89	35.47
5	*Pseudomonas* JK-5 +ToLCV	27.06	25.24	22.64
6	*Pseudomonas* 206(4) +ToLCV	46.25	33.99	45.66
7	*Pseudomonas* JK-16 +ToLCV	41.26	27.55	42.64
8	*Pseudomonas* 212(1) +ToLCV	29.36	23.59	21.50
9	*Pseudomonas* B-15 +ToLCV	41.65	29.70	43.77
10	*Enterobacter* E-21 +ToLCV	17.85	26.56	41.50
11	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	40.76	31.43	43.01
12	Healthy control (No rhizobacteria , no ToLCV)	8.25	17.07	44.90
13	Diseased control (only ToLCV)	-	-	-

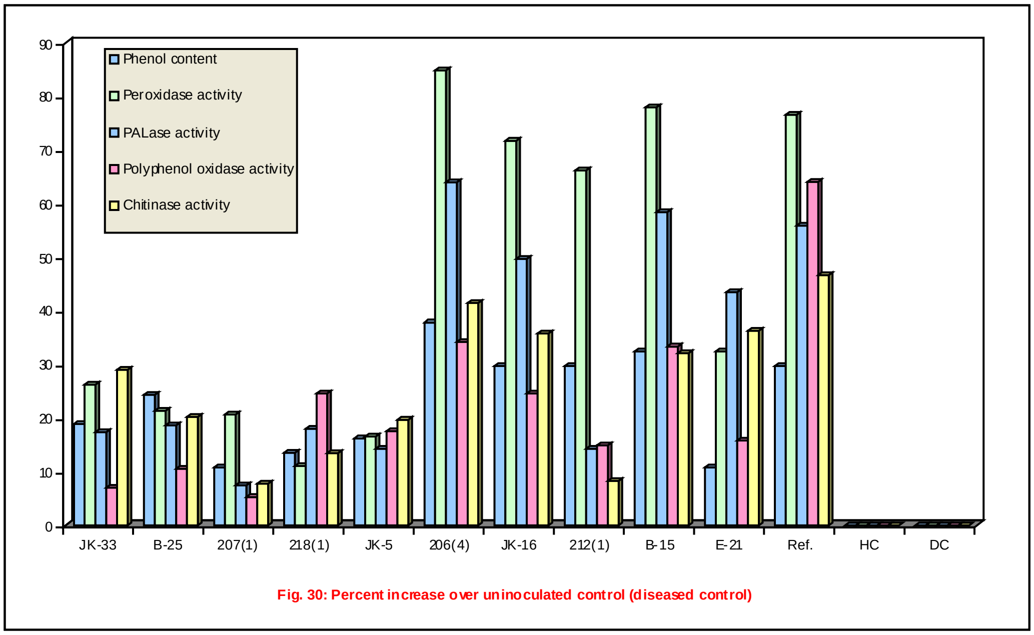


Fig. 30: Percent increase over uninoculated control (diseased control)

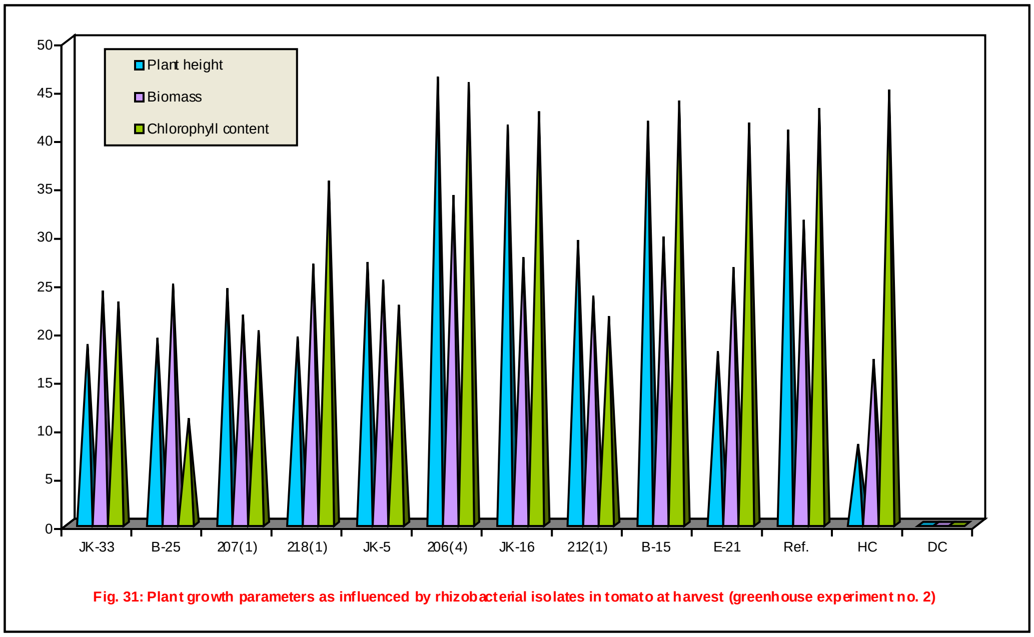


Fig. 31: Plant growth parameters as influenced by rhizobacterial isolates in tomato at harvest (greenhouse experiment no. 2)

Increased activity of peroxidases have been implicated in a number of physiological functions that may contributed to resistance including exudation of hydroxyl cinnamyl alcohol into free radical intermediates (Gross, 1980) and lignification (Walter, 1992). Peroxidase is also associated with deposition of phenolic compounds into plant cell walls during resistance interactions (Graham and Graham, 1991).

Inoculation of tomato plants with *Pseudomonas* sp.206(4) also resulted in the higher synthesis of polyphenol oxidase (34.21 per cent higher than the diseased control),whereas the reference strain showed 64.03 per cent higher PPO activity than the diseased control (Table 40, Fig. 30).The role of polyphenol oxidase (PPO) in disease resistance is to oxidize phenolic compounds to quinones,which are often more toxic to microorganisms than the original phenols and the enzyme itself is inhibitory to viruses by inactivating the RNA of the virus (Vidhyasekaran, 1988).Similar kind of induced higher PPO activity was noticed by Kandan *et al* (2005), when *P. fluorescens* strain CHAO was applied alone or in combination with other strains to tomato seeds for controlling tomato spotted wilt virus in tomato.

Inoculation of tomato plants with *Pseudomonas* sp. 206(4) resulted in the highest synthesis of phenylalanine ammonia lyase (63.97% higher than the diseased control). Phenylalanine ammonia lyase (PALase) plays an important role in the biosynthesis of various defence chemicals in phenyl propanoid metabolism (Daayf *et al.*, 1997). PALase activity was induced during plant-pathogen and plant-pest interactions (Ramanathan *et al.*, 2000; Radjacommare, 2002; Kandan *et al.*, 2002; Bhaarathi *et al.*, 2004 and Harish, 2005). Thus, higher induction of peroxidase and PALase and phenols might have reduced disease incidence and increased disease control in all the rhizobacteria treated plants.

Similar results were obtained by Kavino *et al.* (2003) who observed control of banana bunchy top virus (BBTV) due to induction of increased peroxidase, PALase and phenol content in banana plants when treated with *Pseudomonas fluorescens viz.,* Pf1 and CHAO alone as well as when amended with chitin. Rajinimala *et al.* (2003) also reported that *Pseudomonas chlororaphis* and *P. fluorescens* treated bittergourd plants, when challenge inoculated with bittergourd yellow mosaic virus (BGYMV), significantly increased peroxidase activity (140.37% more than the unioculated control) which led to reduction in disease incidence.

Another fascinating observation was the enhanced chitinase activity in rhizobacteria treated plants (7.77 - 46.63% higher than the diseased control). This increase in chitinase activity might have prevented the damage caused by viral pathogen and, thus, increased the per cent disease control in all the rhizobacteria treated plants. Synthesis and accumulation of PR proteins have been reported to play an important role in plant defence mechanisms. Chitinases, which are classified under PR-3 have been reported to associate with resistance in plants against pests and diseases (Maurhofer *et al.*, 1994 and Van Loon, 1998).

Chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infections, Chitosan was shown to inhibit the systemic propagation of viruses and viroids throughout the plant and to enhance the host's hypersensitive response to infection.They are reported to control a number of plant viruses like potato virus X, tobacco mosaic and necrosis virus, alfalfa mosaic virus, peanut stunt virus and cucumber mosaic virus (Pospieszny *et al.*, 1991., Chirkov., 2002).

Based on the initial screening, three bacterial isolates *viz.,* B-15, 206 (4) and JK-16 were selected for further characterization and biocontrol studies. Application of this rhizobacterial isolate mixture (206(4) + B-15+ JK-16) along with chitosan significantly reduced ToLCV severity, achieving a disease severity reduction of 87.5 per cent at 75 DAI to 100 per cent at 45 DAI in glasshouse condition. In field condition, this treatment showed 93.30 per cent and 84.60 per cent disease severity control in kharif and summer seasons respectively.Vasanthi *et al.* (2010) also observed that tomato leaf curl virus infected plants were significantly lower (25%) with less symptom severity and delayed symptom expression in *Pseudomonas* sp. VPT10 with chitin treated tomato plants as compared to non-bacterized control plants. Postma *et al.* (2009) observed control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan with a reduction in number of diseased plants by 50-100 per cent.

Murphy *et al.* (2003) observed significant protection against CMV in tomato when plants were treated with PGPR combination formulated with chitosan.Wisniewska-Wrona *et al* (2007), reported that the oligomers and partially degraded products of chitosan totally retarded (100 per cent) the lucerne mosaic virus (AlMV) and inhibited (32 per cent) the growth of resistant tobacco mosaic virus (TMV).

