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Antifungal proteins: An ecofriendly approach for sustainable alternative of biocontrol against the disease-causing agents in plants

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ABSTRACT

The chemical fungicides applied to counter the diseases associated with annual and perennial crops are creating a major concern by affecting the environment adversely. Furthermore, improper and inadequate application of these fungicides leads to a process of co-evolution that develops resistance in fungal pathogens against these compounds. This current scenario has aggravated the search for alternative disease management strategies and/or safer antifungal agents that could substitute the current fungicides with bio-fungicides. Fungicides of biological origin are the botanicals proteins remain present in different plant parts and seems to be involved in either constitutive or induced resistance to pathogenic fungal attack and thus play a vital role in plant defense system against pathogenic fungi through controlling their spread. A great number of antifungal peptides and proteins have already been reported, with more are being discovered almost daily. Till now, 17 families of antifungal proteins have been identified that have a high potential for therapeutic applications in agriculture for biocontrol of pathogenic microbes that protect plants against diseases.

Keywords: Antifungal proteins; Bio-pesticides; Biological control; Fungicide

INTRODUCTION

Fungal pathogens are considered as the most notorious group of pathogens as they have higher pathogenicity that not only causes disease epidemics but also causes more intense year-to-year incessant loss than other pathogens (Singh, 2018). The worldwide disease estimate suggests that the pathogenic fungi are responsible for around 60-65% of major crop diseases (Passari *et al.,* 2016) including rusts, smuts, mildews, blight, etc. (Kumar, 2018). With increase in the number of crop pathogens, the application of synthetic agrochemicals in conventional agriculture became common agriculture practice but at the same time, large-scale use of these synthetic fungicides has been reported to cause acute toxicity, require longer degradation period, show bio-magnification, harmful effect on food and health, high cost and environmental hazards (Patel *et al.,* 2014). The Continuous applications of these fungicides is also developing resistance in fungal-pathogens and thus making them more hazardous.

The current legislation of many countries worldwide is not allowing the use of synthetic fungicides before fruit harvest as well as on the crops under organic agriculture practices. Additionally, the awareness of consumers also demands the organically fructified vegetable and fruits free from chemical fertilizers (Feliziani *et al.,* 2015). On one side we need to control the disease in order to increase the crop yield but at the same time the excessive use of chemicals is contaminating our environment therefore, there is a need of sustainable and effective control of plant disease by implementing the green technology with the use biological pest control measures. For this there is an urgent need to explore new fungicides and control tools that have a broad range of structural classes and selective mode of action with lesser side effects (Sinha *et al.,* 2017). Bio-pesticides are getting more attention to control plant pathogens as they are naturally occurring substance and work in eco-friendly manner without any disruption to the ecosystem (Mishra *et al.,* 2015). Bio-fungicides can be grouped into different categories including naturally occurring antifungal compounds (Gerwick and Sparks, 2014).

The use of antifungal proteins (AFPs) isolated from microbes and plants evidences the efficacy of exogenous application of AFPs for control of fungal infections in plants (Theis *et al.,* 2005). However, the spraying of plant extracts or purified protein solutions in large quantities might be of concern in term of production, cost, effectiveness and allergenic to workers. The current review attempts to provide an overview on the PR proteins, their classification, role in respect to antifungal activities and brief application of antifungal proteins.

ANTIFUNGAL PROTEINS (AFPs)

These are the low molecular weight proteins and peptides produced as defence proteins against fungal infection that appear to be involved in either constitutive or induced resistance to pathogenic fungal attack (Hegedüs and Marx, 2013). AFPs are the group of broad-spectrum plant defense proteins that play a key role in plant defense against pathogenic fungi by preventing or limiting their spread (Lacerda *et al.,* 2014). Generally, these proteins are race or species specific and shows, a range of antifungal activity *viz.* inhibition of cell-wall synthesis, its structural disruption, liberation of chitin oligosaccharides from cell wall, damage to cellular ribosomes, inhibition of cell cycle and DNA synthesis, disruption of membrane structure, membrane channel or pore formation leading to leaking of cytoplasm and fungal cell lysis, adversely affect the fungal hyphae growth etc. (Perfect, 2017). There are hundreds of AFPs are known today, with more being discovered almost daily (Solanki *et al.,* 2018). Some examples of AFPs are PR-1 proteins, 1,3, β-glucanases, chitinases, chitin-binding proteins, thaumatin-like (TL) proteins, defensins, cyclophilin-like protein, glycine/histidine-rich proteins, ribosome-inactivating proteins (RIPs), lipid-transfer proteins (LTPs), killer proteins (killer toxins), protease inhibitors, defensin like proteins,

deoxyribonuclease, embryo-abundant protein and other proteins (Ng, 2004; Sels *et al.,* 2008, Wong, *et al.,* 2010; Yan *et al.,* 2015). The nomenclature of these AFPs is based on their origin, nature, mechanism of action, structure or similarity to a known type protein (Sagaram *et al.,* 2012). These AFPs are also classified under pathogenesis-related (PR) proteins (Golshani *et al.,* 2015), the term coined by Antoniw *et al.* (1980). The PR proteins are the group of plant-encoded proteins induced by different stress stimuli and play an important role in plant defense against pathogens (Jain and Kumar, 2015).

