

**Review Article**

**SOLANUM PROTEASE INHIBITORS AND THEIR THERAPEUTIC POTENTIALITIES: A REVIEW**

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**ABSTRACT**

Protease inhibitors (PIs) are diverse group of proteins with low molecular weight that are ubiquitous in all life forms. PIs are reducers of the physiological activity of proteases and fascinated the attention of biotechnological researchers. In the evolutionary course, plants have developed diverse adaptive mechanisms of defence against various unfavourable conditions including that of predators and pathogens. Phylogenetic relationships among diverse PI families like serpin, Bowman-Birk, cereal trypsin/ $\alpha$ -amylase inhibitor, proteinase inhibitor I, proteinase inhibitor II and cystatin have been evaluated. PIs evolution seems to occur through multiple interacting mechanisms and not commonly seen with other co-evolving molecules. Interaction of PIs produced by host organisms and the invasive/dietary protease of pathogens or parasites or predators, leads to a phylogenetic 'arms race' of rapid structural modulation in both interacting proteins. Further, the high rate of retention of gene duplication and inhibitory domain multiplication results the PI as potential model system to trace the basic evolutionary process of functional diversification. The mode of action of PI is either *via* inactivating the hydrolytic enzymes or depolarization of cell membrane of the pathogens thereby inhibiting its growth and invasion. Generally, PIs possess significant number of disulfide bonds due to cysteine residues that provide them resistance to heat, extremes of pH, and proteolysis. However, PIs have been extracted and purified only from few monocots and dicots plants. Currently, PI genes were used for developing insect-resistant transgenic plants for crop improvements. Classification of PIs over the last several years has been based on structural-functional relationships. This review bridges the gap between the folkloric uses of *Solanum* PIs, their diversity and biological potentialities.

**Keywords:** *Solanum*, Protease inhibitors, Biological potentialities, Diversity, Characterization

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**INTRODUCTION**

Plant PIs are small proteins with a molecular mass range of 4 to 85 kDa. Generally plant PIs are present in significant level in storage tissues, contributing up to 10% of the total protein content. The first report of PI in nature was revealed by Fermi and Pernossi [1] and are widely distributed in diverse tissues of animals, plants and microbes [2]. Many angiosperm families like Fabaceae, Poaceae, Cucurbitaceae, Solanaceae showed their presence with diverse structural forms. Mostly, PIs have been proven as effective defence molecules against pests and pathogens in *in vivo* experimentals and also reported their optimal expression in transgenic crops. Most PIs bond with specific active site of the proteases resulting in the formation of stable protease inhibitor complex and there by inactivating the catalytic reaction either by competitive or non-competitive mode of reactions. PIs have multiple functions by interfering with the proteolytic activity of the target proteases in the organisms. Diverse types of PIs have been isolated, characterized and evaluated for their biological potentials [3]. Mostly, PIs are produced in the organisms in response to various stresses such as pathogens, insects, wounding, and environmental parameters like salinity, heavy metals and temperature. The microbicidal mode of action of PI is either *via* inactivating the hydrolase enzymes or depolarization of plasma membrane of the invading organisms thereby inhibiting its growth and development. The insecticidal role of PIs is based on their inhibitory activities against the digestive enzymes of predators and pathogens resulting in to critical shortage of essential molecules or by interfering with important biochemical or physiological processes such as moulting or replication. Further, PIs play an important role in regulation of cell cycle, cell death, differentiation and immunity reactions [4].

Similarly, PIs have long been used as antinutritional, antimetastatic and anti-inflammatory molecules. The physiological functions of many plant PIs are largely inferred from their developmental and tissue specific expression patterns. The most extensively analyzed protease inhibitors in plants were serine and cysteine protease inhibitors. Miller *et al.*, [5] reported some small *in vivo* PIs derived

from the modification process of multidomain precursors. In addition, many plant PIs do not inhibit their own proteases but have specificities against animal or invading microbial enzymes. *Solanum* taxa showed this unique molecule with wide variations in terms of structure and function.