All the plants treated with the combination of rhizobacteria and chitosan showed elicitation of enzyme activities and phenolics compounds. A significant increase in phenol content, peroxidase, polyphenol oxidase, PALase and chitinase activity was observed in all the treatments when compared to healthy control and diseased control. Plants inoculated with rhizobacterial mixture + chitosan have registered the highest level of phenolics (74.35 per cent higher than in the disease control) and defense enzymes compared to other treatments. Inoculation of tomato plants with rhizobacterial mixture + Chitosan also resulted in the highest synthesis of phenylalanine ammonia lyase (70.58 per cent higher than the diseased control) (Table 42 and Fig 32). Phenylalanine ammonia lyase (PALase) activity has been observed to be induced during plant -pathogen and plant - pest interactions (Harish, 2005) and is known to play an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.,* 1997). Though all the treatments induced biosynthesis of peroxidase, the rhizobacterial mixture + chitosan showed the highest peroxidase activity which was 116.44 per cent more than the diseased control (Fig 32). Liana *et al* (2011) in an earlier study observed control of tobacco mosaic virus by *Bacillus* sp. through induced systemic resistance in tobacco as evidenced by increased levels of defense enzymes such as phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase and pathogenesis-related (PR) proteins in tobacco.

Another fascinating observation was the enhanced chitinase activity in rhizobacteria-treated plants (69.26 per cent higher than the diseased control). This increase in chitinase activity might have prevented the damage caused by viral pathogen and, thus, reduced the disease severity. Inoculation of tomato plants with rhizobacterial mixture + chitosan also resulted in the highest synthesis of polyphenol oxidase activity (122.60 per cent higher than the diseased control) (Fig 32). PPO has been shown to be inhibitory to viruses by inactivating the RNA of the virus (Vidhyasekaran, 1988). Nandeeshkumar *et al* (2008) observed enhanced activation of catalase, PALase, peroxidase, PPO and chitinase level in sunflower when seeds were treated with 5 per cent chitosan for controlling downy mildew. In field condition during summer season, this treatment (rhizobacterial mixture + chitosan) exhibited increased level of phenol, peroxidase, PALase, PPO and chitinase by 44, 97.29, 59.31, 63.39, 49.03 per cent respectively over disease control (Table 45, Fig.37). Ting *et al* (2007) found that combination of chitosan and *Cryptococcus laurentii* resulted in a synergistic inhibition of the blue mold rot caused by *Penicillium expansum* in apple. Liu *et al* (2007) found that chitosan treatment induced a significant increase in the activities of PPO, peroxidase and enhanced content of phenolic compounds which controlled the grey mold and blue mold in tomato. Chirkov *et al* (2001) reported callose, ribonuclease and β-1,3 glucanase induction in potato plants as defense response against potato virus X (PVX) when plants were sprayed with chitosan solution (1 mg/ml). Similar kind of inhibition was reported on tomato leaves when treated with chitosan and challenge inoculated with potato spindle tuber viroid (Pospiezny, 1997). Thus, it has been proved that PGPR treatment along with chitosan significantly inhibited virus accumulation and systemic propagation of virus inside the treated plants.

Though all the treatments tested in this investigation induced biosynthesis of defense molecules, rhizobacterial mixture + chitosan showed the highest induction of defense molecules in both glasshouse and field conditions.

The rhizobacterial inoculation resulted in significant reduction in the population of the vector. In the present study, *Pseudomonas sp.* 206(4) reduced the population by over 74.99 per cent when compared to the diseased control (Fig. 34). This is in conformity with the finding of Sikora and Murphy (2005) who also observed that rhizobacteria controlled cucumber mosaic virus in tomato by reducing the population of its vector, aphids by 80 per cent. The rhizobacterial mixture + chitosan reduced the vector population by over 66.79 per cent when compared to the diseased control (Fig. 35).

Table 42: Per cent increase in ISR molecules as influenced by rhizobacterial isolates and chitosan at 45 DAS (greenhouse experiment no.3)

Sl. No.	Treatments	Per cent increase over uninoculated control (healthy control)				
		Phenol content	Peroxidase activity	PALase activity	Polyphenol oxidase activity	Chitinase activity
1	*Pseudomonas* B-15 +ToLCV	17.94	52.63	35.29	26.08	22.94
2	*Pseudomonas* JK-16 +ToLCV	12.82	46.05	29.41	23.47	15.58
3	*Pseudomonas* 206(4) +ToLCV	20.51	73.68	41.17	41.73	27.70
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	30.76	89.47	52.94	62.60	32.03
5	*Pseudomonas* B-15+ Chitosan +ToLCV	41.02	85.52	52.94	81.73	57.14
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	33.33	63.81	47.05	76.52	54.11
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	48.71	93.42	58.82	107.82	60.17
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan + ToLCV	74.35	116.44	70.58	122.60	69.26
9	Chitosan +ToLCV	10.25	21.71	11.76	19.13	8.22
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	25.64	50.01	35.29	56.52	19.04
11	Chemical control +ToLCV					
12	Diseased control (only ToLCV)					

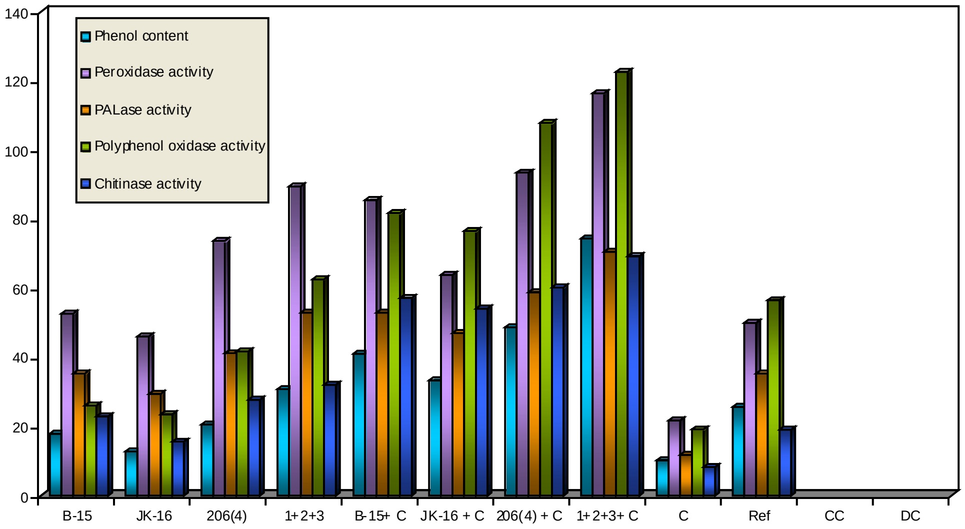


Fig. 32: Per cent increase in defense molecules as influenced by rhizobacterial isolates and chitosan at 45 DAS (greenhouse experiment no. 3)

Fig. 32: Per cent increase in defense molecules as influenced by rhizobacterial isolates and chitosan at 45 DAS (greenhouse experiment no. 3)

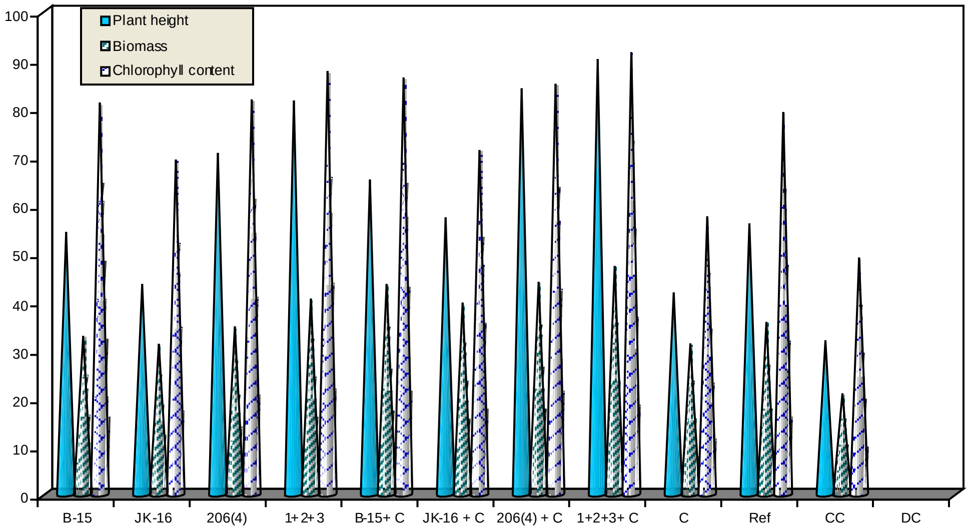


Fig. 33: Plant growth parameters as influenced by rhizobacterial isolates and chitosan in tomato at harvest (greenhouse experiment no. 3)

Fig. 33: Plant growth parameters as influenced by rhizobacterial isolates and chitosan in tomato at harvest (greenhouse experiment no. 3)

Table 43: Per cent increase in plant growth parameters as influenced by rhizobacterial isolates and chitosan in tomato at 45 DAS (greenhouse experiment no.3)

Sl. No.	Treatments	Per cent increase over inoculated control		
		Plant height	Biomass	Chlorophyll content
1	*Pseudomonas* B-15 +ToLCV	54.31	32.76	81.04
2	*Pseudomonas* JK-16 +ToLCV	43.53	31.15	69.28
3	*Pseudomonas* 206(4) +ToLCV	70.68	34.76	81.69
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	81.46	40.52	87.58
5	*Pseudomonas* B-15+ Chitosan +ToLCV	65.08	43.55	86.27
6	*Pseudomonas* JK-16 + Chitosan +ToLCV	57.32	39.64	71.24
7	*Pseudomonas* 206(4) + Chitosan +ToLCV	84.05	43.94	84.96
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	90.08	47.26	91.50
9	Chitosan +ToLCV	41.81	31.25	57.51
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	56.03	35.64	79.08
11	Chemical control +ToLCV	31.89	20.89	49.01
12	Diseased control (only ToLCV)	-	-	-

Hanafi *et al.* (2006) also observed that the inoculation of tomato plants with *Bacillus subtilis* BS strain induced resistance of plants to *Bemisia tabaci* under both saline and non-saline soils, which, in turn, led to reduced incidence of tomato leaf curl virus (ToLCV) disease. Similarly, Murphy *et al.* (2000) also observed, in a field trial, that whitefly density was significantly reduced in four of the PGPR treatments leading to reduced disease severity of tomato mottle virus in tomato.