PR proteins are basically protein expressed in response to salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) as a part of systemic acquired resistance (SAR) in plants (Ferreira *et al.,* 2007; Golshani *et al.,* 2015). The PR proteins have been classified into various families (**Table 1**) based on the sequence homology and biological activity of the induced defense proteins (Golshani *et al.,* 2015). Additionally, the isoelectric points of the PRs are further considered as an important parameter for sub group of classification (Sudisha *et al.,* 2012). The migration of these PRs on native polyacrylamide gel electrophoresis (PAGE), reaction with specific antisera and mRNA probes (Choi *et al.,* 2015) can also be considered as an important classification criterion. On the basis of above-mentioned parameters, there are seventeen different groups of PRs and other non-classified PR proteins (Selitrennikoff, 2001; Ferreira *et al.,* 2007; Sels *et al.,* 2008; Sinha *et al.,* 2014).

Table 1: Families of pathogenesis-related proteins and their functional properties.

S. No.	PR-Protein family	Properties
1.	PR1	Antifungal
2.	PR2	β-1 3-glucanases
3.	PR3	Chitinases (Class I, II, IV, V, VI, VII)
4.	PR4	Chitinases (Class I, II)
5.	PR5	Thaumatin-like proteins
6.	PR6	Proteinase inhibitor
7.	PR7	Endoproteinase
8.	PR8	Chitinase (Class III)
8.	PR9	Peroxidase
10.	PR10	Ribonuclease-like proteins
11.	PR11	Chitinase (Class I)
12.	PR12	Defensin
13.	PR13	Thionin
14.	PR14	Lipid-transfer protein
15.	PR15	Oxalate oxidase
16.	PR16	Oxidase-like
17.	PR17	Antifungal and antiviral

PR-1 proteins

The adequate amount of protein found to be accumulated upon invasion of pathogens in numerous plants (Hegde and Keshgond, 2013), although, the exact mechanism of action is not known (Ferreira *et al.,* 2007; Sinha *et al.,* 2014). The protein with 15 to 17 kDa mass and structural homology with cysteine-rich proteins is reported to have antifungal properties at micro-molar level against number of plant pathogenic fungi, including *Uromyces fabae*, *Phytophthora infestans* and *Erysiphe graminis* (Niderman *et al.,* 1995; Doehlemann and Hemetsberger, 2013). PR-1 proteins are further sub-divided into acidic and basic PRs (Sudisha *et al.,* 2012). The acidic PR-1 proteins are soluble in acidic buffers and have comparatively low (1416 kDa) molecular weights (Mohamed and Sehgal, 2018). These acidic PRs are reported from tobacco, tomato, barley, maize, parsley and also plants belonging to Graminae, Solanaceae, Chenopodiaceae and Amaranthaceae families and found to be effective against *Phytophthora sojae, Fusarium oxysporum* and *Colleto-trichum trifolii* infections (Sudisha *et al.,* 2012). The basic PR-1 proteins or PRB-1b contains a hydrophobic N-terminal region of 30 amino acids as a signal peptide for the translocation to the endoplasmic reticulum (Sudisha *et al.,* 2012). The PRB-1b protein isoforms have been identified in maize, *Arabidopsis* and celery (Sinha *et al.,* 2014).

PR-2 proteins

These proteins with β-1,3 glucanase activity are also known as β-glucanases and catalyses the endotype hydrolytic cleavage of 1, 3 β-D-glucosidic linkages in β-1,3 glucans (Antoniw *et al.,* 1980; Gupta *et al.,* 2013). The chitinase and β-1,3-glucanase have been isolated from pea pods exhibiting synergistic effect against fungal spore germination. The AFP have been found in a wide variety of plants, including tomato, wheat, barley, pearl millet, rice, chickpea, soybean, maize, tobacco, peas and *Arabidopsis* (Cote *et al.,* 1991; Kim and Hwang, 1997; Lacerda *et al.,* 2014). Selitrennikoff (2001) on the basis of amino acid sequence, PR-2 proteins are grouped into four classes. Class-I are basic proteins of 33 kDa reported from plant vacuoles. Classes II and III are acidic proteins of 36 kDa and do not have the C-terminal extensions but contain a signal for targeting into the vacuoles (Nawrot *et al.,* 2014). The class IV glucanases were isolated from tobacco species with molecular weight of 35 kDA. Class I and II glucanases were found 50250 times more potent in degrading β-1,3 glucan substrate then class III and IV (Selitrennikoff, 2001; Balasubramanian *et al.,* 2012). A β-1, 3-glucanase of 65-66 kDa consisting of three sub-units with non-covalent bond conjugate has been isolated from *Jatropha curcas* using column chromatography. The protein with isoelectric point of 8.3 possessed antifungal activity against *Rhizoctonia solani* and *Gibberella zeae* under in vitro conditions (Wei *et al.,* 2005).