***Solanum* group**

*Solanum* (Solanaceae), a group of annual or short lived perennial herbaceous weeds, distributed throughout the temperate and tropical regions of the world. *Solanum* contributes the largest and most complex genus with more than 2,000 species. *Solanum* species represent nearly 1% of the world's angiosperm flora, which might bear tribute to its great antiquity and an extraordinary rate of speciation. This huge diversity makes *Solanum* an interesting life form in terms of its evolutionary and economic stand point of view. Examples of food plants in *Solanum* were potato, eggplant, naranjilla, jasmine nightshade and others [6]. Many species of *Solanum* were used as medicine to cure digestive, intestinal problems like stomach-ache, diarrhoea, piles, dysentery and also for various skin problems such as sores, boils, cuts, wound, bruises, fever and malaria, headache and rheumatism. Some species were stimulants whereas others have sedative properties. Furthermore, many species were employed against respiratory tract disorders such as cough, sore throat, bronchitis, asthma and urinary problems. Most of the medicinal attributes of the species was due to the presence of steroidal glycoalkaloids [7]. Similarly, *Solanum* species shows insecticidal and fungicidal properties. *S. nigrum*, the black nightshade, a noxious weed but effectively inhibit the gut proteinases of pests and could potentially be used in generating insect resistant transgenic plants.

***Solanum* protease inhibitors**

Generally, PIs are classified, based on the enzyme type they inhibit such as serine protease inhibitors and others [8]. The classification is also performed based on sequence homology like kunitz-type inhibitors. Further, they are also grouped in to different families based on their mass, architecture, number of disulphide bridges,

isoelectric points and mechanism of action. *Solanum* species represents repository of protease inhibitor (PIs) diversity.

Serine Protease inhibitors (SPI) represent the largest family of inhibitors distributed throughout in plants. They are low-molecular mass proteins (3–25 kDa) that inhibit trypsin and/or chymotrypsin. SPIs are classified into different classes on the basis of amino acid sequence and mechanism of interaction including  $\alpha$ -helical,  $\beta$ -sheet and a/b proteins, as well as small disulfide rich proteins. Based on their mechanisms of action, three types of SPIs were recognized like canonical, non-canonical inhibitors and serpins. SPIs such as trypsin, elastase, chymotrypsin are the most intensively studied groups [9].

Potato serine protease inhibitor (PSPI) is a heterodimeric double-headed crystal kunitz-type SPI. PSPI show a  $\beta$ -coiled structure with two reactive site loops comprising Phe75 and Lys95 residues [10]. It is further sub-divided into Potato inhibitor type-I (PI-I) and type-II (PI-2) families. PI-II showed inhibition against trypsin,  $\alpha$ -chymotrypsin and elastase. The potato (*Solanum tuberosum*) tuber is a rich source of SPIs and carboxypeptidase inhibitors. Potato inhibitor I, is a small pentameric molecule of 8 kDa protein. It is an inhibitor of chymotrypsin, subtilisin and trypsin. Further, potato also contains two other homologous SPIs such as PCI and PTI which are also homologous with inhibitor aubergine isolated from the exocarps of *Solanum melongena* and also from tomato and potato leaves [11].

SPIs from leaves were best analyzed from Solanaceous species, i.e., when leaves were wounded mechanically or through insects initiates the synthesis of diverse SPIs [12]. The newly synthesized inhibitors show a high degree of homology with potato I and potato II inhibitors already reported from potato tubers [13, 14].

Ryan and Balls [15] isolated potato inhibitor type-I from tubers of potato followed by Wingate *et al.*, [16] from tomato fruits and leaves