Herman *et al.* (2008) noticed that the presence of plant growth-promoting rhizobacterium, *Bacillus* sp. exhibited substantial tolerance to green peach aphid infestations in bell pepper production. In field trials, 47.7–56.1 per cent reduction in leafolder incidence was noticed in the *Pseudomonas fluorescens* treatment containing chitin, and its efficacy was equivalent to the standard fungicide and insecticide treatments (Commare *et al.,* 2002). Sudhakara *et al*. (2011) observed that *Bacillus subtilis* BS3A25 formulation proved highly effective in reducing the aphid population and CMV incidence in tomato as compared to a commercially available insecticide. Senthilraja (2010) found chitin-based bioformulation of *Beauveria bassiana* and mixture of *Pseudomonas fluorescens* B2 +TDK1 + Pf1 (amended with or without chitin) applied through seed, soil and foliar spray effectively reduced the incidence of leafminer and collar rot in groundnut compared to individual bioformulation and control treatments both under glasshouse and field conditions.

The leaves of rhizobacteria-treated and untreated tomato plants, were tested for the presence of the virus through semi quantitative PCR assay. In general, a slight reduction in the viral inoculum load was observed in all the treated plants. At both 45 DAS and 75 DAS, the reference strain, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed the least viral inoculum.

In case of combined application of PGPR and chitosan, almost all the treatments exhibited a significant amount of viral load after 10th cycle of the PCR, except rhizobacterial mixture + chitosan*, Pseudomonas* sp. 206(4) and chemical control treatment which showed the least viral titre which was not detected after 10th cycle of the PCR at 75 DAS.The diseased control plant exhibited highest amount of viral load after 10th cycle of the PCR.The rhizobacterial mixture + Chitosan treatment showed the least viral inoculum both in glass house and field conditions.

Zehnder *et al.* (2000) followed ELISA method to detect the viral load (cucumber mosaic virus) in PGPR treated cucumber plants. Even in field trials, they observed significantly lower ELISA values in all PGPR treatments than in the disease control, with a concomitant decrease in disease severity. Detection of viral DNA (tomato mottle virus) using Southern dot blot analysis was correlated with disease symptoms severity rating as well as tomato fruit yields (Murphy *et al.,* 2000). Malathi *et al.* (2011) also detected Rice tungro virus particles from infected rice leaves by RT- PCR based diagnosis. Kavino *et al.* (2007) also reported, plants treated with mixture of bacterial strains (*P. fluorescens* Pf1, *P.fluorescens* CHAO and *B.subtilis* EPB22) exhibited very low titre value of banana bunchy top virus in banana as compared to uninoculated control by ELISA. Kandan *et al* (2005) , reported minimum titre value of tomato spotted wilt virus (TSWV) in tomato by ELISA , both in glass house and field conditions when plants were treated with *P. fluorescens* either alone or in mixture.

Murphy *et al* (2003) used the PGPR combinations formulated with chitosan and used this biopreparation against control of CMV in tomato. He found, in each biopreparation treatment, the level of CMV accumulation were significantly lower as compared to control plant which was detected by ELISA.

Since the selection of biocontrol agents against any pathogen must be based on their antagonistic properties as well as the plant growth promoting abilities, the PGPR strains were evaluated for their ability to promote plant growth.

In addition to suppressing the viral disease, all the rhizobacterial isolates promoted growth and biomass of tomato. In general, challenge inoculation with the viral pathogen significantly decreased growth and fruit yield of tomato. There was significantly improved growth and fruit yield in all the plants treated with rhizobacterial isolates at all intervals of time. This may be due to higher production of phytohormones by the rhizobacterial isolates.

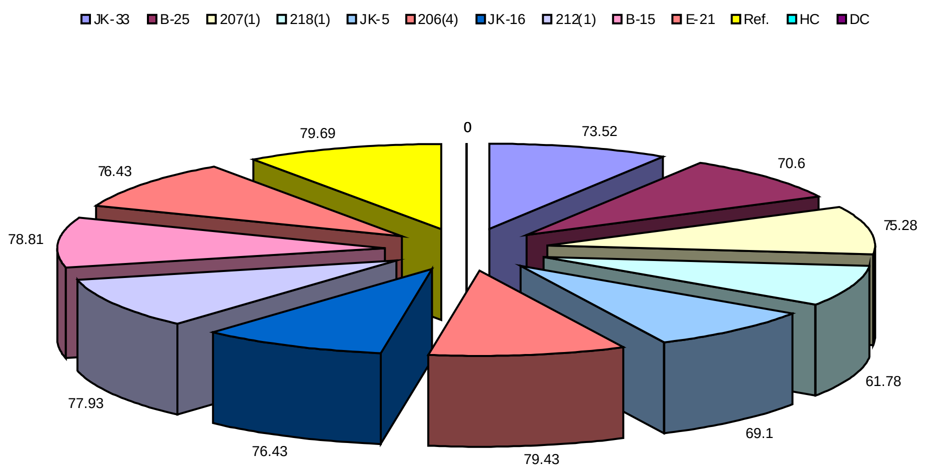


Fig. 34: Effect of inoculation of rhizobacteria on the population of *Bemisia tabaci* on tomato plants (greenhouse experiment no. 2)

Fig. 34: Effect of inoculation of rhizobacteria on the population of Bemisia tabaci on tomato plants (greenhouse experiment no. 2)

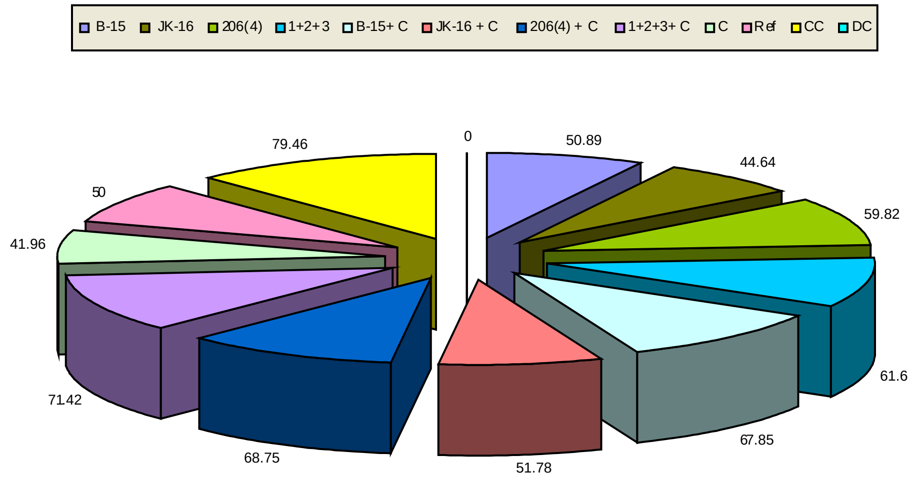


Fig. 35: Effect of inoculation of rhizobacteria and chitosan on the population of *Bemisia tabaci* on tomato plants (greenhouse experiment no. 3)

Fig. 35: Effect of inoculation of rhizobacteria and chitosan on the population of Bemisia tabaci on tomato plants (greenhouse experiment no. 3)

Some of these strains had also stimulated growth and yield of tomato in earlier investigations (Earnapalli, 2005; Kirankumar, 2007).

Pseudomonas 206(4) significantly improved growth components of tomato, when compared to the remaining rhizobacterial isolates. It increased the plant height by 46.25 percent, biomass by 33.99 percent compared to the viral pathogen inoculated control (Table 41 and Fig. 31). *P. fluorescens* strains have been reported to increase plant growth by producing plant growth regulators like gibberellins, cytokinins and indole acid, which can either directly or indirectly promote plant growth and development. However, enhanced growth promotion depends on the strain, method of application and concentration of the inoculum used (Dunne *et al*., 1998; Nandakumar *et al.,* 2001a; Radjacommare *et al.,* 2002; Ramamoorthy *et al*., 2001).

Increased growth, biomass and yield as a result of bacterization with rhizobacterial strains have been previously reported (Alstrom, 1987; Dileepkumar and Dubey, 1991, Yeole and Dube, 1997, Jagadish, 2006 and KiranKumar, 2007) and have been implicated to be due to an increase in the production of plant growth substances (Gaskins *et al.,* 1985; Sivaprasad *et al.,* 2003 and Earnapalli, 2005).

In general, there was an increase in the total chlorophyll content up to 45 DAS and declined at harvest. This may be due to partitioning of photosynthesis towards reproductive parts (sink) from sources like stem and leaves.

Among all the treatments, *Pseudomonas* 206(4)) inoculated plant leaves recorded the highest chlorophyll content. Its inoculation increased chlorophyll content in tomato leaves by over 45.66 percent when compared to the diseased control (Table 41 and Fig. 31). The remaining rhizobacterial isolates also showed increase in chlorophyll content.The increased chlorophyll content must have been as a result of enhanced stomatal conductance, photosynthesis and transpiration (Levi and Krikun, 1980). Enhanced chlorophyll content in tomato leaves when inoculated with PGPR strains in presence of TMV has also been reported by Kirankumar (2007).

Success of PGPR as microbial inoculants for agricultural crops depends on their effective formulation to deliver a critical number of viable microorganisms consistently (Paau, 1988). Ideal formulations are expected to have an extended shelf-life, be feasible for large-scale production and convenient for field application. Seed treatment with PGPR formulations usually delivers a high number of viable microbes and allows application of the inoculants directly in the root zone. Apart from microbial inoculants, application of biopolymers like chitin or chitosan to farming soils have also been found to increase crop yields (Li *et al*., 1997). Chitin and its derivatives are biologically degradable and, therefore, could be the supplements of choice in PGPR inocula.