The PR-2 proteins at micro-molar levels (50 mg/ml) found active against a wide number of fungi viz., *Rhizoctonia solani*, *Candida albicans, Aspergillus fumigatus* in *in-vitro* (Yan *et al.,* 2015). The antifungal activity of PR-2 proteins has been convincingly demonstrated by a number of in-vitro enzyme and whole-cell assays using a transgenic plant over-expressing the protein (Veluthakkal and Dasgupta, 2010). The antifungal activity of PR-2 proteins that hydrolyze the structural 1,3 β-glucan present in the fungal cell wall, particularly at the glucan exposed hyphal apex of filamentous molds, results into weakening of cell wall that leads to cell

lysis and consequently cell death (Gamir *et al.,* 2017).

PR-3 proteins

PR-3 proteins are endo-chitinases that cleaves glycosidic bond between N-acetyl glucosamine (NAG) monomers resulting in a weakened cell-wall rendering fungal cells osmotically sensitive. PR-3 proteins have molecular weight between 26 and 43 kDa (Nielsen *et al.,* 1997; Stoykov *et al.,* 2015). PR-3 chitinases have been divided into five groups; class I, II, IV, VI and VII. (Selitrennikoff, 2001; Demain, 2014). Class I have molecular mass of 32 kDa, contain an N-terminal cysteine-rich domain of 40 amino acids and a highly conserved central region of chitin binding hevein-like domain separated by a hinge region (Rathore and Gupta, 2015). Class II proteins are identical to class I but lacks hevein-like domain and have molecular mass of 27-28 kDa (Kesari *et al.,* 2015). Class IV are similar to class I chitinases but are significantly smaller due to major deletions in the four-chitin binding domain (CBD) (Sudisha *et al.,* 2012). Class VI only representative identified from sugarbeet lacks four out of eight cysteines in the CBD and has the longest spacer regions with 135 amino acids, out of which 90 are prolines (Martínez-Caballero *et al.,* 2014). Class VII is the only known representative present in rice and these chitinases lacks CBD but has high homology with cDNA of the Class IV PR-3 proteins (Patel and Goyal, 2017). Chitinases isolated from plants like tobacco, cucumber, beans, peas, grains etc. showed potent antifungal activity against various plant pathogens, including *Trichoderma reesei, Alternaria solani, Alternaria radicina, Fusarium oxysporum, Rhizoctonia solani, Guignardia bidwellii, Botrytis cinerea* and *Coprinus comatus* (Kumar *et al.,* 2018). Smilarly, Shenoy *et al.* (2006) reported a chitinase antifungal proteins with molecular weight of 29 kDa isolated from the bulbs of the Indian squill; *Urginea indica*. The protein had sequence similarity to the sequences Class II chitinase of *Hordeum vulgare* and lacks Lys-rich N-terminal domain typical of Class I chitinases. It showed antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani*.

PR-4 proteins

PR-4 are 13-14.5 kDa chitin-binding proteins and have been classified into two classes (Selitrennikoff, 2001). Class I have sequence similarity to hevein and belong to the super family of chitin-binding lectins whereas, class II lack the chitin-binding hevein-like domain (Mendoza-Figueroa *et al.,* 2014). These proteins have been isolated from potato, tobacco, barley, tomato etc. and are effective against variety of plant pathogens viz., *Trichoderma harzianum, Fusarium culmorum, Fusarium graminearum* and *Botrytis cinerea* (Singh *et al.,* 2014). The mechanism of action of class I proteins includes binding to nascent β-chitin results in disruption of cell polarity and integrity with concomitant inhibition of growth leading, to inhibition of hyphal growth (Bormann *et al.,* 1999; Roncero *et al.,* 2016). Fungi inhibited by these proteins include *Paecilomyces variotii, Aspergillus* spp., *Fusarium oxysporum, Neurospora crassa, Botrytis cinerea* and *Alternaria brassicola* (Pusztahelyi *et al.,* 2015; Yan *et al.,* 2015).

PR-5 proteins

These are 22 kDa proteins also known as thaumatin-like proteins (TLPs) remains localized in roots, corolla, flower buds and tissues of over-ripen cherries additionally, seeds of several cereals are known to contain TLPs. An antifungal TLP of 20 kDa has been isolated from *Phaseolus vulgaris* using affinity and ion exchange chromatography found effective against *Fusarium oxysporum, Pleurotus ostreatus* and *Coprinus comatus* (Ye *et al.,* 1999). Similarly, Wang and Ng (2004) isolated and purified an antifungal protein of 10 kDa through DEAE-cellulose and Affinity-gel chromatography from *Pleurotus eryngii*. The N-terminal sequences of protein showed similarity with AFP isolated from *Lyophyllum shimeiji* and showed resemblance to thaumatin and TLPs. The protein inhibited mycelial growth of *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

PR-5 proteins have been isolated from corn, soybean, rice, wheat, tobacco, tomato, pumpkin, beans, barley, flax and many other plants (Selitrennikoff, 2001; Gohel *et al.,* 2006). TLPs share significant amino acid sequence homology within the fruits of the tropical plant *Thaumatococcus daniellii* (Kumar and Venkatesh, 2014) found to be extremely soluble and accumulates vigorously in selective tissues or sub cellular and extracellular compartments of cells (Gupta *et al.,* 2015). The mechanism of action for these proteins is not fully known but have number of hypothetical observation where these retarding the fungal growth (Ibeas *et al.,* 2000; Liu *et al.,* 2010). For example, several TLPs are known to cause permeability changes in fungal cell-wall without affecting the protoplasts (Van der Weerden *et al.,* 2013).

