

Indigenous and Recombinant Chitinases as Biocontrol Agents against Plant Pathogens: A Comparative Study

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ABSTRACT

An increase in urbanization and industrialization has increased the demand of rapid and sustainable agricultural practices. Alternate practices offering better agricultural productivity are necessary to meet the world food demand. Biological control offers a suitable substitute towards chemical pesticides which have their drastic effects on both man and environment. Several lytic enzymes play a key role in these alternative strategies on control of pathogens and pests. Chitinase is one such enzyme complex that has been effectively used in biological control. These enzymes breakdown chitin efficiently into *N*-acetylglucosamine and its oligomers called chitooligosaccharides. Chitin forms the exoskeleton of arthropods, nematodes and cell wall component in fungi. Due to this, chitin has been targeted for the development of biological control agents against plant pathogens. Plants have known to implicate chitinases in defence against plant pathogens thus chitinases provide an alternate solution to harmful chemicals to combat plant pathogens. This review throws light on the current status of agriculture and further deals with chitinases native to many plants and genetically modified ones, which have been designed in defence against many plant pathogens.

Keywords: antifungal, biopesticide, chitin, genetic modification

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AGRICULTURE: PAST AND PRESENT

Agriculture is the cultivation of foods and products useful to man through the process of farming. Agriculture began around 10,000 B.C. and played an important role in the development of human civilizations in the past (Craig 1993). Agriculture basically comprises of a variety of techniques and specialities including both sustainable agriculture and exhaustive farming. Crops of world are constrained to less than 100 species of plants and are cultivated by man from several centuries (Food and Agriculture Organization of the United Nations 2007). Agricultural foods include cereals, fruits, vegetables, etc. whereas the agricultural products include fibres, fuels and raw materials. Agriculture is the main source of food and raw materials to industries and hence, plays an important role in a nation's economy. The major crops which have to be cultivated on large scale to meet the consumer demand are:

- Pulses: Black gram, soybean, cowpea, rice bean, green gram, red gram, etc.;
- Cereals: Wheat, paddy, barley, ragi, bajra, etc.;
- Oilseeds: Groundnut, safflower, coconut, oil palm, sunflower, sesamum, etc.;
- Commercial crops: Sugarcane, turmeric, chillies, tobacco, cotton, etc.;

- Plantation crops: Tea, coffee, rubber, cardamom, cinnamon, clove, nutmeg, etc.;
- Horticultural crops: Mango, pineapple, grapes, apricot, apple, cherries, tomato, okra, cabbage, cauliflower, radish, turnip, carrot, etc.

Along with the above, high-yield varieties of common staple crops like wheat and rice have been introduced as a part of the Green Revolution. In the 20th century, there has been an amplified usage of synthetic fertilizers and pesticides which initially increased the yield of crops. Despite the demand of agriculture, there has been a significant depletion in its workforce due to industrialization. Agricultural productivity is facing diminution not only due to a reduced workforce but also due to man-made and natural disasters. Modern techniques like plant breeding, use of pesticides and fertilizers have increased the output of crops but have led to problems like soil erosion and resistant pests and pathogens. It has also caused a great imbalance in the delicate ecosystem which needs to be balanced for our very own existence. Due to the excess use of chemical pesticides and fertilizers, there has been around 40% degradation in the world's soil (Sample 2007). Several epidemic diseases like the stem rust on wheat have reduced yield of essential crops due to pesticide tolerance. Similarly, fungal attack on potatoes in 1840's in Ireland and insect attack on cotton in

Table 1 Methods employed for the control of various pests and pathogens.

Pest/Pathogen	Crop affected	Chemical agent	Biological agent	References
Fungal pathogens causing root diseases	Soybean	Hexaconazole	<i>Bacillus pumilus</i> GB34	Joshi and Gardener 2006
<i>Rhizoctonia solani</i>	Food crops	Kitazin	<i>Trichoderma virens</i> GL21	Roberts <i>et al.</i> 2004
<i>Xanthomonas campestris</i>	Beans	Tricyclazole	<i>Gliocladium virens</i> (a.k.a. <i>Trichoderma virens</i>) GL-21	Roberts <i>et al.</i> 2004
<i>Botrytis cinerea</i>	Vegetable crops	Chlorothalonil	<i>Streptomyces griseoviridis</i> strain K61	Holmalahti <i>et al.</i> 1992

1960's in Mexico initiated the development of alternate agents for the control of pathogens.