In addition to suppressing the viral disease, rhizobacterial isolates + chitosan greatly improved plant growth, chlorophyll content, biomass, fruit yield with extended shelf life of fruits (Table 44 and Fig. 36). This may be due to higher production of phytohormones by the rhizobacteria. *Pseudomonas* sp. B-40 had stimulated growth and yield of tomato in earlier investigations (Earnapalli 2005; Kirankumar, 2007). Inoculation of rhizobacterial mixture + Chitosan increased the plant height by 90.08 percent, biomass by 47.26 percent and chlorophyll content by over 91.50 percent compared to the virus inoculated control under glasshose condition (Table 43 and Fig. 33). In field condition, this treatment exhibited increased plant height by 68.09 percent, biomass by 95.58 percent, chlorophyll by 34.08 percent and fruit yield by 287.99 percent as compared to the viral pathogen inoculated control in kharif season (Table44, Fig. 36) whereas, in summer season there was 44.51, 109.58, 45.48 and 226.12 per cent increase in plant height, biomass, chlorophyll and fruit yield respectively (Table 46 and Fig. 38). At very high vector virus pressure condition in summer season, there was 30.57, 102.82, 40.49 and 256.89 per cent increase in plant height, biomass, chlorophyll and fruit yield respectively (Table 48 and Fig.40). The shelf life of fruits also got extended due to rhizobacterial and chitosan treatment.The extended shelf life was 89.06 per cent and 68.62 per cent in kharif and summer season respectively.

Chitin supplemented peat formulation of *Bacillus subtilis* AF1 increased the emergence and dry weight of pigeon pea seedlings by 29 and 33 per cent, in comparision to an increase of 21 and 30 per cent, respectively by *Bacillus subtilis* AF1alone in field condition both in kharif and rabi season (Manjula and Podile , 2005).

Table 44: Per cent increase in plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during *kharif* 2011

Sl. No.	Treatments	Per cent increase over inoculated control				
		Plant height	biomass	Fruit yield	Chlorophyll content	Shelf life
1	*Pseudomonas* 206(4) +ToLCV	58.89	67.25	194.32	30.19	65.62
2	*Pseudomonas* B-15 +ToLCV	49.69	51.78	174.45	26.09	59.37
3	*Pseudomonas* JK-16 +ToLCV	44.17	48.08	134.49	25.5	53.12
4	*Pseudomonas* (206(4) +B-15+JK-16) +ToLCV	60.73	82.63	230.34	33.2	84.37
5	*Pseudomonas* 206(4) + Chitosan +ToLCV	63.19	83.24	250.01	31.42	75.01
6	*Pseudomonas* B-15+ Chitosan +ToLCV	55.21	71.52	220.52	29.05	65.62
7	*Pseudomonas* JK-16 + Chitosan +ToLCV	52.76	64.12	189.30	28.46	56.25
8	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	68.09	95.58	287.99	34.08	89.06
9	Chitosan +ToLCV	42.94	44.37	106.98	21.95	50.01
10	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	48.46	54.86	189.95	28.76	59.37
11	Chemical control +ToLCV	25.15	20.93	111.79	17.51	15.62
12	Diseased control (only ToLCV)					

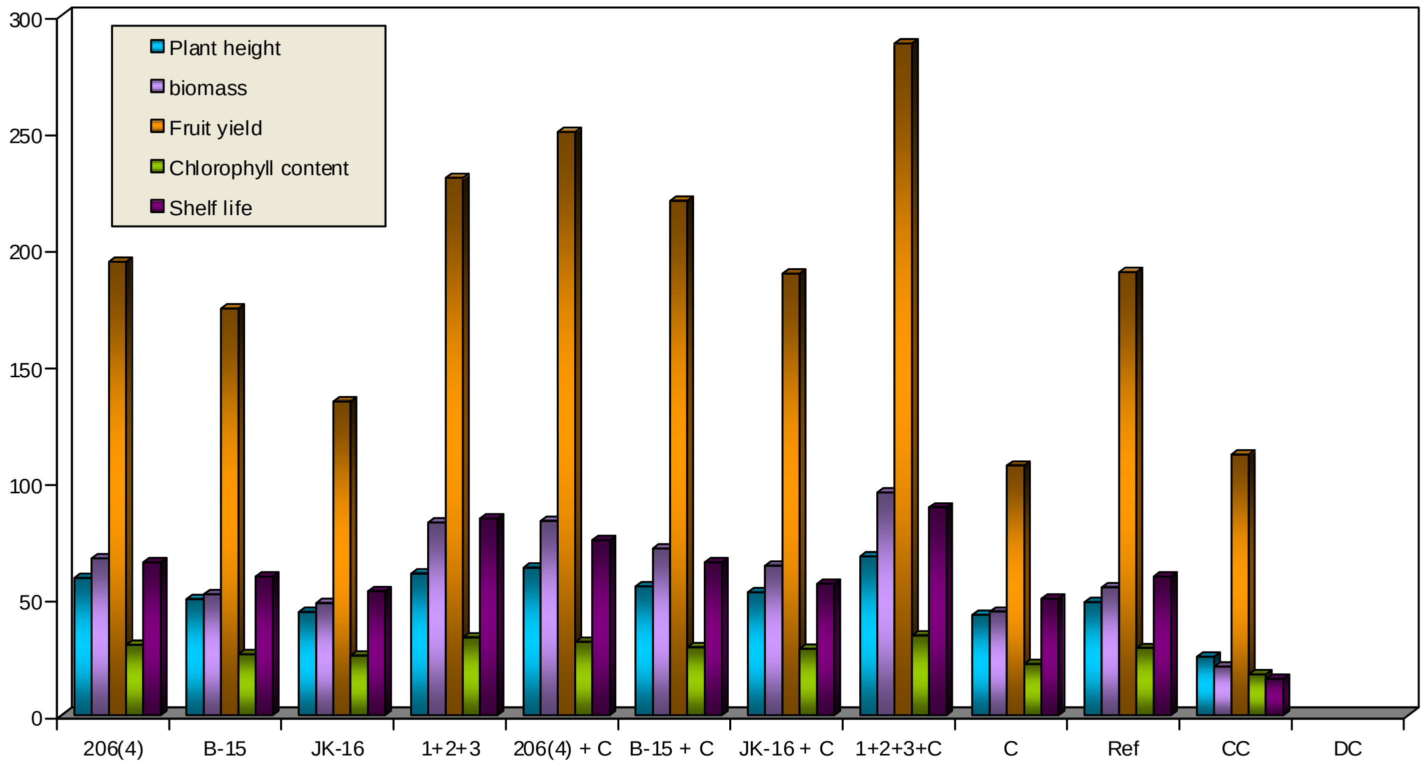


Fig. 36: Plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during kharif 2011

Fig. 36: Plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during kharif 2011

For achieving success in plant growth promotion and crop protection, there must be effective root colonization by the introduced microorganisms. Here, an attempt was made to visualize the extent of root colonization by the inoculated rhizobacteria through Scanning Electron Microscopy. SEM pictures revealed that tomato roots inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan showed locally very dense population of bacteria. Here, the cells were encapsulated within a massive network of root derived material. Roots inoculated with *Pseudomonas* sp. 206(4) but without chitosan contained lesser bacterial cells. The synergistic effect of chitosan when applied together with the bacterial inoculant is intriguing. Here, the chitosan has stimulated the population of *Pseudomonas* sp.

The results of present investigation showed that chitosan supplementation resulted in an increase in the antagonists on tomato roots. The fact that chitosan is a rich N-source as well as a C-source may be significant here. The increased number of antagonists on the roots are probably explained by the role of chitosan as a nutrient source. These findings are completely supported by Postma *et al*, (2009) who observed substantially higher number of bacterial cells (*Lysobacter enzymogenes* 3.1T8) in cucumber roots by scanning electron microscopy which enhanced the biocontrol efficacy of *L. enzymogenes* 3.1T8 against crown rot in cucumber.

The cellular structures of the healthy, treated (*Pseudomonas* sp. 206(4) + chitosan) and diseasd plants were studied by transmission electron microscopy (TEM) which showed the subcellular changes which occurred inside the cells due to ToLCV and rhizobacterial inoculation. In case of diseased cells, there was complete loss of subcellular architecture. Cells got completely shrunken with moon shaped chloroplasts whereas at some part of the cells, there was complete loss of chloroplasts observed. But, in case of treated leaves, cells appeared somewhat close to normal cells. Some extent of distortion was seen in chloroplasts whereas mitochondria, endoplasmic reticulum and other organells appeared to be normal. Healthy plant cells appered normal with intact histological structure.

The leaves of diseased control plants recorded the least chlorophyll content due to the breakdown of chlorophyll pigments by the pathogen as clearly shown by the study. Manners and Scott (1985) also observed that one of the earliest alternations in leaf tissue due to pathogenic infection was the selective breakdown of chloroplast polysomes. The reduced chlorophyll content in diseased control treatment could also be attributed to the inhibition of chloroplast development by the pathogen as reported by Robert and Wood (1982), Kirankumar (2007) and Patil (2010) also observed reduced chlorophyll content in the leaves of tomato plants challenge inoculated with TMV and bhendi plants inoculated with BYVMV.

Histopathological studies were conducted to study the changes occuring due to ToLCV infection as well as to study to what extent these changes are reduced by inoculation with the biocontrol agent. The histopathological studies revealed more anatomical destruction in leaves of diseased plants as compared to treated and healthy plants. There were more intercellular spaces. The palisade cells were markedly reduced in size, highly distorted and lost their columnar compact nature. However, the treatments receiving *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp. B-15 + Chitosan retained almost the same histological structure as exhibited by healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight distortion with reduction in their compact nature. The reduction in size of leaf tissues and their destruction may be due to metabolic changes in tissues causing hypotrophy in palisade and spongy parenchyma cells. Similar observations were made by Singh (1971) in infected papaya leaves, Mishra and Singh (1973) in CMV affected chilli leaves,Ehara Mishawa (1975) in CMV infected tobacco, Alok *et al.* (1986) in tomato infected by TMV and Singh and Rathi (1996a) in pigeonpea infected by sterlity mosaic virus.