PR-6 proteins

These are grouped under proteinase inhibitors and are most-stable defense proteins regulated developmentally and induced in response to insect and pathogen attack (Sudisha *et al.,* 2012). The proteinase inhibitors are classified into three major group based on the proteinase-substrates of pathogens they inhibit i.e., serine, cysteine and aspartate/metallo-proteinase inhibitors (Sudisha *et al.,* 2012). Serine proteinase inhibitors have potential to react with different proteinase (Doares *et al.,* 1995; Dobó *et al.,* 2016). Cysteine proteinase inhibitors are 12-16 kDa in mass and inhibit papain and cathepsin proteinase (Koiwa *et al.,* 1997; Jimenez-Sandoval *et al.,* 2017). The members of the metallo-proteinase have been found in tomato and potato and cleave exopeptidase produced by pathogenic fungi (Van Loon, 1999; Adhikari *et al.,* 2017).

PR-7 proteins

These endo-proteinase are exclusively characterized in tomato that degrades cell wall proteins and carries out the hydrolysis of chitin along with glucan (Goldman and

Goldman, 1998; Haran *et al.,* 1996). The protein assumed to have accessory action to antifungal potential and has many homologous sequences that shows relevance with subtilisin like protease responsible for disease resistance response to pathogen in tomato (Tornero *et al.,* 1997; Shahid *et al.,* 2015). However, the mechanism of action and its homologues are not clearly understood.

PR-8 proteins

It is categorized under chitinase class III group of pathogenesis-related proteins with supplementary lysozyme function (Metraux *et al.,* 1989; Fister *et al.,* 2016) characterized in cucumber, tobacco, chickpea and *Arabidopsis* (Sudisha *et al.,* 2012). The proteins show sequence homology and substrate specificity-based differences and occurs in both acidic and basic forms. Hevamine is the best characterized PR-8 chitinase from tobacco (Neuhaus, 1999; Van Loon, 1999; Tam *et al.,* 2015). It has been reported that PR-8 exhibit lysozyme activity and disrupt gram-positive bacteria (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001).

PR-9 proteins

This group includes PR proteins which exhibits peroxidase activity. These are heme-containing-glycoproteins catalysis oxidation of hydrogen peroxide (H_2O_2) into diverse range of organic and inorganic substrates (Sudisha *et al.,* 2012). Several isoforms of protein occur in plants and animals involved in a range pathogen related and non-related physiological defense processes (Sudisha *et al.,* 2012). Three broad classes of plant peroxidases have been identified based on their localization and action. Class I includes cytochrome-c and ascorbate peroxidase; Class II comprises an extra cellular fungal like peroxidase whereas, class III contains Hrp C (an extra cellular plant peroxidases) (Passardi *et al.,* 2005). More than sixty-isoforms of peroxidase have been isolated and purified from different sources under both abiotic and biotic stress conditions. The plants PR-9 peroxidases have two structural domains with a central heme group and conserved catalytic sites where isoleucine and phenylalanine are commonly involved in substrate binding (Sudisha *et al.,* 2012). The two catalytic domains containing the proximal and the distal heme binding regions are created from ten helices and three sheets like secondary structure. Amino acids like arginine, asparagine and aspartate are associated in the peroxidase specific catalysis (Ziadi *et al.,* 2001; Suklavoic *et al.,* 2003; Falade *et al.,* 2017).

Lignin forms an extensive network of aromatic structures with cross links in plant cell-walls and thus confers mechanical strength. Peroxidase reinforces the plant cell wall by catalyzing deposition of lignin and this peroxidase driven lignification increases during the fungal infection and wounding (Lagrimini, 1991; Verma and Dwivedi, 2014). Further, the reactive oxygen species like H_2O_2 released as by-product of cell wall lignifications are also toxic to pathogens (Thordal-Christensen *et al.,* 1997; Karkonen and Kuchitsu, 2015) and can act as intracellular messengers to trigger other defense responses such as synthesis of other pathogenesis related proteins (Levine *et al.,* 1994; Camejo *et al.,* 2016). A peroxidase of 34 kDa with antifungal activity toward *Fusarium solani, M. arachidicola* and *Pythium aphanidermatum* has been isolated from lima bean seeds (Wang *et al.,* 2009a; 2009b). Similarly French bean legumes produce a 37 kDa peroxidase and exhibit inhibitory activity on mycelia growth of *Botrytis cinerea, F. oxysporum,* and *M. arachidicola* (Ye and Ng, 2002a).

PR-10 proteins

It includes intracellular defense proteins with ribonuclease like activity and structure. The synthetization of these proteins are induced in response to pathogens attacks in potato, asparagus, bean, rice and pearl millet (Sudisha *et al.,* 2012). They are acidic in nature without any peptide and are intracellularly localized (Shivkumar *et al.,* 2000; Hillwig *et al.,* 2011). Significant homology exists between various members of the PR-10 group of pathogenesis-related proteins (Vidhyasekaran, 2002; Hwang *et al.,* 2003; Agarwal and Agarwal, 2014). Ribonucleases exhibiting antifungal activity have been isolated from American ginseng, ginseng, and sanchi ginseng (Lam and Ng, 2001; Ng *et al.,* 2002; Wang and Ng, 2001; Shin *et al.,* 2015, Im *et al.,* 2016). Deoxy-ribonucleases probably acts by hydrolyzing DNA of invading foreign organisms. A 30-kDa asparagus DNase with a novel N-terminal sequence eliciting antifungal activity against *Botrytis cinerea* has been isolated (Wang and Ng, 2001; Vriens *et al.,* 2014; Yan *et al.,* 2015).