as a consequence of wounding [17]. It is a double headed molecule, each subunit possess an S-S bond capable of inhibiting trypsin and  $\alpha$ -chymotrypsin. Potato inhibitor II were reported from the leaves, flowers, fruit and phloem of Solanaceous species [18] and was also purified from tomato [19]. Purified PIs from *S. nigrum* was also characterized and evaluated in terms of its medicinal potentialities [20]. Kunitz-type PIs, the most common type reported from cereals, legumes and in members of Solanaceae. Stress inducible trypsin inhibitor was found in potato tubers (*S. tuberosum*) [21]. Metallo-carboxypeptidase and aspartyl protease inhibitor have been identified in tomato and potato (table 1). Potato leaves also contain potato type I and type II inhibitors induced as a consequence of wound. The carboxypeptidase inhibitors isolated from potato were small sized inhibitors and are stabilized by many disulfide bridges [22]. Potato tuber protein consists a major storage protein patatin, and also some low kDa PIs which can be classified in to ten different groups. [23, 24]. The most common inhibitors belong to serine proteases of Kunitz-type inhibitors (KTI), potato protease inhibitors I and II (PIN I, PIN II) and Bowman-Birk inhibitors (BBIs). KTIs were also other best characterized inhibitors. These vacuolar proteins were abundant in potato tubers and represent a complex diverse group of proteins [25]. Metallo carboxypeptidase inhibitor (MCPIS) includes metallo-carboxy peptidase inhibitor and  $\alpha$ -cathepsin-D inhibitor family. They were small protein inhibitors isolated from potato and tomato consisting of 38-39 amino acids with 3 S-S bonds with molecular mass of 42 kDa [26]. MCPIS competitively inhibit a broad spectrum of carboxy peptidases from microbes and animals but not the serine carboxy peptidases from yeast and plants [27]. These inhibitors also inhibit the activity of thermolysin, matrilysin, neutrophil, collagenase, interstitial collagenase, atrolysin C, stromelysin, carboxypeptidase A, and TNF- $\alpha$  convertase. The potato carboxypeptidase inhibitor extracted from *S. tuberosum* inhibits thermolysin metallo-carboxypeptidase.

**Table 1: Diversity of PIs from *Solanum* species**

Type	Source	Target protease	References
Proteinase inhibitor II	<i>Solanum tuberosum</i>	Trypsin, Chymotrypsin	Greenblatt <i>et al.</i> , [28]
Cathepsin D inhibitor	<i>Solanum tuberosum</i>	Cathepsin D, Trypsin	Strukelj <i>et al.</i> , [29]
Kunitz cysteine peptidase inhibitor 1	<i>Solanum tuberosum</i>	Cysteine proteases	Gruden <i>et al.</i> , [30]
Chymotrypsin inhibitor 1	<i>Solanum tuberosum</i>	Trypsin, Chymotrypsin	Richardson [31]
Potato peptidase inhibitor II inhibitor unit 1	<i>Solanum tuberosum</i>	Trypsin, Chymotrypsin	Keil <i>et al.</i> , [32]
Tomato peptidase inhibitor II inhibitor unit 1	<i>Solanum lycopersicum</i>	Trypsin, Chymotrypsin	Graham <i>et al.</i> , [33]
Tomato peptidase inhibitor II inhibitor unit 2	<i>Solanum lycopersicum</i>	Trypsin, Chymotrypsin	Barrette-Ng <i>et al.</i> , [34]

Potato, inhibitor I was regulated by wounding and developmental process while, tobacco inhibitor I protein was related with senescence only. [35]. Potato protein, potamin-1 (PT-1) isolated from the water-soluble fraction of potato tuber (*S. tuberosum* L. cv. Gogu valley) shows protease inhibitory and antimicrobial activity [36]. This PT-1 was a 5.6 kDa trypsin-chymotrypsin protease inhibitor, with 62% homology to serine protease inhibitor. Potamin-1 had the ability to inhibit trypsin, chymotrypsin, and papain. These SPIs have diverse roles such as anti-inflammatory, tissue repair and host defence [37]. Valueva *et al.*, [38] studied the time course and the composition of proteinase-inhibiting proteins accumulation from the potato tubers diffusates and its elicitation using salicylic, jasmonic, and arachidonic acids. Jasmonic and arachidonic acids activated the accumulation of the chymotrypsin inhibitors in the tubers in response to the injury, whereas salicylic acid inhibited this process which suggest its role in the lipoxygenase metabolism of signal transduction of the anti-injury defense system in the dormant potato tubers.