The studies also revealed reduction in insoluble polysaccharides in palisade and spongy parenchyma cells of diseased plants as compared to healthy plants, which showed rich concentration of polysaccharides in the cells. The treatments receiving *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp. B-15+Chitosan showed almost the same concentration of polysaccharides as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight reduction in concentration of polysaccharides in palisade and spongy parenchyma cells. The reduction in polysaccharides may be due to varied metabolism in infected tissues.

Table 45: Per cent increase in ISR molecules as influenced by rhizobacterial isolates and chitosan at 45 DAS in field conditions during summer 2012

Sl. No.	Treatments	Per cent increase over uninoculated control (healthy control)				
		Phenol content	Peroxidase activity	PALase activity	Polyphenol oxidase activity	Chitinase activity
1	*Pseudomonas* B-15+ Chitosan +ToLCV	20	34.23	28.96	27.67	18.26
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	24	50.45	44.82	46.42	22.11
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	44	97.29	59.31	63.39	49.03
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	12	23.42	22.75	14.28	15.86
5	Diseased control (only ToLCV)	-	-	-	-	-

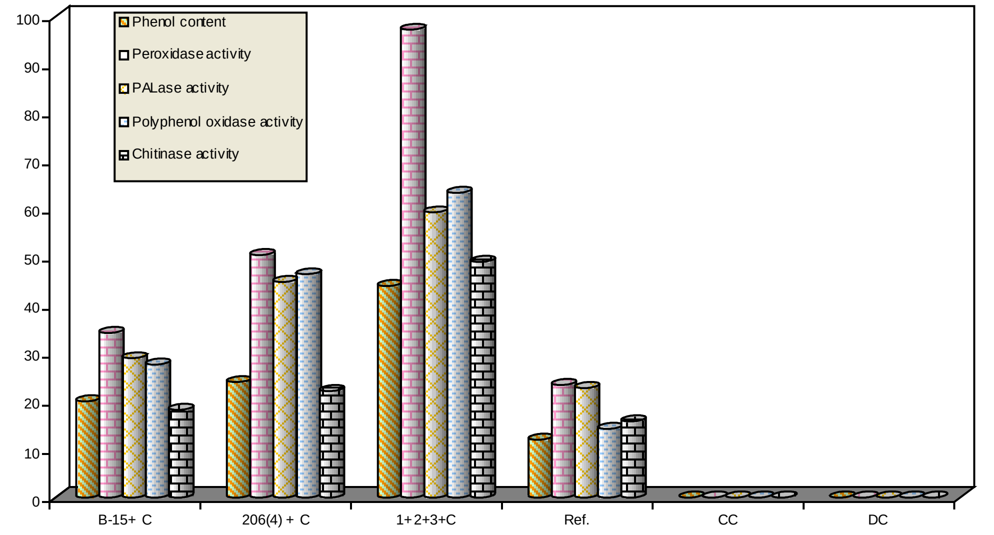


Fig. 37: Per cent increase in ISR molecules as influenced by selected rhizobacterial isolates and chitosan at 45 DAS in field conditions during summer 2012

Fig. 37: Per cent increase in ISR molecules as influenced by selected rhizobacterial isolates and chitosan at 45 DAS in field conditions during summer 2012

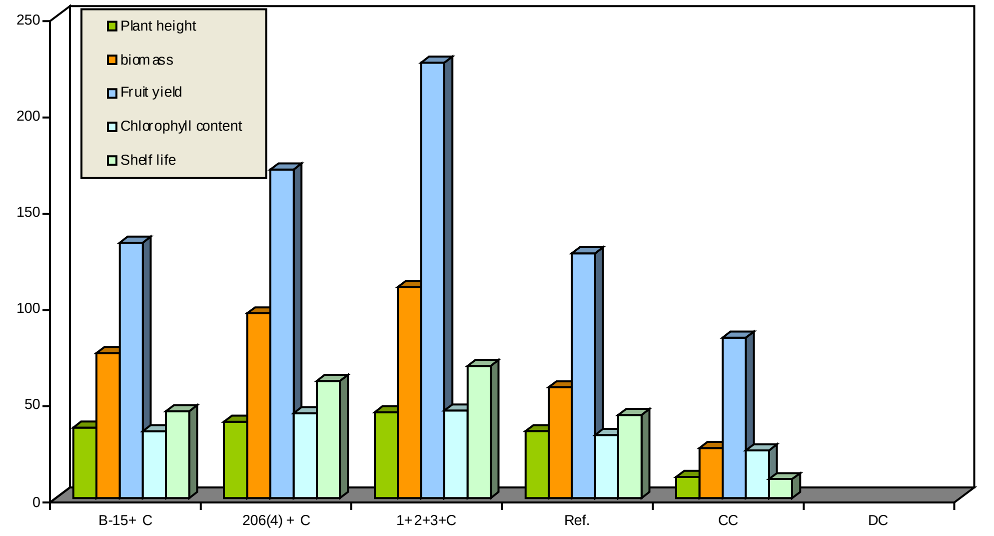


Fig. 38: Plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during summer 2012

Fig. 38: Plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during summer 2012

Table 46: Per cent increase in plant growth, biomass and fruit yield as influenced by rhizobacterial isolates and chitosan in tomato at harvest in field conditions during summer 2012

Sl. No.	Treatments	Per cent increase over inoculated control				
		Plant height	biomass	Fruit yield	Chlorophyll content	Shelf life
1	*Pseudomonas* B-15+ Chitosan +ToLCV	36.58	75.34	132.58	34.69	45.09
2	*Pseudomonas* 206(4) + Chitosan +ToLCV	39.63	95.89	170.64	44.02	60.78
3	*Pseudomonas* (206(4) +B-15+JK-16) + Chitosan +ToLCV	44.51	109.58	226.12	45.48	68.62
4	Reference strain (*P. fluorescens* NCIM 2099) +ToLCV	34.75	57.53	127.09	32.65	43.13
5	Chemical control +ToLCV	10.97	26.02	83.22	24.78	9.80
6	Diseased control (only ToLCV)	-	-	-	-	-

Table 47: Per cent increase in ISR molecules as influenced by best rhizobacterial isolate and chitosan at 45 DAS

Sl. No.	Treatments	Per cent increase over uninoculated control (healthy control)				
		Phenol content	Peroxidase activity	PALase activity	Polyphenol oxidase activity	Chitinase activity
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	13.04	19.84	12.19	34.31	35.20
2	Diseased control (only ToLCV)	-	-	-	-	-

Table 48: Per cent increase in plant growth, biomass and fruit yield as influenced by best rhizobacterial isolate and chitosan in tomato at harvest

Sl. No.	Treatments	Per cent increase over inoculated control				
		Plant height	biomass	Fruit yield	Chlorophyll content	Shelf life
1	*Pseudomonas* 206(4) + Chitosan +ToLCV	30.57	108.82	256.89	40.49	22.72
2	Chemical control +ToLCV	10.82	50.98	58.62	28.22	6.81
3	Diseased control (only ToLCV)	-	-	-	-	-

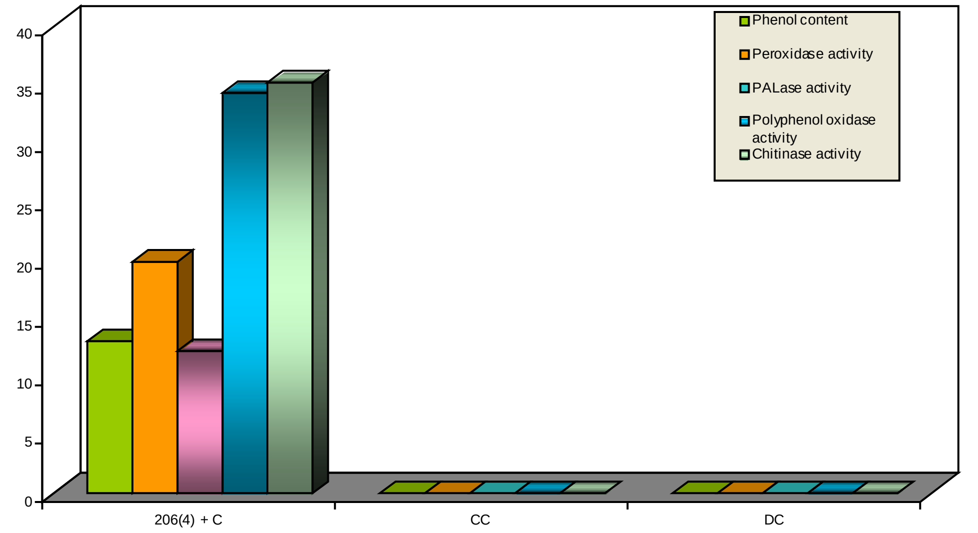


Fig. 39: Per cent increase in ISR molecules as influenced by selected rhizobacterial isolates and chitosan at 45 DAS in field conditions during summer 2012

Fig. 39: Per cent increase in ISR molecules as influenced by selected rhizobacterial isolates and chitosan at 45 DAS in field conditions during summer 2012

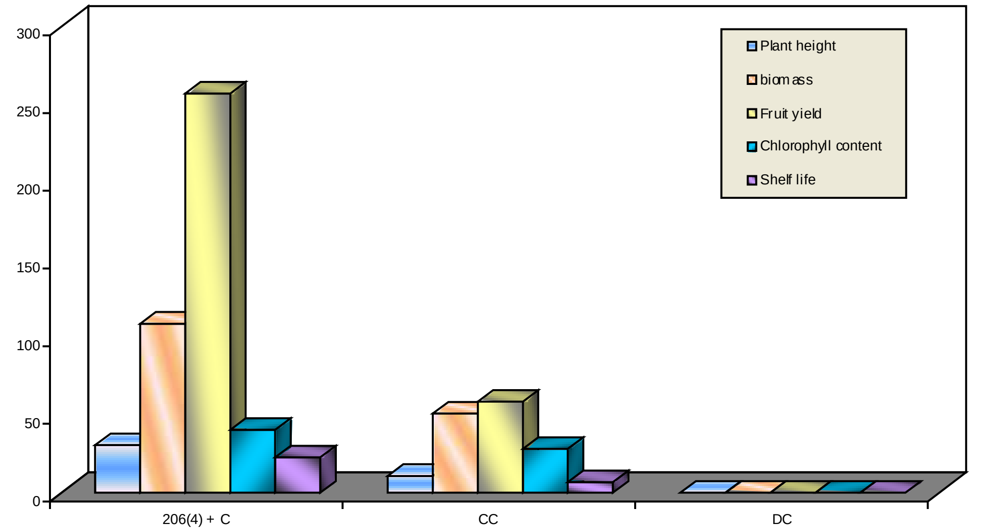


Fig. 40: Plant growth, biomass and fruit yield as influenced by best rhizobacterial isolate and chitosan in tomato at harvest in field conditions

Fig. 40: Plant growth, biomass and fruit yield as influenced by best rhizobacterial isolate and chitosan in tomato at harvest in field conditions

Several workers have reported significant decrease in total sugars, reducing sugars and non-reducing sugars in diseased leaves (Khurana, 1970; Singh, 1973 and Johri, 1975).