PR-11 proteins

These group of proteins have higher affinity towards zinc and are unique group of proteins as they don't show resemblance with any known chitinase group (Sudisha *et al.,* 2012). Firstly, identified in tobacco and have only known molecular homologue in pepper. The ultraviolet radiation and viral infection trigger the synthesis of 18 kDa protein that lacks characteristic chitin binding domain (Heitz *et al.,* 1994; Bravo *et al.,* 2003; Hamid *et al.,* 2013).

PR-12 proteins

These are the defensins, small (5 kDa) basic antimicrobial peptides of 41-54 amino acids containing eight cysteine including one C-terminus residues, for ease detection. The structure comprises a triple stranded sheets and one helix, stabilized by disulfide bonds (Sudisha *et al.,* 2012). These proteins have been identified in various plant species especially found located in the peripheral cell layers/ xylem (Lacerda *et al.,* 2014). These proteins are generally not found within the healthy tissues but accumulate systemically after localized fungal or bacterial infection (Pennecks *et al.,* 1996; Lv *et al.,* 2016). The polypetide sequence-based classification suggests four groups of defensins (Conceiaco and Broaekart, 1999; Cools *et al.,* 2017). Group I (morphogenic defensins) causes morphological changes in susceptible fungi, group-II includes proteins that inhibits fungal growth without

morphological changes, group III inhibits α-amylases only under *in vitro* conditions, while group IV is exclusive in terms of antifungal specificity and structure (Segura *et al.,* 1998; Rautenbach *et al.,* 2016). PR-12 have a broad spectrum as they check the growth of wide range of plant fungal pathogens, including *Botrytis cinerea, Alternaria brassicola, Fusarium culmorum, Fusarium oxysporum, Fusarium solani* and *Candida albicans* at micro-molar levels (Selitrennikoff, 2001; Yan *et al.,* 2015).

PR-13 proteins

These are cysteine rich 5 kDa proteins isolated from roots and leaves of oats, rye, maize, tomato, barley and papaya (Sudisha *et al.,* 2012). These proteins have constitutive expression upon pathogen infection and are classified into four classes on the basis of number of cysteine residues and disulfide bonds (Sudisha *et al.,* 2012). The class I with eight cysteine residues purothionin/hordothionin/barley leaf thionin. Class II thionins lacks cysteine number three and six-viscotoxin, class III lacks cysteine number two and eight whereas, class IV thionins lack cysteine two (Ji *et al.,* 2015). The precursor for thionins is comparatively larger (15 kDa) with some conserved regions as 61-tyrosine and 65-glycine including six-cysteine residues which are absolutely conserved (Plattner *et al.,* 2015). The crystal structure of protein is compact and stabilized by three to four disulfide bonds. Thionins are amphipathic with phospholipid-binding sites and remains distributed in the cell walls, vacuoles and protein bodies (Olendo *et al.,* 1999; Svetlana *et al.,* 2012). Antifungal action includes direct protein-membrane contacts through electrostatic interaction of cationic thionin and anionic phospholipids in fungal membranes, ensuing in pore formation or a specific interaction with certain lipid domain (Asano *et al.,* 2013).

PR-14 proteins

Lipid transfer proteins (LTPs) have been classified as PR-14 group of pathogenesis-related proteins. These proteins are lipid shuttlers between cell-organelles. LTPs are small globular proteins (8.7 kDa) of 90 amino acids with four alpha helices stabilized by same number of disulfide bonds with a central tunnel-like hydrophobic cavity that may accommodate a wide variety of lipids which helps in lipid loading and transfer (Olendo *et al.,* 1999; Salminen *et al.,* 2016). Velazhahan *et al.* (2001) purified a 25 kDa antifungal protein from the seeds of pearl millet. The N-terminal sequences of the protein showed homology to non-specific LTPs of cotton, wheat and barley. The purified LTPs inhibited mycelial growth of *Trichoderma viride* and *Rhizoctonia solani*.

These proteins have also been isolated from a number of plants, bacteria, animals that may play several roles in vivo including, defense against pathogens. The proteins are also capable in transferring phospholipids between membranes and showed effectivity against a number of pathogens (Selitrennikoff, 2001). The actual mechanism of action is not known but these proteins insert themselves into the fungal cell membrane subsequently, the central cavity forms a pore that efflux's the intracellular ions and causes fungal cell death (Selitrennikoff, 2001; Finkina, *et al.,* 2016). Furthermore, LTPs transfer acyl monomers for synthesis of cutin and this extracellular lipophilic coating covers aerial surface of plants which protect the plants from pathogens (Kader, 1996; Wong *et al.,* 2010). *Brassica campestris* seeds produce a 9.4 kDa LTP with potent antifungal activity against *M. arachidicola* and *F. oxysporum* in dose dependent manner (Lin *et al.,* 2007). A number of nonspecific small LTPs (ns-LTPs) exhibited both antibacterial and antifungal properties *in vitro* have been studied. Some ns-LTPs in pollen, latex, vegetables, fruits, and nuts are allergens (Egger *et al.,* 2010).