Aspartic proteinase inhibitor corresponding to the group cathepsin D inhibitors (CDIs) was studied in *S. tuberosum* comprising 15 isoforms with significant sequence similarity [39]. The two isoforms of CDI were reported includes PDI (Protein disulfide isomerase) and NDI (naphthalene-diimide) which shows inhibitory activity against aspartate proteinases [40].

Kazal family are double headed inhibitors which inhibit trypsin and chymotrypsin simultaneously and were identified by Kazal *et al.* [41]. Kazal protein domain is an evolutionary conserved region of serine protease inhibitors. Six types are recognized. With ten consensus variable contact positions and the 19 possible variants at each of these positions were reported. Kazal 1 domains often occur in tandem arrays of small  $\alpha$ + $\beta$  fold containing three disulfide bonds capable of inhibiting trypsin and elastase inhibitor, avian ovomucoid, acrosin, elastase inhibitor via the amino terminal region of this domain binds to the active part of its target proteases, thus inhibiting their function. Kazal 2 domain is an indicative of SPI of Merops inhibitor groups comprising I1, I2, I17 and I31. This was also noticed in the extracellular part of agrins, which are not PIs. Kazal PIs reported from *S. tuberosum* and tomato shows fungicidal activity against *Phytophthora infestans* [42]. Trypsin inhibitors were the well characterized PIs forms stable complex with trypsin at 1:1 molar ratio for inhibiting the enzymatic action.

Aberoumand [43] evaluated qualitative phytochemical analysis of *S. indicum* and reported the presence of polyphenols, saponins, alkaloids, phytic acid, saponins and PIs. Inhibitory potential of trypsin inhibitor was 10.6 TIU/g. Studies on *S. stramonifolium* also revealed the presence of peptides (or protein) in the seed aqueous extract that act as potential protease inhibitors [44].

### Purification and characterization of *Solanum* PI

PIs have immense applications from plant protection to antiviral/anticancer. Hence, the discovery of novel PIs with new properties are of great interest. Park *et al.*, [45] isolated and purified Kunitz-type serine protease inhibitor from potato (*S. tuberosum*) using chromatographic techniques. Sritanyarat *et al.*, [46] isolated and characterized iso-inhibitors of the potato protease inhibitor I family from the latex of *Hevea brasiliensis*, that strongly inhibits subtilisin A. PIs from *S. aculeatissimum* was characterized and was a potential source of many biological properties such as analgesic, anti-inflammatory, anti-hypertensive and anti-microbial. Double-headed protease inhibitor was isolated, purified and characterized including its kinetics from the fruits of *Solanum aculeatissimum* (SAPI) [47]. Kunal *et al.*, [48] isolated, purified and characterized a 20 kDa protein, named PotHg, showing hemagglutination activity from tubers of Indian potato, *S. tuberosum*. The biochemical activity of many individual plant KTIs from potato has been analysed extensively and represents the largest KTI family [49]. Further, the KTI family showed unique sequence variation, including many nonsynonymous substitutions and loops, which appear to translate into functional diversity in the plants.

### *Solanum* PI genes

PLANT-PIs is a document created to unravel the information on plant PIs and related genes. For each PI, links to sequence databases were reported together with an abstract of the functional features of the molecule. 351 plant PIs+several iso-inhibitors were deposited in the data bank.

Five diverse Kunitz PI (KPI) group B genes were characterized by Speranskaya *et al.*, [50] from the diploid genome of the non-potato species *S. palustre*. From cultivated potato accessions, 3 genes share a similarity index of 99% to KPI-B genes and 2 genes showed about 96% identity. KPI-B2 and KPI-B4 proteins from *S. palustre* contain conserved residues that involved to trypsin and chymotrypsin-specific binding sites of KPI-B, respectively. Analysis of inhibition of trypsin and chymotrypsin by Spls-KPI proteins, five of them were produced in *E. coli* purified using Ni-sepharose resin and ion-exchange chromatography. The recombinant Spls-KPI-B inhibited trypsin; K(i) values ranged from 84.8 (Spls-KPI-B4), 345.5 (Spls-KPI-B1), and 1310.6 nM (Spls-KPI-B2) to 3883.5 (Spls-KPI-B5) and 8370 nM (Spls-KPI-B3). Further, Spls-KPI-B1 and Spls-KPI-B4 inhibited chymotrypsin. Results suggest that regardless of substitutions of key active-center residues both Spls-KPI-B4 and Spls-KPI-B1 were functional trypsin-chymotrypsin inhibitors.