Intensive agriculture which is practiced in several parts of the globe is associated with decreased soil quality. The monocultures typically used in intensive agriculture lead to a low biodiversity and subsequently increase the number of pests, which are controlled through excessive use of pesticides thus causing an increment in the herbicide resistant weeds and insects (Acquaah 2002). A loss in biodiversity is also attributed to degradation of land caused by deforestation, desertification, soil erosion, mineral depletion and chemical degradation. The UN-FAO report cites land management is the main reason behind degradation and that 1.5 billion people rely upon the degrading land. Although the "Green Revolution" significantly increased rice yields in Asia, yield increases have not occurred in the past 15–20 years (Tilman *et al.* 2002). Pesticide use has increased since 1950 to 2.5 million tons annually worldwide, yet crop loss from pests has remained relatively constant (Pimentel *et al.* 1996). The Agrochemicals Policy Group, an apex body of 200 crop protection companies, has indicated that agricultural loss in 2007 due to pests was estimated at Rs. 140,000 million and that sensible pesticide use could decrease the losses and enhances productivity (Agriculture-Biz and Commodities, Business Line, Business Daily from Hindu, May 01, 2008).

Agriculture in India and other developing countries rely mostly on traditional methods like growing seasonal crops and rotation of crops. Apart from the reasons mentioned above, agriculture productivity in India has not been very convincing due to dependability on seasonal monsoons for irrigation. Mr S. Kumarasamy, Chairman, Agrochemical Policy Group, indicated that loss of crops in India was due to usage of only 1/4th of the total cropped area of 180 million ha which is treated with chemicals. The level of pesticide use in India was 480 g/ha, while it was 17000 g in Taiwan, 17000 g in Japan, and 4500 g in the US. Correspondingly, crop yields in India were the lowest. However, Dr K.S.R.K. Murthy, President, Plant Protection Association of India, Hyderabad, quoted that pesticide use can be advocated only when right agricultural practices were followed (Agriculture-Biz and Commodities, Business Line, Business Daily from Hindu, May 01, 2008).

The economic loss in agriculture due to pests and pathogens is the most significant factor in comparison to those caused by natural calamities. Among the insect pests, *Helicoverpa armigera* causes severe damage to important crops such as cotton, pulses, vegetables and sunflower in India (<http://www.dacnet.nic.in>). Additionally legumes such as groundnut, cowpea, etc. are prone to attacks by soil-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* and others. Sugarcane, *Saccharum officinarum* is one of the largely cultivated cash crops grown all over India. The major insect pests on sugarcane include *Tryporyza novella* and *Pyrilla perpusilla* and the fungal pathogens include *Colletotrichum falcatum*, *Ustilago scitaminea* and others. Sunflower (*Helianthus annuus* L.), an important oilseed plant in food industries to produce vegetable oils, is a good example of plants threatened with pests and pathogens, particularly fungi leading to a significant decrease of crop yield. *Sclerotinia sclerotiorum* causes head and stem rot in sunflower. It infects about 400 species of sunflower and is an aggressive pathogen (Steadman *et al.* 1994). Control of this fungal pathogen is of importance due to wide range of its infections in different

plants (especially oilseed plants like sunflower and canola). **Table 1** enlists the chemical and biological agents employed for the control of major pests and pathogens. Farmers are ultimately left with few practical and economic methods that can increase crop yield.

Sir Albert Howard, one of the pioneers of organic farming had argued that long time use of synthetic fertilizers and pesticides would lead to decreased soil fertility (Sir Albert and Wendell Berry 2007). In the recent years, there has been an increasing awareness of the importance of sustainable agriculture. One of the pioneers of this movement has been the European Union Commission which was the first body to certify Organic food in 1991 and brought about reforms in its Common Agricultural Policy (CAP) in 2005 (www.ec.europa.eu). The increased importance given for organic farming has re-established research in alternative technologies like pest management and selective plant breeding. The recent technology to join this force is the genetically modified foods. Alternate methods employing reduced usage of chemical pesticides and fertilizers are the need of the hour. This brings the focus on the usage of bioinsecticides and biopesticides which would be cost effective, ecofriendly and thus increase crop yield. The biological control strategies become effective by targeting the biological components present in the pests or pathogens. Increased awareness of biological control methods is crucial to assist and practice sustainable agriculture (Wang *et al.* 1999a, 1999b). Biological control has its own successful equivalence and it is interesting to note that scientists from the nineties are exploring multi-pronged approaches to pest control. The biochemicals used as pesticides are environmentally safe, selective and specific in their action and easily biodegradable. In addition, their cost and time of production is low as compared to chemical based control measures, and they can be used in combination with other control measures in integrated pest management programs. The highlight of their use is that they are very rarely harmful to other plants and non-target organisms.