PR-15 proteins

These are oxalate-oxidases with sequence similarities to wheat germins (Zhang *et al.,* 1995). The molecular weight is 22-25 kDa and are isolated from germinating barley, corn, oat, rice, rye and other cereals and dicot plants (Sudisha *et al.,* 2012). The protein has heteropentameric secondary structure and are secreted into the extra cellular spaces (Chipps *et al.,* 2005). These proteins are glycoprotein in nature, protease resistant, superoxide dismutase insensitive and are responsible for generation of reactive oxygen species (ROS) immediately after pathogen infection (Xu *et al.,* 2003).

PR-16 proteins

The proteins are oxalate oxidase like protein and has been isolated from barley (Wei *et al.,* 1998) and hot pepper during the resistance response to bacterial and viral infection. The nature of PR-16 is similar to PR-15 but with comparatively higher molecular weight (100 kDa) (Ferreira *et al.,* 2007).

PR-17 proteins

This category of proteins has been characterized from cDNA of barley, its only representative and the central C-terminal part of the deduced amino acid sequence has five highly conserved domains. They share sequence similarities with aminopeptidases from the eukaryotes and thermolysins from bacteria suggesting a proteolytic like activity (Tam *et al.,* 2015). Proteins of PR-17 family shows affinity towards zinc and therefore, is similar to zinc metalloproteinases. The C-terminal A to E domains are highly conserved and are similar to thermolysins. Domain A has protein kinase C phosphorylation site and B has conserved similarities with aminopeptidases (Christensen *et al.,* 2002; Sudisha *et al.,* 2012).

UNCLASSIFIED PATHOGENESIS-RELATED PROTEINS

The novel PR-proteins having antifungal activity are being discovered at a rapid pace but have not been catalogued yet under above groups. A brief account of some of these proteins are given below.

Cyclophilin-like protein

Cyclophilins are a highly conserved proteins and are

intracellular receptors for cyclosporine. The protein is a mutase, catalyses *cis-trans* isomerization of imide bonds in polypeptides and are involved in protein folding and cell communication (Pliyev and Gurvita, 1999; Piotukh *et al.,* 2005). High molecular weight cyclophilins binds and activates steroid receptors (Cunningham, 1999; Silverstein *et al.,* 1999; Piotukh *et al.,* 2005).

Cyclophilins also promotes assembly of multiprotein complexes that comprises a protein kinase or a phosphoprotein phosphate or both (Cunningham, 1999; Dawar *et al.,* 2017). The proteins have an extensive distribution among bacteria, plants and animals (Ostoa-Saloma *et al.,* 2000; Dawar *et al.,* 2017). Cyclophilin-like antifungal proteins have been isolated from black eyed pea (Ye and Ng, 2001) and chickpea (Ye and Ng, 2002b). Similarly, the protein mungin (18 kDa) from mung bean (*Phaseolus mungo*) with significant sequence homology to cyclophilins was found to inhibit α- and β-glucosidases *in vitro* and exhibited resistance against *Rhizoctonia solani, Fusarium oxysporum, Botrytis cinerea* and *Coprinus comatus* (Ye and Ng, 2000; Tang *et al.,* 2014).

Glycine/histidine-rich proteins

These proteins are extensively composed of glycine and histidine, comprising over 80% of these amino acids. The proteins are found to inhibit the most common human pathogen *Candida albicans* (Dae-Hee *et al.,* 1998) although the mechanism of action is not known.

Ribosome-inactivating proteins (RIPs)

RIPs are RNA N-glycosidases that depurinate rRNA, resulting in protein synthesis inhibition (Barbieri *et al.,* 1993; Hwu *et al.,* 2000, Zhu *et al.,* 2018). Roberts and Selitrennikoff (1986) extracted RIPs from barley seeds (*Hordeum vulgare*) that showed growth retardation of *Trichoderma reesei* at a minimal concentration (120 µg/ml). Plant RIPs inhibit mammalian, bacterial, fungal and plant protein synthesis under both *in vitro* and *in vivo*. RIPs have been classified into three types. Type-I are single-chain N-glycosidases with molecular masses of 11 to 30 kDa, type-II contains two chains, a cell-binding lectin (B chain) and N-glycosidase (A chain), with molecular masses of 60 kDa whereas, type 3 comprises four chains organized as two dimers of type-II RIPs. RIPs have been isolated from a number of plants species viz., *Mirabilis expansa, Pisum sativum, Momordica charantia, Ricinus communis, Viscum album* and many others (Rust *et al.,* 2017). As far as the mechanism of action is concerned, studies on type-II RIPs suggests the cell-binding B chain (lectin) binds to fungal cells, forming a channel allowing the N-glycosidase to enter into cells, resulting in RNA damage (Zhang *et al.,* 1999; Xia and Sui, 2000; Khan and Khan, 2011).