18 clones representing copies of 4 Kunitz-type PI group B genes (PKPI-B) obtained by PCR cloning of *S. tuberosum* L. cv. Istrinskii) genomic DNA were sequence analyzed by Speranskaya *et al.*, [51]. 3 novel genes such as PKPI-B1, PKPI-B2, and PKPI-B10 represented by five, two, and seven clones, respectively. The remaining four clones were related to the PKPI-B9 gene. These data show that at least four PKPI-B encoding genes are harbored in the genome of the cultivar. Their analysis reveals that variability of PKPI-B encoding genes in potato was limited and could be explained by cross-hybridization events in the ancestor forms rather than by random mutagenesis.

Xu *et al.*, [52] suggested that PIs offer insect resistance in transgenic plants but their endogenous functions remain undefined. Expression analysis of a PI II (PIN2) from *S. americanum* from phloem tissue of stems, roots and leaves suggesting its novel endogenous role. Two cDNAs encoding PIN2, SaPIN2a and SaPIN2b, from cDNA library were isolated using tomato PIN2 cDNA as hybridization probe. SaPIN2a shows 73.6% identity to SaPIN2b and further confirmed by Southern blot analysis. Northern blot analysis reveals that the two genes were wound inducible flowers. SaPIN2a was expressed more in stems. *In situ* hybridization analyzes on stem showed that SaPIN2a mRNA is expressed in companion cells and some sieve tubes. Western blot analysis using SaPIN2a-specific antibodies showed SaPIN2a accumulation in stems, leaf midribs and fruits. Immuno-histochemical localization, using these antibodies, revealed SaPIN2a expression in external and internal phloem of stem and further confirmed by immuno electron microscopy. The study suggests the role of SaPIN2a in proteolysis in the phloem.

Krinitina *et al.*, [53] studied group A Kunitz-type PIs (KPI-A) of potato involved in protecting them from pests and pathogens. KPI-A fragments were cloned, amplified, sequenced, and analyzed from the subgenera *Potatoe* sect. *Petota* (5 genes from *S. tuberosum* ssp. *andigenum* and 2 genes from *S. stoloniferum*) and *Solanum* (5 genes from *S. nigrum*), and their consensus sequences were established. 97–100% identity was seen among these sequences and the KPI-A sequences of *Petota* (cultivated potato *Solanum tuberosum* ssp. *tuberosum*) and *Etuberosum* (*S. palustre*). The interspecific diversity of KPI-A did not exceed its intraspecific variation. The distribution of highly variable and conserved sequences in the mature protein-coding area was the same in all the species. In *S. dulcamara*, *S. lycopersicum*, and *Mandragora officinarum* the same primers are unable to amplify the homologous genes and the phylogenetic analysis grouped *S. lycopersicum* separately from other species like *S. nigrum*. The cluster comprises of species of the sections *E. tuberosum* and *Petota*.

Although *S. nigrum* is resistant to the strains of the oomycete *Phytophthora infestans*, which causes dreadful diseases of Solanaceae, the amino acid sequences encoded by *S. nigrum* KPI-A differed slightly, if at all, from their counterparts of cultivated potato, which is susceptible to *P. infestans* infection.

Hartl *et al.*, [54] analyzed four different Serine PI profile of *S. nigrum*. Transcript and activity characterization showed tissue-specific and insect-elicited accumulation patterns. Stable and transient gene silencing of SPIs displayed different specificities for target proteinases: the novel SPI2c revealed high specificity for trypsin and chymotrypsin, while two other SPI2 homologs were highly active against subtilisin. Field and lab analyzes of SPIs to display herbivore- and gene-specific defensive properties, with dissimilar effects on closely related species. No visible developmental phenotype in SPI-silenced plants, suggesting that SPIs do not involve in regulating endogenous proteases.