Fungal plant pathogens are also effectively controlled with microorganisms and have been a field of study for more than 70 years (Howell 2003). There are numerous strains of yeast and mold species that have been isolated for their capability to antagonize plant pathogens and their biological products are commercially available in the market (Punja and Utkhede 2003). It is however essential to understand the mechanism of action of biocontrol agents so that they can be effectively used. The mechanisms of numerous biocontrol agents have been studied but not fully elucidated. Various mechanisms have been described which suggests competition for space and nutrients, mycoparasitism, antibiosis or elicitation of plant defences which contribute simultaneously or sequentially (Janisiewicz and Korsten 2002). The biocontrol agents include plant products, microorganisms, microbial enzymes, enzyme inhibitors and antibiotics. Plant products like Pathogenesis Related (PR) proteins are known to inhibit various plant pathogens and have been found to be effective biocontrol agents (Datta and Muthukrishnan 1999). The class actinobacteria represent the maximum number of antimicrobial producing organism (Berdy 2005). Amongst these, *Micromonospora* spp. reported by Conn *et al.* (2008) was able to activate key enzymes in the systemic acquired resistance (SAR) or the jasmonate/ethylene pathways and hence, played an important role in defense against plant pathogens. Similarly, *Micromonospora* sp. M39 isolated from mangrove rhizosphere soil

exhibited antifungal activity against the rice blast pathogen *Pyricularia oryzae* MP0292 by producing metabolites (Ismet *et al.* 2004). Similarly, antimicrobial metabolite was isolated and characterized from *Nocardia levis* MK-VL_113 which displayed antimicrobial potential against a host of plant pathogens as reported by Kavita *et al.* (2010). *Paenibacillus polymyxa* strains produced two types of peptide antibiotics that were effective against fungal and bacterial plant pathogens (Beatty and Jensen 2002; Raza *et al.* 2009). In addition, *Serratia plymuthica* IC14 also exhibited multiple antifungal mechanisms by the formation of proteases, antibiotics and siderophores (Kamensky *et al.* 2003).

TARGETS AND AGENTS FOR BIOLOGICAL CONTROL

Plants when exposed to pathogens such as fungi and viruses produce proteins which are upregulated by other small molecular weight molecules. These plant proteins called pathogenesis-related (PR) proteins exhibit antifungal activity. The mechanisms of antifungal action of few groups of PR proteins have been clearly identified (Selitrennikoff 2001). These proteins can be used as agents for biological control.

Cell wall is a common target for biological control and lytic action can be mediated through the action of enzymes like chitinase, β -1,3-glucanase and proteases popularly known as mycolytic enzyme complex (Roberti *et al.* 2008). Specific cell wall components can serve as target sites and few common structural elements offer targets for wide-spectrum biocontrol agents. One such component is chitin, which is a structural element in insects, arthropods and fungi. Thus, insects and fungi share a key structural polymer, chitin which is absent in plants and mammals. Therefore, chitin synthesis or degradation offers an attractive target for biological control. At the same time, it is beneficial to develop a common biocontrol agent against both insects and fungi. Chitin is a major component in the lining of the insect's digestive tract and in the cell wall of fungi. Ingestion of chitinolytic enzymes may significantly reduce growth and inhibit the development of insects and fungi. To understand the mechanism of the biocontrol agent namely chitinases, a perspective of the substrate on which it acts and the types of chitinases present in nature would give an insight about its application. Chitin is composed of repeating units of *N*-acetyl glucosamine (GlcNAc). The individual chitin chains within the microfibrils are linked by hydrogen bonds between the carbonyl and amino groups of *N*-acetylglucosamine. In nature, chitin occurs in three forms known as α -, β -, and γ -chitin (Ruiz-Herrera 1991). In α -chitin, the adjacent chains are arranged in an anti-parallel orientation, whereas in β -chitin the chains are arranged in a parallel manner. In γ -chitin, the chains are grouped in sets of three strands where one chain is anti-parallel and the other two are arranged in parallel direction. The chitin microfibrils of α -chitin are tightly packed and this form is stabilized by a number of inter-chain hydrogen bonds. This is the most thermodynamically stable form of chitin, which is also the most abundant form (Merzendorfer and Zimoch 2003). Insect cuticles are primarily composed of α -chitin, which confers the highest degree of strength and stability. In contrast to α -chitin, β - and γ -chitins are less tightly packed and form a greater number of hydrogen bonds with water resulting in a greater degree of hydration relative to α -chitin. β -chitin is more susceptible to enzymatic degradation due to its above said properties (Merzendorfer and Zimoch 2003). Both β -chitin and γ -chitin therefore, form a more flexible and soft chitinous structure. Chitin in fungal cell walls is sensitive to treatments with dilute HCl or chitinase. The sensitivity of the fungal cell wall to lytic enzymes has been exploited by using chitinase-producing bacteria to control plant-pathogenic fungi (Kamil *et al.* 2007).