2S albumins

These are glutamine rich low molecular weight storage proteins with similar physicochemical properties present in monocotyledonous and dicotyledonous seeds (Youle and Huang, 1981; Horax *et al.,* 2010). The proteins have two unequal subunits linked together with disulfide bridges derived from a single precursor polypeptide (Krebbers *et al.,* 1988; Khan *et al.,* 2016). The smaller subunits contain the antifungal properties (Terras *et al.,* 1993; Moreno and Clemente, 2008). An antifungal peptide (5 kDa) with sequence homology to storage 2S albumins has been isolated from seeds of the passion fruit (*Passiflora edulis*) showed, antifungal activity against *Aspergillus fumigatus, Fusarium oxysporum* and *Trichoderma harzianum* (Pelegrini *et al.,* 2006). Similarly, a 2S albumins homologue has also been isolated from chilli (*Capsicum annuum*) seeds (Ribeiro *et al.,* 2007; Meneguetti *et al.,* 2017).

Lectins

Glycoproteins that recognize and bind reversibly to carbohydrate moieties of complex glycoconjugates, inhibit fungal conidial germination, alter germ tubes and thus inhibits hyphal growth (Allen *et al.,* 1973; Lotan *et al.,* 1975; Mirelman *et al.,* 1975; Santos *et al.,* 2012). Plants generally produce lectins as a part of their defense mechanism against pathogenic fungal species (Keen, 1992; Jandú *et al.,* 2017) for example a chitin binding lectin having antifungal properties has been studied from stinging nettle rhizome, *Urtica dioica* (Broekaert *et al.,* 1989). Antifungal-lectins are also been reported from *Phaseolus vulgaris* (Ye *et al.,* 2001) and in *Amaranthus viridis* (Kaur *et al.,* 2006). The 14.5 kDa mannose binding lectin from *Dendrobium findlayanum* exhibits antifungal activity against *A. alternata* and *Colletotrichum* sp. (Sattayasai *et al.,* 2009). Similarly, a 30 kDa lectin with antifungal activity against major phytopathogens has been isolated from leaves of *Withania somnifera* (Ghos, 2009).

Embryo-abundant protein- like proteins

Also known as late embryogenesis abundant proteins (LEA) are hydrophilic stress proteins, produced under the most desiccated conditions. These mitochondrial proteins are found abundantly in seeds and accumulates in drought tolerant organisms (Tolleter *et al.,* 2007). The antifungal protein ginkbilobin (13 kDa) from *Ginkgo biloba* seeds (Wang and Ng, 2000) with N-terminal sequence homology to white spruce embryo-abundant protein, exhibited strong antifungal action against *B. cinerea, Coprinus comatus, F. oxysporum, M. arachidicola* and *R. solani*.

Polygalacturonase inhibiting protein-like activity

These proteins remain associated with the cell wall inhibited fungal endopolygalacturonases (Bergmann *et al.,* 1994; Liu *et al.,* 2017). A 36-kDa protein, with N-terminal sequence homology to polygalacturonase-inhibiting proteins without polygalacturonase inhibiting activity, was purified from small brown-eyed cowpea seed. The protein exerted antifungal activity against *M. arachidicola* (Tian *et al.,* 2013). The severity of damage caused by *Phytophthora capcisi* was reduced in transgenic tobacco plants expressing pepper

polygalacturonase-inhibiting protein (Wang *et al.,* 2013).

Puroindolines

The puroindolines are 13-kDa basic wheat endosperm proteins with a tryptophan-rich domain and five disulfide bonds. The proteins might be the membrane toxins with a role in defense against microbial pathogens (Charnet *et al.,* 2003; Sanders *et al.,* 2017). Dubreil *et al.* (1998) reported the antifungal activity of these proteins against *Alternaria brassicola, Ascochyta pisi, Fusarium culmortun,* and *V. dahliae*. Transgenic rice with constitutive expression of puroindoline genes (pinA and/or pinB) showed fewer symptoms in response to the rice blast (*Magnaporthe grisea*) and sheath blight fungus (*R. solani*) (Krishnamurthy *et al.,* 2001).

Killer proteins (Killer toxins)

Killer toxins are glycosylated proteins produced by yeast and some other fungal species that bind to specific receptors on the surface of their target microorganism to kill them through a target-specific mode (Mannazzu *et al.,* 2019). The molecular weights of killer proteins (toxins) range from 1.8 to >150 kDa. The production of killer protein is prevalent among yeasts and near about hundred yeast killer species have been identified till date. The examples of most well-characterized killer toxins are K1, K2, and K28 of S. cerevisiae, PaKT of *Wickerhamomyces anomalus*, PMKT and PMKT2 of *Pichia membranifaciens*, Kpkt of *Tetrapisispora phaffii, zymocin* of *Kluyveromyces lactis* and HM-1 of *Cyberlindnera mrakii* (Kasahara *et al.,* 1994; Schmitt *et al.,* 1996; Magliani *et al.,* 1997; Santos and Marquina, 2004; Santos, *et al.,* 2007; Orentaite *et al.,* 2016; Gier *et al.,* 2017). Killer strains have biocontrol potential against various phytopathogens such as *Colletotrichum gloeosporioides* (Lima *et al.,* 2013), *Penicillium digitatum, P. italicum* and *B. cinerea* (Platania *et al.,* 2012; Parafati *et al.,* 2016). *Debaryomyces hansenii* can be used to control the growth of *Monilinia fructicola* and *Monilinia fructigena* (Grzegorczyk *et al.,* 2017). Despite of their wide diversity, the mechanism of killer toxins is a two-step, in the first step killer toxin binds to specific cell surface receptors of target organism. In second step, killer proteins kill the cells by various mechanism including permeabilization and disruption of cell wall synthesis, inhibition of DNA synthesis, disruption of K+ channel activity, inhibition of (1,3) β-glucan synthesis, or by halting the cell cycle (Kimura *et al.,* 1997, 1999; Ahmed *et al.,* 1999; Suzuki and Shimma 1999; Eisfeld *et al.,* 2000).