Fischer *et al.*, [55] studied diverse families from PIs of potato cultivars. The functional diversity was analyzed by sequencing 9,600 cDNA clones originated from 10 mature potato cultivar tubers. 120 unique inhibitor cDNA clones were screened by homology searches. 88 inhibitors represented novel sequence variants. Kunitz type inhibitors (KTI), potato protease inhibitors I and II (PIN), pectin methylesterase inhibitors, metallo-carboxypeptidase inhibitors and defensins were common among them. 23 inhibitors were functionally characterized and among them eleven were pharmacological relevant proteases. The purified recombinant proteins were evaluated for their inhibitory activities on trypsin. Members of the KTI and PIN families inhibited pig pancreas elastase,  $\beta$ -Secretase, Cathepsin K, HIV-1 protease and potato 5-lipoxygenase.

Heibges *et al.*, [49] revealed that kunitz-type enzyme inhibitors of potato were polymorphic small proteins. 55 variable DNA sequences from mature cultivars like Provita and Saturna analysed by expressed sequence tags showed high sequence similarity to KPis.

The frequency of Kunitz-type inhibitor ESTs in Provita was 4 folds higher than in Saturna tubers, and none of the Provita ESTs was identical to any of the Saturna ESTs. The phenogram of the deduced amino acid sequences of the inhibitors resulted A, B and C homology groups. Group A derived from Provita ESTs and group B each other show more similarity than group C. Non-conservative amino acid substitutions and insertion/deletion polymorphisms reveal functional differentiation among members of the gene family. 21 genes for Kunitz-type enzyme inhibitors (6, 9, 6 for group A, B and C respectively) were estimated to exist in the potato genome. Genetic mapping and the identification of bacterial artificial chromosome clones showing more than one member of the gene family indicated that most inhibitor genes of groups A, B and C were grouped in a cluster that maps to a single region potato chromosome III.

Anna *et al.*, [56] studied the effect of recombination on gene polymorphism encoding Kunitz-type PIs in *Solanum*. 12 different genes encoding KPis from *S. tuberosum* and *S. palustre* were analyzed revealing their mosaic structural features. According to Zawala and Baldwin [57]. Trypsin PI gene expression in *Nicotiana attenuata* was up regulated during pest infection. Moulin *et al.*, [58]

examined the gene expression of PIs, specifically trypsin inhibitors, in the leaf extract of *Capsicum baccatum* var. pendulum inoculated with Pepper yellow mosaic virus. Revina *et al.*, [59] isolated Subtilisin PI gene from potato tubers. The inhibitor has no effect on trypsin, chymotrypsin, and the cysteine proteinase papain. The N-terminal sequence of the protein consists of 19 amino acid residues and was highly homologous to sequences of the group C of the subfamily of potato Kunitz-type PIs (PKPIs-C). By cloning PCR products from the genomic DNA of potato, a gene denoted as PKPI-C2 was isolated and sequenced. The N-terminal sequence (residues from 15 to 33) of the protein encoded by the PKPI-C2 gene is identical to the N-terminal sequence (residues from 1 to 19) of the isolated protein PKSI. Thus, the inhibitor PKSI was seemed to be encoded by this gene. Pouvreau *et al.*, [60] isolated and sequenced the gene of the most abundant PI in potato cv. Elkana which show 98% identity with potato serine PI (PSPI), of the Kunitz family. Antibodies were raised against the two most abundant isoforms of PSPI. The binding of these antibodies to PSPI isoforms and PIs from different groups of PI in potato showed 70% resemblance to the protease inhibitors present in potato juice.