The chitin synthesis and degradation pathways have been extensively studied (Tokura and Hamura 2007). The key enzymes involved in chitin synthesis which offer targets for biological control are chitin synthase and gluco-

samine phosphate synthase. Chitin synthesizing systems characteristically include chitin-degrading enzymes also, where the nascent polymers are incorporated in the existing meshwork after its degradation. Chitin is thus hydrolyzed by the synergistic and consecutive action of endochitinases, exochitinases and *N*-acetylglucosaminidases. Chitin metabolism can be targeted at degradation of pre-formed chitin and inhibition of key enzymes like chitin synthase or other enzymes of chitin or cell wall metabolism. Lysis of the host structure by secretion of extracellular lytic enzymes is one of the important mechanisms that are involved in the antagonistic activity of biocontrol agents (Mathivanan *et al.* 1998; Kim *et al.* 2002). Similarly, fungal pathogens can be controlled by action of mycolytic enzymes through the lysis of cell walls, hyphal tips and germ tubes (Kunz *et al.* 1992).

CHITINASES

Plant chitinases form the major component of the pathogenesis related proteins which are induced during pathogenic attack on the plants and they play an important role in the defence mechanism against plant pathogens (Schlumbaum *et al.* 1986; Broglie *et al.* 1991; Collinge *et al.* 1993). In recent years, soil-borne microorganisms that produce chitinases are considered as potential biocontrol agents against fungi and nematodes which cause diseases of agricultural crops. Chitinases also play an important role in nature in the recycling of carbon and nitrogen thus maintaining an ecological balance. Chitinases could be used directly in field applications or indirectly by introducing the chitinase genes in the resident rhizoflora or in plants.

Chitinases are present in plants, seeds, stems, tubers and flowers and they are tissue specific and regulated during the development of the plant. Plant chitinases are induced by the attack of phytopathogens as pathogenesis-related (PR) proteins in plant self-defence or by contact with elicitors such as chitoooligosaccharides and play an important role in self-defence against pathogens. Chitinase genes respond to an attack by potential pathogens, elicitor treatments, and stress conditions (Boller *et al.* 1986). Other chitinases are reported to participate in important physiological processes of plants such as embryogenesis and ethylene synthesis. Simultaneous induction of chitinase and α -1,3-glucanase has been implicated in defence of plants against pathogens (Wessels and Sietsma 1981). It has been reported that both chitinase and α -1,3-glucanase activities increased co-ordinately in tomato when inoculated with *Fusarium solani*, and that action of the enzymes was synergistic (Benhamou *et al.* 1990). Likewise, chitinase and α -1,5 glucanase from pea pods act synergistically in the degradation of isolated fungal cell walls (Mauch *et al.* 1988). The physiological concentrations of chitinase and α -1,3-glucanase can effectively inhibit growth of many potentially pathogenic fungi (Schlumbaum *et al.* 1986; Mauch *et al.* 1988). Chitinase stimulation in plants during fungal attack as well as *in vitro* inhibition of fungal growth has been established (Schlumbaum *et al.* 1986; Linthorst 1991; Arlorio *et al.* 1992; Collinge *et al.* 1993; Herrera-Etrela and Chet 1999; Ano *et al.* 2003) and numerous chitinase genes have therefore been isolated and characterized from various plant species. Chitinases from numerous plants have been studied along with their structure, pathogen specificity and antifungal properties but their exact role and mechanism in plant defence requires further investigation.