Nucleic acid concentration was more in palisade and spongy cells of diseased leaves as compared to healthy leaves. The manifestation of increase in nucleic acid content may due to combined effect of host and viral nucleic acid. Usually, concentration of virus in infected cells increases rapidly upon infection, thus, increasing their nucleic acid concentration in such cells. The treatments receiving *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp. B-15+ Chitosan showed almost similar nucleic acid content as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight increase in nucleic acid content in palisade and spongy parenchyma cells. Similar results have been obtained by Joshi and Dubey (1974) for nucleic acids in CMV infected chilli leaves, Russo and Martell (1975) in tobacco infected nuclei, Channarayappa *et al.* (1992) for nucleic acids and chloroplasts of tomato cells by ToLCV, Singh and Rathi (1996b) on the presence of foreign ribonucleo protein in cytoplasm and nucleus of virus infected pigeonpea and Johri (1975) who reported increase in total nucleic acid in virus infected host tissues of papaya.

There was a reduction in protein content in palisade and spongy parenchyma cells of infected plants compared to healthy plants. It may be due to degradation of host protein or reduction in synthesis of protein subsequent to viral infection since virus uses host cell contents for replication. The plants treated with *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp. B-15+ Chitosan showed almost the same concentration of proteins as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight reduction in concentration of proteins in palisade and spongy parenchyma cells. This is in accordance with the findings of Singh *et al*. (1977) and Sun (1985) who reported reduction of protein content by 41 per cent in diseased papaya fruits.

In general, histochemical analysis revealed increase in nucleic acid and decrease in insoluble polysaccharides and also protein content due to ToLCV infection.

Thus, the present study has come out with the growth promoting *Pseudomonas* 206(4), as the most effective biocontrol agent in reducing the disease severity of ToLCV in tomato and the beneficial effects of the rhizobacterial mixture could be further augmented when combined with chitosan. The higher biocontrol of the ToLCV disease could be due to following attributes:

a. Induction of systemic resistance by triggering various defense molecules.

b. Bringing about the least histopathological changes in polysaccharides, proteins and nucleic acid.

c. Significant reduction in the virus titre in treated leaves as evidenced by Semi-quantitative PCR studies.

Thus, this combined treatment would be of significance as a component of integrated disease management of ToLCV in tomato.

6. SUMMARY AND CONCLUSIONS

In the present study, an attempt was made to evaluate 50 rhizobacterial isolates against ToLCV disease on tomato to select the promising ones and elucidate their mechanisms of biocontrol.The promising isolates were further tested in combination with chitosan to study the biocontrol efficacy both under pot and field conditions. The ability of the promising isolates to promote plant growth with and without chitosan was also assessed. The salient features of the finding are outlined below.

1. As many as 50 rhizobacterial isolates obtained from the culture collection of the Departments of Agricultural Microbiology and Biotechnology, UAS, Dharwad were screened *in vivo* for the control of ToLCV in tomato under glasshouse conditions. The results have clearly indicated that out of 50 isolates, 11 isolates controlled the viral disease severity completely. While, nineteen strains controlled the disease severity moderately to an extent of 50 per cent. Out of 50, ten promising isolates were selected for studying their plant growth promotion and induction of systemic resistance (ISR) activity. The rhizobacterial isolates selected were B-15, B-25, 212 (1), 206(4), 207 (1), JK-33, JK-5, JK-16, E-21 and 218 (1).
2. Based on the morphological and biochemical traits, the isolates were tentatively identified. Out of ten selected isolates, three isolates (218(1), JK-33 and 207(1)) belonged to fluorescent *Pseudomonas*, six (B-15, B-25, 212 (1), 206(4), JK-5, JK-16) belonged to non-fluorescent *Pseudomonas* and one (E-21) was identified as *Enterobacter* sp.
3. *Pseudomonas* 206(4) controlled ToLCV disease severity to the maximum extent of 57.15 per cent followed by *Pseudomonas* B-15 and *Pseudomonas* JK-16 which controlled the disease by 42.86 per cent.
4. The ISR activity of the rhizobacterial isolates was assessed by estimating defense molecules such as phenols, peroxidase, polyphenol oxidase, chitinase and PALase enzymes. The results showed that all the isolates controlled the disease by inducing systemic resistance in tomato plants.
5. All the ten rhizobacterial isolates increased phenol content in tomato leaves when compared to the diseased control. *Pseudomonas* 206(4) showed the maximum phenol content (37.80 per cent higher than the diseased control).
6. All the isolates triggered biosynthesis of peroxidase.The Plants which received *Pseudomonas* 206(4) exhibited the highest peroxidase activity which accounts to 84.82 per cent higher than the diseased control.
7. There was induction in biosynthesis of polyphenol oxidase in all the isolates. Again, the plants inoculated with *Pseudomonas* 206(4) exhibited the highest polyphenol oxidase activity which accounts to 34.21 per cent higher than the diseased control.
8. All the rhizobacterial isolates enhanced the accumulation of PALase in tomato leaves. The plants inoculated with *Pseudomonas* 206(4) exhibited maximum PALase activity (63.97 per cent higher than the diseased control).
9. All the rhizobacterial isolates showed the increased activity of chitinase also in tomato leaves. The plants inoculated with *Pseudomonas* 206(4) exhibited maximum chitinase activity (41.45 per cent higher than the diseased control).
10. All the rhizobacterial isolates resulted in significant reduction in the whitefly population. *Pseudomonas* 206(4) showed the highest reduction in the insect population (74.99 per cent less than that of the diseased control).
11. All the isolates were tested for the presence of the virus through semi quantitative PCR assay. Inoculation of *Pseudomonas* 206(4) resulted in the least virus concentration.
12. The presence of ToLCV in affected leaves was confirmed by PCR amplification using ToLCV specific coat protein primers. In order to further confirm the diagnosis results, the amplified products were eluted and cloned in PTZ57R/T cloning vector, transformed in *E.coli* (DH5α) and sequenced. The sequencing results gave the 98 per cent similarity with Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment DNA A.

13. Out of the ten promising isolates, *Pseudomonas* 206(4) emerged as the best organism in plant growth promotion. It increased the plant height by 46.53 per cent and biomass by 33.99 per cent, when compared to the pathogen inoculated control.
14. The influence of rhizobacterial isolates on chlorophyll content of tomato was also assessed. The results indicated that *Pseudomonas* 206(4) increased chlorophyll content in tomato leaves by over 45.66 per cent at 45 DAS when compared to the diseased control.
15. Based on biocontrol potential and plant growth promotion, three efficient isolates 206(4), B-15 and JK-16 out of ten were further evaluated in combination with and without chitosan both under glasshouse and field condition.
16. The treatment receiving 206(4)+ Chitosan reduced the disease severity by 75 percent wheras the treatment receiving rhizobacterial mixture + Chitosan exhibited 87.50 per cent ToLCV severity control under glasshouse condition.
17. The best treatment was further evaluated under field condition in two consecutive seasons and consistent results obtained. The rhizobacterial mixture + Chitosan showed 93.34 per cent severity control under kharif season and 84.60 per cent severity reduction under summer season.
18. The ISR activity of the rhizobacterial isolates in combination with chitosan was assessed by estimating defense molecules such as phenols, peroxidase, polyphenol oxidase, chitinase and PALase enzymes. The results showed that the combination of chitosan and *Pseudomonas* sp. resulted in synergistic effect on ISR activity.
19. All the rhizobacteria+chitosan treated isolates increased phenol content in tomato leaves when compared to the diseased control. Rhizobacterial mixture (206(4)+B-15+JK-16) + Chitosan showed the maximum phenol content (65.78 per cent higher than the diseased control) in glasshouse condition and 44.00 per cent higher than the diseased control under field condition.
20. All the combined treatments induced biosynthesis of peroxidase. The plants which received rhizobacterial mixture + Chitosan exhibited the highest peroxidase activity which accounts to 110.59 per cent higher than the diseased control in glass house condition and 63.34 per cent higher than the diseased control in field condition
21. There was induction in biosynthesis of polyphenol oxidase in all the rhizobacteria+chitosan treated plants. Plants which received rhizobacterial mixture + Chitosan exhibited the highest polyphenol oxidase activity which accounts to 95.65 per cent higher than the diseased control in glasshouse condition and 63.39 per cent higher than the diseased control in field condition
22. All the combined treatments enhanced the accumulation of PALase in tomato leaves. The plants inoculated with rhizobacterial mixture + Chitosan exhibited maximum PALase activity (71.76 per cent higher than the diseased control) in glass house condition and 59.31 per cent higher than the diseased control in field condition.
23. All the rhizobacterial isolates with chitosan showed the increased activity of chitinase in tomato leaves. The plants inoculated with rhizobacterial mixture + Chitosan exhibited maximum chitinase activity (69.26 per cent higher than the diseased control) in glass house condition and 49.03 per cent higher than the diseased control in field condition.
24. All the rhizobacterial isolates + Chitosan, resulted in significant reduction in the whitefly population. The rhizobacterial mixture + Chitosan showed the highest reduction in the insect population (74.41 per cent less than that of the diseased control at 75 DAI).
25. All the isolates were tested for the presence of the virus through semi quantitative PCR assay. The rhizobacterial mixture + Chitosan showed the least virus concentration both in glass house and field condition.
26. Out of the twelve treatments, rhizobacterial mixture + Chitosan emerged as the best treatment in plant growth promotion also. It increased the plant height by 86.42 per cent and biomass by 41.27 per cent and chlorophyll content in tomato leaves by 91.50 per cent at 45 DAS when compared pathogen inoculated control in glasshouse condition.