APPLICATION OF ANTIFUNGAL PROTEINS

Novel biofungicides

Antifungal proteins are considered as agrichemical biofungicides. They have a high potential for agricultural therapeutic application for biocontrol of pathogenic microbes. They have the potential to be eco-friendly-alternatives for harmful pesticides and may promote sustainable agriculture practices towards green farming (Bonaterra *et al.,* 2012). Antifungal protein from *Aspergillus giganteus* on rice leaves has been used to control infection by *Magnaporthe grisea* (Vila *et al.,* 2001). An antifungal protein from *A. giganteus* also protected tomato plants from infection by *F. oxysporum* (Theis *et al.,* 2005).

Genetically modified crops

AFPs genes have been introduced into agriculture crops to uplift the plant defense system against pathogenic fungal invasions. A wide range of transgenic crops with expression of antifungal proteins showed augmented resistance against pathogenic fungi. Recombinant DNA technology nowadays allows to incorporate two or more antifungal gene in specific crop that results in effective and broader-spectrum disease control mechanism over single gene strategy (Chen *et al.,* 2016). Recombination of antifungal genes in various cash crops viz., banana, cotton, groundnut, mustard, potato, rice and tomato has been carried out to create genetically modified crops exhibiting increased fungal pathogen resistance (Cletus *et al.,* 2013). The overexpression of class I chitinase and β-1, 3-glucanase (PR-3 and PR-2 family, respectively) from tobacco in tomato achieved greater resistance for fungal pathogens. Similarly, a transgenic carrot plant containing the same genes has shown a high level of resistance against major fungal pathogens of carrots. Additionally, constitutive overexpression of tobacco class I, PR-2 and PR-3 transgenes in potato plants enhanced their resistance to *Phytophthora infestans* (Bachmann *et al.,* 1998). Similar results from co-expression of chitinase and β-1, 3-glucanase in plant disease resistance are reported by Kombrink *et al.* (2001). Increased resistance to crown rust disease in transgenic Italian ryegrass expressing the rice chitinase gene was demonstrated (Takahashi *et al.,* 2005). Other alternative strategy includes the gene-engineering of PR-5 for improvement of crop disease resistance through potent plasmolyzing and antifungal effect. Overexpression of cloned rice thaumatin-like (PR-5) gene in transgenic rice plants improved resistance to *Rhizoctonia solani* causing sheath blight disease in eco-friendly manner (Datta *et al.,* 2001). Similarly the gene chitinase could be used to enhance fungal resistance in tobacco, rice, clover and tea crops (Kirubakaran *et al.,* 2007). However, commercial cultivation of genetically modified crops has been governed by various statutory bodies. Nevertheless, the safety issue of genetically modified crops has been addressed and possible solution has been proposed (Ghosh *et al.,* 2013; Wang, *et al.,* 2014).

Food preservatives

The demand for preservatives from natural sources has been amplified in recent years and therefore, water-soluble antifungal seed proteins are being used as preservatives not only in bakery but also in other food processing industries. This may be the most immediate application of plants AFPs although; the issue of potential allergenicity of plant proteins to human being remains a concern (Mirabella *et al.,* 2014; Axel *et al.,* 2017). A case study revealed that water-soluble

extracts from *Amaranthus* seed has potent antifungal activity against *Penicillium roqueforti*, a fungus isolated from contaminated bread (Rizzello *et al.,* 2009). This fungus is a major food spoiler and is somewhat resistant to chemical antifungal preservatives. Therefore, AFPs sourced from edible seeds seem to be promising and low-cost food preservatives for food industries.

CONCLUSION

The application of synthetic agrochemicals is a common agriculture practice in our conventional agriculture to counter the damage caused by pathogenic fungal plant diseases. The applications of these synthetic chemicals have various environmental and economic issues like high toxicity, poor target selectivity, resistance against pathogen, longer degradation period and are not cost effective. Therefore, there is a need of antifungal proteins from natural substances to control the plant pathogens as they work in eco-friendly manner without disrupting the ecosystem. The cost of extraction, purification, formulation, stability and on farm exposure of these antifungal proteins is the major concerns but if we draw our attention towards the cost and benefit ratio the benefits are always greater. Further, there is a need and scope for the implementation of strategy that can generate huge mass of these AFPs in a cost-effective manner.

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