The chitin metabolism in insects is not well understood despite its biological significance compared to the chitin biosynthetic pathway in insects or other invertebrates (Cohen 2001). The chitin biosynthetic pathway begins with the cleavage of trehalose by trehalase and the formation of glucose (Merzendorfer and Zimoch 2003). The last step in the chitin biosynthetic pathway is catalyzed by chitin synthase (EC 2.4.1.16), which catalyzes the polymerization of chitin from activated UDP-*N*-acetylglucosamine (UDP-GlcNAc) monomers. The two primary enzymes responsible for the degradation of chitin in insects are chitinases (EC

3.2.1.14) and β -N-acetylglucosaminidases (EC 3.2.1.30). These enzymes play an important role during ecdysis during which endochitinases act upon the cuticle to release chitooligosaccharides which are later hydrolysed to N-acetylglucosamine by exochitinases. The monomer is reused to synthesize a new cuticle. The chitinases found in insects play a defensive role against the pathogens which act upon them. The chitinases in insects are regulated by hormones during transformation of the larvae. Insect chitinases can be inhibited by chitinase inhibitors like allosaminidin (Koga *et al.* 1997). The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* produce several chitinases which may have a variety of different functions. Some of these chitinases are important cuticle-degrading enzymes and act synergistically with proteases to hydrolyze insect cuticle (St. Leger *et al.* 1996; Lubeck *et al.* 2008).

The fungal cell wall is composed of 1, 3- β - and 1, 6- β -glucan, chitin, mannan and proteins. There is branching and extensive cross-linking between chitin, glucan and other cell wall components. During growth and morphogenesis there is a breakage within these polymers by chitinases or glucanases. The chitinases found in fungi are intracellular and extracellular (Takaya *et al.* 1998; Kim *et al.* 2002). Chitinases play a role in fungal and yeast morphogenesis. In *Saccharomyces cerevisiae* chitin is localized to the junction between mother and daughter cells and a secreted chitinase is required to separate the newly formed daughter cells (Kuranda and Robbins 1991). They are involved in spore germination and in hyphal branching of fungi (Gooday *et al.* 1990).

In natural environments, fungi rely on a range of secreted hydrolytic enzymes to supply the small molecules required for their nutritional needs. Secreted chitinases have been studied from numerous fungal species in the perspective of degradation of environmental chitin. Entomopathogenic fungi secrete chitinases that are believed to be involved in digestion of the insect cuticle (St. Leger *et al.* 1996). Secreted chitinases and other hydrolytic enzymes including proteases and glucanases are associated with autolysis of fungal cells in nutrient limiting conditions.

Bacteria produce chitinases to make use of chitin polymer to fulfil their nutritional requirements. Various genera like *Serratia*, *Chromobacterium*, *Klebsiella*, *Bacillus*, *Streptomyces*, etc. produce chitinases. Other microorganisms like *Micromonospora* sp., *Microbispora rosea*, *Actinoplanes philipinensis*, *Nocardioidea* sp. also contribute significantly in reducing soil-borne fungal pathogens (Coombs *et al.* 2004; El-Tarabily *et al.* 2006; Hirsch and Valdes 2010). *Bacillus cereus* also exhibited antifungal activity against *Fusarium solani*, *Fusarium oxysporum* and *Pythium ultimum* when grown on shellfish chitin waste as reported by Chang *et al.* (2007). Chitinase from *Bacillus thuriensis* subsp. *aizawai* exhibited lytic activity against the cell walls of six phytopathogenic fungi and inhibited the mycelial growth of *Fusarium* sp. and *Sclerotium rolfsii* (de la Vega *et al.* 2006).

CHITINASES IN BIOCONTROL

It has been demonstrated previously that *Bacillus cereus* strain 65, isolated as an endophyte from mustard (*Sinapis*) plant, inoculated into cotton seedlings is able to reduce the root rot disease caused by *Rhizoctonia solani* (Pleban *et al.* 1997). *Paenibacillus* sp. D1 isolated from the effluent treatment plant of a seafood processing industry exhibited broad spectrum tolerance towards a number of pesticides at concentrations higher than recommended for field applications. Chitinase of *Paenibacillus* sp. D1 was found to be more tolerant than the organism itself and this will help in the Integrated Pest Management (IPM) to reduce the usage of chemicals as reported by Anil *et al.* (2009). Several isolates of *Enterobacter cloacae* are reported to exhibit biocontrol activities against *Pythium* spp. and against *Fusarium* wilt of cucumber besides other plant diseases caused by fungal pathogens. *Enterobacter aerogenes* B8 significantly reduced