A drastic reduction in chlorophyll content was observed in diseased control plants.These treatments were further evaluated in field condition in two consecutive seasons.

27. Rhizobacterial mixture + Chitosan treatment increased the plant height by 68.09 per cent and biomass by 89.86 per cent and chlorophyll content in tomato leaves by 34.02 per cent at 45 DAS when compared to diseased control in kharif season.
28. In summer season also this treatment exhibited increase in plant height by 45.73 per cent , biomass by 109.58 per cent and chlorophyll content by 45.48 per cent at 45 DAS when compared to diseased control.
29. The influence of rhizobacterial isolates and chitosan on yield of tomato was also assessed. The results indicated that the fruit yield due to inoculation with rhizobacterial mixture + Chitosan was increased by 286.46 percent as compared to the viral pathogen inoculated control in kharif season and 226.12 per cent during summer. The shelf life of fruits also got extended due to rhizobacterial and chitosan treatment.The extended shelf life was increased by 89.06 per cent and 68.62 per cent in *kharif* and summer season respectively, when compared to disease control.
30. Application of chitosan or the bacterial inoculant alone was not very effective.
31. Histopathological studies revealed more anatomical destruction in diseased plants as compared to treated and healthy plants. There were more intercellular spaces. The palisade cells were markedly reduced in size, highly distorted and lost their columnar compact nature.
32. Histochemical studies also revealed reduction in insoluble polysaccharides and proteins in palisade and spongy parenchyma cells of diseased plant as compared to healthy plant. The plants treated with *Pseudomonas* 206(4) +chitosan and *Pseudomonas* B-15+ chitosan contain almost same level of insoluble polysaccharides and proteins in palisade and spongy parenchyma cells as shown by healthy plants. In case of *Pseudomonas* 206(4) and *Pseudomonas* B-15 treated plants,a slight decrease was observed.
33. Histochemical analysis also revealed increase in nucleic acid content in pathogen inoculated control plants as compared to healthy plants. Nucleic acid concentration in treated plants with *Pseudomonas* 206(4) +chitosan and *Pseudomonas* B-15+ chitosan was almost same as shown by healthy plants.But, incase of *Pseudomonas* 206(4) and *Pseudomonas* B-15 treated plants, a slight increase was observed.
34. Histochemical studies clearly indicated that rhizobacteria+chitosan biopreparation is more effective in controlling ToLCV as compared to rhizobacteria alone.
35. Studies for assessing the root colonization ability of the most efficient PGPR strain by Scanning Electron Microscopy(SEM) revealed that, roots inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan showed locally very dense populations and the cells were encapsulated within a massive network whereas, roots inoculated with *Pseudomonas* sp. 206(4) but without chitosan also contained bacterial cells, but much less .The numbers of *Pseudomonas* sp. 206(4) on the roots were substantially increased with co-inoculation of chitosan .
36. Transmission Electron microscopic (TEM) examination of the partially purified preparation of ToLCV revealed the presence of isometric and pentagonal with single and paired Gemini virus particles, with a dimension of 20 × 30 nm to 24 × 30 nm and the particles exhibited the characteristic shape of a geminivirus.
37. The subcellular changes which occurred inside the cells due to ToLCV and rhizobacterial+chitosan inoculation were also studied by transmission electron microscopy (TEM). There was complete loss of subcellular architecture within diseased cells and moon shaped chloroplast observed. But, in treated leaves, cells appeared somewhat closer to normal cells and only some extent of distortion was seen in chloroplasts.
38. Thus, the present study has indicated *Pseudomonas* 206(4) as the most efficient biocontrol agent in suppressing the ToLCV disease severity in tomato through ISR activity, besides promoting plant growth and fruit yield.

However, the rhizobacterial mixture (206(4)+ B-15+JK-16) +chitosan was the most effective treatment both in glasshouse and field conditions.

Although the rhizobacterial mixture and chitosan did not protect plants completely from infection by ToLCV, the treatment did significantly reduce ToLCV-induced symptom severity and yield losses. Hence, it can be recommended as a component of integrated disease management systems. Considering the cost of chitosan (Rs 62.50 per formulation) and its positive effects in the suppression of ToLCV as well as in plant growth promotion, this treatment is cost effective and can be adopted by farmers, after testing under natural conditions of vector and virus pressure in multilocations. The B:C (benefit cost) ratio for the best treatment worked out to be 3.89 as compared to 3.21 in the conventional method.The detailed cost of cultivation and net returns are furnished in Appendix- VI.

Future line of work

1. Inclusion and testing of the promising PGPR isolates in the integrated disease management (IDM) schedule for viral diseases.
2. Combination studies involving the promising PGPR strains with other microbial inoculants such as *Azospirillum,* P-solubilizer etc.
3. Testing of these PGPR strains under natural conditions of virus and vector pressure, including a detailed study on the economics of the technology.
4. Novelty of these isolates to be tested through sequencing of full length 16s r-DNA.

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Appendix- Ia: Loading dye composition

Loading dye (6x)	0.25% bromophenol blue
	40% (w/v) sucrose in water

Appendix- Ib: Ethidium bromide

10 mg/ml in distilled water. Stored at 4°C in dark bottle.

Appendix- Ic: Recipe for 1 per cent Agarose gel (40 ml)

Agarose	400 mg
1× TAE	40 ml
EtBr (10 mg/ml)	2 µl

Appendix- Id: 50x TAE composition

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

Total volume 1000 ml with double distilled water.

Appendix- II: Ligation reaction recipe

Plasmid vector PTZ57 R/T	3 µl
Purified PCR fragment	9.0 µl
10x ligation buffer	6.0 µl
Deionized water	11.0 µl
T4 DNA ligase, 5U	1.0 µl
Total	30 µl

Appendix- III: Restriction Recipe

Vector DNA	0.5 µl
Enzyme *ECOR* I +*Hind* III (10U)	0.5+0.5 µl
10x (*ECOR* I+*Hind*III) buffer "D"	2.0 µl
1x BSA	1µl
Sterile water	5.5µl
Total	10µl

Appendix IV: Media composition

A. Nutrient agar

Peptone	5.0 g
Beef extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	6.8 -7.2

B. Rose Bengal agar

D-glucose	10.0 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulkphate hydrate ($MgSO_4.7H_2O$	0.5 g

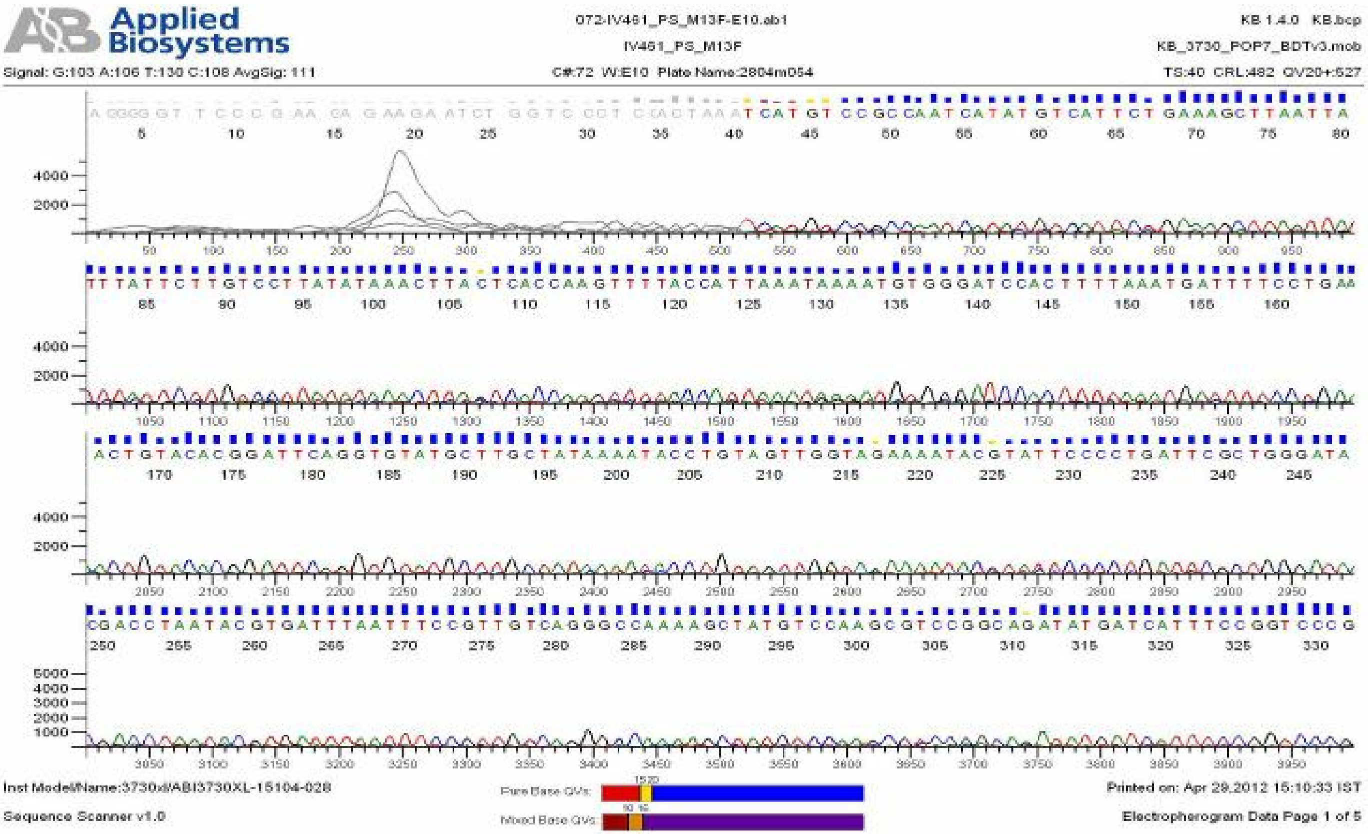
C. Kuster's agar

Starch	10.0 g
Casein	0.3 g
KNO_3	2.0 g
NaCl	2.0 g
K_2HPO_4	2.0 g
$MgSO_4$	4.3 g
$CaCO_3$	0.20 g
$FeSO_4.7H_2O$	0.01 g
Agar	18 g
Distilled water	1000 ml
pH	7.2