infections of apple crown and root rot caused by *Phytophthora cactorum* (Uthkede *et al.* 1986). Some strains of *Enterobacter agglomerans* were efficient in the control of plant diseases caused by different bacterial and fungal pathogens. Mutation in the chitinase gene reduced the biocontrol effect indicating that the indigenous chitinase was more effective than the mutated chitinase (Chernin *et al.* 1995). Chitinases from *Paenibacillus illinoisensis* KJA-424 also suppressed the symptom of damping-off cucumber seedlings caused by *Rhizoctonia solani* in greenhouse trials (Jung *et al.* 2003). Trial applications of chitinase positive *Pseudomonas* strains effectively reduced sheath blight disease in rice and promoted plant growth and increased yields (Nandakumar *et al.* 2001). Incubation of fungal pathogens with *Streptomyces* chitinase caused their lysis. Similar results were reported in the chitinolytic enzymes from *Streptomyces albidoflavus* NRRL B-16746 which had significant levels of activity under alkaline conditions (Broadway *et al.* 1995; Gupta *et al.* 1995). Bioassays indicated that the purified chitobiosidases significantly reduced spore germination and germ tube elongation of *Botrytis cinerea* and *Fusarium oxysporum*. The occurrence of sunflower head and stem rot disease by *Sclerotinia sclerotiorum* was greatly reduced by the use of soil borne *Streptomyces* sp. in green house studies. This paves the path for developing transgenic plants harbouring the gene(s) responsible for the biocontrol like chitinases, proteases and glucanases (Baniasadi *et al.* 2009).

Plant chitinases have been useful in biological control as out of four chitinases from the European chestnut plantlets (*Castanea sativa* Mill.), three displayed antifungal activity against chestnut blight fungus *Cryphonectria parasitica* (Vannini *et al.* 1999). Similarly chitinase with antifungal activity was isolated from Mung bean (*Phaseolus mungo*) seeds (Ye *et al.* 2005). It was also reported that acidic chitinases are mainly involved in the biocontrol of plant pathogens while basic chitinases form a part of the pre-formed defence in sorghum (Huang and Backhouse 2006).

Cell wall lysis by mycoparasites has been suggested to correlate with the biological control of plant pathogenic fungi. There have been several reports on the chitinolytic system of the mycoparasitic fungus *Trichoderma harzianum* (de la Cruz *et al.* 1992; Harman *et al.* 1993; Lorito *et al.* 1993; Haran *et al.* 1995). *Trichothecium roseum* was demonstrated to be a mycoparasite, capable of parasitizing *Sclerotinia sclerotiorum* (Huang and Kokko 1993). The purification and characterization of a chitinase from a strain of *Trichothecium roseum* and its effects on fungal mycelia were assayed using *Alternaria alternata*, *Fusarium moniliforme* and *Magnaporthe grisea*. Such chitinases may be useful for the production of transgenic microorganisms with superior biocontrol capabilities and of transgenic plants with high resistance to plant pathogenic fungi. Chitinases from *Trichoderma* and *Rhizopus* strains were reported to degrade the cell walls of *Fusarium solani* and also inhibit its microconidial germination (Nguyen *et al.* 2008). Yasir *et al.* (2009) reported that bacteria present in vermicompost had inherent antifungal activity due to chitinolytic activity which inhibited the growth of various plant pathogens. Vermicompost with various bacterial communities can be used as a biopesticide which would also provide nutrients to the plants. Most of the chitinases which have been reported in literature have been tested against pathogenic plant bacteria and fungi only at the laboratory levels (Asran-amal *et al.* 2010). Novel antifungal and antibacterial chitinases along with important characters like ability to grow in acidic or alkaline conditions, salinity, etc would benefit in making potential biocontrol agents. The stability of these chitinases or the organisms having the above mentioned features would be put to test in actual field conditions where there is constant variation due to various environmental factors. Elaborate studies need to be carried out in this aspect which would help in making a greener and cleaner environment.

RECOMBINANT CHITINASES

A major limitation to the development of mycoinsecticides is a longer response time after application. Improvements in the effectiveness of mycoinsecticides may be obtained by optimizing the preparation or through genetic modification. Genetic modification through the expression of foreign chitinase gene(s) in another organism or the plant itself would help in developing resistant cultivars against plant pathogens.