D. King's B agar medium

Peptone	16.0 g
K_2PO_4	1.6 g
$MgSo_4$	1.6 g
Glycerol	1000 ml
Agar	18.00
Distilled water	1000 ml
pH	6.5 – 7.0

Appendix Va: Sequencing result



Applied Biosystems

072-IV461_PS_M13F-E10.ab1

IV461_PS_M13F

C#:72 W:E10 Plate Name:2804m054

KB 1.4.0 KB.bcp

KB_3730_POP7_BDTv3.mob

Signal: G:103 A:106 T:130 C:108 AvgSig: 111

TS:40 CRL:482 QV20+:527

A GGGG GT T CCCG AA CA G AAGA ATCT GGTC CCT C CACTAAATCATGT CCGCCAATCATATGTCATTCTGAAAGCTTAATTA

TTTATTCTTGTCCTTATATAAACTTACTCACCAAGTTTTACCATTAAATAAAATGTGGATCCACTTTTAAATGATTTTCCTGAA

ACTGTACACGGATTCAGGTGTATGCTTGCTATAAAATACCTGTAGTTGGTAGAAAATACGTATTCCCCTGATTCGCTGGGATA

CGACCTAATACGTGATTTAATTTCCGTTGTCAGGGCCAAAAGCTATGTCCAAGCGTCCGGCAGATATGATCATTTCCGGTCCCG

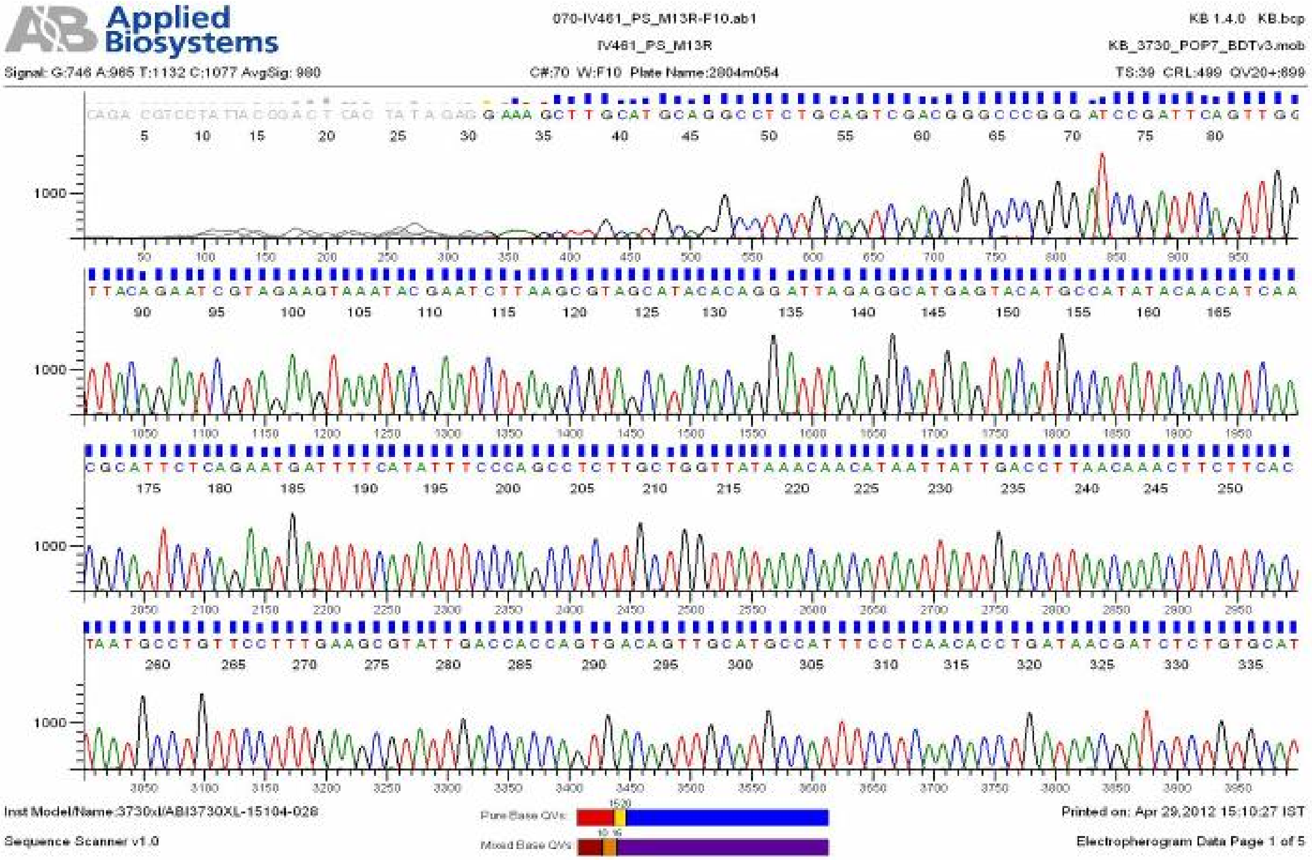
Inst Model/Name:3730xl/ABI3730XL-15104-028

Sequence Scanner v1.0

Printed on: Apr 29,2012 15:10:33 IST

Electropherogram Data Page 1 of 5

Appendix Vb: Sequencing results



Applied Biosystems

070-IV461_PS_M13R-F10.ab1

IV461_PS_M13R

KB 1.4.0 KB.bcp

KB_3730_POP7_BDTv3.mob

Signal: G:746 A:965 T:1132 C:1077 AvgSig: 980

C#:70 W:F10 Plate Name:2804m054

TS:39 CRL:499 QV20+:899

Inst Model/Name:3730xl/ABI3730XL-15104-028

Sequence Scanner v1.0

Printed on: Apr 29,2012 15:10:27 IST

Electropherogram Data Page 1 of 5

Appendix VI: Cost of cultivation of irrigated tomato (per ha)

Sl. No.	Particulars	Following conventional method (`)	Following the technology developed in this research programme (consortium of B-15, JK-16 and 206(4) + chitosan) (`)
1	Field preparation	6,000	6,000
2	Sowing and transplanting	7,000	7,000
3	Weeding	10,000	10,000
4	Irrigation	8,750	8,750
5	Plant protection chemicals	12,500	-
6	Cost of microbial inoculants and chitosan	-	6,625
7	Chemical fertilizer	8,000	8,000
8	Labour	13,000	13,000
9	Staking transport and other miscellaneous expenses	6,000	6,000
10	Grand total (cost of cultivation)	71,250	65,375
11	Gross return	3,000,00	3,19,800
	Yield	50 ton	53.30 ton
	Selling price	6/kg	6/kg
12	Net return	2,28,750	2,45,425
13	B:C	3.21	3.89

PLANT GROWTH PROMOTING RHIZOBACTERIA-INDUCED SYSTEMIC RESISTANCE AGAINST ToLCV DISEASE IN TOMATO (*Lycopersicon esculentum* Mill.)

SHEFALI MISHRA **2012** **Dr. K. S. JAGADEESH**
MAJOR ADVISOR

ABSTRACT

Fifty rhizobacterial isolates were screened against ToLCV disease in tomato. Based on the disease severity control, ten isolates were selected for further studies. The presence of ToLCV in affected leaves was confirmed by both transmission electron microscopy and sequencing of viral coat protein specific gene. The mechanism of virus control was elucidated. All the isolates induced systemic resistance in tomato plants. These plants also showed the highest reduction in the insect population. The green house experiment revealed *Pseudomonas 2*06(4) as the most promising isolate. Semi quantitative PCR analysis revealed lower viral load accumulation in the plants inoculated with *Pseudomonas 2*06(4). The plants inoculated with this isolate also recorded maximum plant height, total biomass, chlorophyll content and fruit yield over the diseased control. Based on biocontrol potential and plant growth promotion, three efficient isolates were further evaluated in combination with chitosan both under glasshouse and field conditions. The rhizobacterial consortia + chitosan emerged as the best treatment in disease severity control, plant growth promotion as well as in increasing the yield both under glasshouse and field conditions in two consecutive seasons. The histopathological studies revealed more anatomical destruction in diseased plants as compared to rhizobacteria + chitosan treated plants. The root colonization ability of the most efficient PGPR strain 206(4) was assessed by SEM, which indicated that the roots inoculated with this isolate in combination with chitosan showed higher population than the isolate without chitosan. The subcellular changes due to ToLCV and rhizobacterial+chitosan inoculation, as studied by TEM , revealed complete loss of subcellular architecture in diseased cells and the cells appeared somewhat closer to normal cells in the treated leaves. Thus, the rhizobacterial consortia and chitosan, significantly reduced ToLCV-induced symptom severity and yield losses, and can be recommended as a component of integrated disease management systems.