An actively antagonistic bacterium that was isolated from a ginseng rhizosphere and identified as a strain of *Pseudomonas stutzeri* could be used as a biocontrol agent against *Fusarium solani*, which causes root rots with considerable losses in many important crops. Genetically bred *P. stutzeri* YPL-1 was obtained by transformation of the bacterium with a broad-host-range vector, pKT230. For genetic development of a multifunctional biocontrol agent by introducing a foreign gene, a genetically bred *P. stutzeri* YPL-1 by transformation with the broad-host-range vector pKT230 was obtained as reported by Lim *et al.* (1991). This study suggests that the introduction of a plasmid-encoded gene into the antagonist will increase biocontrol efficiency by combining its lytic action with other biocontrol abilities. Kirubakaran and Sakthivel (2007) overexpressed a chitinase gene from barley in *E. coli* and reported that the purified chitinase exerted broad-spectrum antifungal activity.

Wiwat *et al.* (1996) transformed *Bacillus thuringiensis* subsp. *israelensis* with chitinase-encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* with the aim of increasing its insecticidal activity, but only low levels of expression were obtained. Regev *et al.* (1996) reported that a recombinant enzyme which encoded a chitinase gene from *Serratia* sp. in *E. coli*, synergistically enhanced the activity of recombinant *B. thuringiensis* δ -endotoxin CryIC against larvae of *Spodoptera littoralis*. The chitinase gene, *Bbchit1* and its upstream regulatory sequence from *Beauveria bassiana* were cloned. The sequence of *Bbchit1* was novel when compared to other reported insect pathogenic fungal chitinases sequences. When the gene was transformed into the genome of *B. bassiana*, three transformants overproduced *Bbchit1*; it enhanced the virulence of *B. bassiana* for aphids as reported by Fang *et al.* (2005). Liu *et al.* (2010) reported the antifungal activity of the recombinant chitinase (ChiA) from *Bacillus thuringiensis* subsp. *colmeri* against *R. solani* and *B. cinerea* which was higher than the antifungal activity against *P. chrysogenum* and *P. piricola*. This chitinase also inhibited the larvae of *Spodoptera exigua* and *Helicoverpa armigera*. The chitin binding domain was shown to play a very important role in the biocontrol activity as the activity decreased when this domain was abolished indicating a specific role for this domain in any chitinase for biocontrol activity.

Prabavathy *et al.* (2006) reported the fusion of the protoplasts isolated from *Trichoderma harzianum*, which exhibited higher chitinase activity than the parent strain and this was used in the biocontrol of *Rhizoctonia solani*. This opens a new area where protoplasts from different asexual fungi could be fused to procure fusion protoplasts having increasing chitinase activity which will in turn increase their biocontrol ability.

A recombinant chitinase from *Pochonia chlamydosporia* inhibited growth of eggs of the nematode *Meloidogyne incognita* and *Bombyx mori*. This suggests that *P. chlamydosporia* could be useful to control nematodes and make an important biocontrol agent (Mi *et al.* 2010).

The role of recombinant chitinolytic enzymes in biocontrol is supported by the work of Lorito *et al.* (1998), who transferred the gene encoding endochitinase from *T. harzianum* (P1) into tobacco and potato and demonstrated a high level and broad spectrum resistance against a number of plant pathogens. Bolar *et al.* (2001) demonstrated enhanced resistance to apple scab incited by *Venturia inaequalis* in transgenic apple plants that had been transformed with genes encoding both endo- and exo-chitinases from *T. atro-*

viride (P1). Recombinant chitinases from wheat were purified and characterized by Arpita *et al.* (2007) which exerted a broad-spectrum antifungal activity against *Colletotrichum falcatum* (red rot of sugarcane) *Pestalotia theae* (leaf spot of tea), *Rhizoctonia solani* (sheath blight of rice), *Sarocladium oryzae* (sheath rot of rice), *Alternaria* sp. (grain discoloration of rice) and *Fusarium* sp. (scab of rye). Similarly, Salami *et al.* (2008) overexpressed a Class III chitinase gene from grapevine which had antifungal activity against *Botrytis cinerea*. Transgenic potato and tobacco plants expressing chitinase from *Trichoderma* have been shown to be highly tolerant or resistant to foliar pathogens (*Alternaria alternata*, *Alternaria solani*, and *Botrytis cinerea*) and to the soil borne pathogen *R. solani*. All transformants had higher chitinase activity than the wild type when grown in glucose-containing medium. *T. harzianum* overproduced chitinase Chit33 without any specific inducer and does not undergo proteolysis. It could inhibit *R. solani* growth under inducing *in vitro* conditions (Limon *et al.* 1999). Datta *et al.* (2001) also reported that in transgenic elite *Indica* rice cultivars; there was consecutive expression of infection induced chitinases, which increased the resistance to sheath blight. Using *Agrobacterium tumefaciens* strain C58, a rice chitinase gene was transformed in spray type *Chrysanthemum* and transgenic lines developed showed enhanced resistance to gray mold (Takatsu *et al.* 1999). This suggests that transgenic plants harbouring chitinase gene(s) from other sources or plants may enhance the resistance of the plants against pests and pathogens. *Chaetomium globosum*, an important biocontrol fungus, suppressed the development of rice blast and wheat leaf rust when its recombinant protein was used as a biocontrol agent (Liu *et al.* 2008). Fan *et al.* (2007) expressed a recombinant chitinase gene from *Beauveria bassiana* in *E. coli* and *Pichia pastoris*. The chitinase degraded the cuticles of insects very efficiently. This facilitates the development of a new cost-effective technique for large-scale production of recombinant chitinases for biocontrol of fungal plant pathogens.

Genetically modified organisms or crops offer enhanced biocontrol with the beneficial traits as compared to the native crops or microorganisms in most of the cases. But the usage of genetically modified organisms or plants has proven to be controversial. The issues addressing food security and environmental impacts still need to be studied in detail.

FUTURE PERSPECTIVES

Chitin metabolism provides an effective target for biocontrol since it is indigenous to pests and fungi. Further investigations would reveal the exact mechanism of role of chitinases, PR proteins and other hydrolytic enzymes in providing complete protection of crops against pests and pathogens. Breeding of plants which express high levels of these hydrolytic enzymes along with resistance gene(s) would provide an alternate choice and thus reduce the usage of excessive pesticides and insecticides. Microorganisms exhibiting these useful traits can also be used in fields for the better yield of disease free crops. It would be interesting to design transgenic plants or organisms which would offer resistance to pests and pathogens by expressing chitinases and other PR-proteins collectively. Besides, integrated pest management using antifungal or insecticidal chitinases in conjunction with other biocontrol agents can be more effective. All such practices would help greatly in the reduction of chemicals and create a greener and cleaner tomorrow besides enhancing agricultural productivity.

LATEST UPDATES

Ge *et al.* (2010) showed that amendment of chitin in the culture medium of *Rhodotorula glutinis* was effective in controlling postharvest grey mould of strawberries by *Botrytis cinerea* as it increased the production of chitinase along with other enzymes. Chitin is an approved biopesti-

cide by EPA which makes it a suitable carrier for different biocontrol agents for the enhanced production of chitinases. Recently, fungal isolates like *Fusarium oxysporum* strain By125, *Nectria haematococca* Bx247, and *Phomopsis* sp. By231 were effective biocontrol agents against Verticillium wilt of cotton as they exhibited 63-69% biocontrol efficacy towards this disease along with increased biomass of 18-62% under greenhouse conditions (Zheng *et al.* 2011). They also had additional advantages like phosphate solubilization, Nitrogen fixation along with cell wall degrading enzymes like cellulose, chitinase and protease. This offers further scope for isolating ideal organisms having these qualities in combating pathogenic bacteria and fungi in addition to promoting plant growth.

Recombinant chitinase (ChiA) from *Bacillus thuringiensis* subsp. *colmeri* 15A3 (Bt. 15A3) was expressed in *Escherichia coli* XL-Blue which inhibited the spore germination of *Penicillium glaucum* and *Sclerotinia fackeliana* efficiently (Liu *et al.* 2010). In addition, the crude ChiA decreased the LC₅₀ of Bt. 15A3 crystal protein against the larvae of *Spodoptera exigua* and *Helicoverpa armigera*. A combination various biocontrol agents can be more effective in combating pests and pathogenesis comparison to their sole application. Transgenic tobacco plants harboring *Autographa californica* nucleopolyhedrovirus chitinase A (AcMNPV ChiA) was found to be permeable to the peritrophic membrane of larvae of two Lepidoptera (*Bombyx mori* and *Heliothis virescens*) and inhibited spore germination and growth of the phytopathogenic fungus *Alternaria alternata* as reported by Maro *et al.* (2010). This enzyme can be produced on a large scale and used as an eco-friendly biocontrol agent in the field of agriculture.

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