### Transgenic plants with *Solanum* PI genes

Proteinase inhibitor II (PIN2) with trypsin and chymotrypsin inhibitory activities belongs to SPI was first reported from tomato and potato [61]. Modern strategy in crop breeding to defend pest/pathogen is by insertion and expression of plant defence proteins. Xu *et al.*, [62] isolated two cDNA clones encoding PIN2, designated *SaPIN2a* and *SaPIN2b* from screening cDNA library prepared from wounded leaves of wild American black nightshade (*S. americanum*) using a tomato *PIN2* cDNA as heterologous hybridization probe. The inhibitors from four families of serine PIs have been induced sequentially in various plants. These families include potato and tomato inhibitors I and II of solanaceous plants [63]. Silvia and Baldwin [64] compared wild type plants and transformed plants with an inverted repeat prosystemin construct (IRSys) to silence the expression of the endogenous *S. nigrum* prosystemin gene. Wild type species elicited with wounding-oral secretions from the larvae of *Manduca sexta* showed the synthesis of trypsin proteinase inhibitors (TPIs) even though prosystemin transcripts were down regulated. The constitutive expression of PIs, which has been reported to occur in storage organs and the reproductive tissues of plants, may fulfil anti-insecticidal as well as other endogenous functions in plants. Plants can differentially perceive various kinds of insect attacks and respond appropriately through activating plant defences including regulation of PIs at transcriptional and post-translational levels. Wound-inducible Pin-II proteinase inhibitors (PIs) were studied extensively for their structural and functional diversity and also their relevance in plant defence against insect pests.

Sin and chye [65] analyzed the expression of proteinase inhibitor II proteins during floral development in *S. americanum*. Further, both *SaPIN2a* and *SaPIN2b* were expressed in floral tissues that was destined to undergo developmental programmed cell death (PCD), suggesting its possible endogenous roles in inhibiting trypsin and chymotrypsin like activities during ontogeny of flowers.

The genetically modified (GM) crops expressing PIs are being tried as an alternative to GM crops with Bt toxins. It is advantageous to introduce PI genes in plants as they are naturally present in plants, so that the chances of adverse effects on human and other animals is eliminated. But many of the GM crops expressing protease inhibitors were not significant as the insects develop resistance by secreting proteases insensitive to inhibitors or by degrading PIs [66]. Available biochemical and molecular evidence indicates that many insects acclimatize to PIs by overproducing existing digestive proteases [67]. Characterization studies of potato PI II reactive site mutants by Beekwilder *et al.*, [68] reported that Potato PI II (PI-2) is composed of two reactive site domains and assessed the role of its two reactive sites with the inhibition of trypsin and chymotrypsin by mutating each of the two reactive sites in multiple ways. Studies proved that the second reactive site strongly inhibits both trypsin and chymotrypsin. Meanwhile, the first reactive site inhibits only chymotrypsin. Lison *et al.*, [69] reported a tomato wound-inducible

protein called jasmonic-induced protein 21 (JIP21) which was a strong chymotrypsin inhibitor belonging to the Serine PIs group.

Potato PI-II gene of potato was introduced into many japonica rice cultivars to produce transgenic rice resistance against insect *in vitro* trials. Wound-inducible PI-II promoter with the first intron of rice actin I gene was capable to give significant expression of PI-II gene in transgenic rice. These transgenic plants were resistant to pink stem borer (*Sesamia inferens*) [70].

### Biological potentialities

#### Antimicrobial

Fungal disease regulation can be executed in both non-pharmacological method ie, by maintaining personal hygiene and also by pharmacological method that can be executed by administering proper antifungal drugs based on the cause of the infecting fungi [71]. PIs are able to affect fungi by inhibiting extracellular and/or intracellular proteases that display important roles in metabolic and infection processes. Park *et al.*, [45] purified AFP-J (antifungal protein) from tubers of *S. tuberosum* cv. L Jopung by using different chromatographic columns. Purified AFP-J strongly inhibited fungal strains like *Candida albicans*, *Trichosporon beigelii*, and *Saccharomyces cerevisiae* and which has 83% homology with SPI belonging to the Kunitz family. Trypsin-chymotrypsin inhibitor from *S. tuberosum* strongly inhibited the pathogenic fungi *C. albicans* and *Rhizoctonia solani* and showed 62% homology with PIs from Kunitz family and interestingly, no lysis of human erythrocytes suggesting the safety of PI as antimicrobial agent. Juleita *et al.*, [72] reported antimicrobial effect of *S. tuberosum* aspartic proteases against *Fusarium solani* and *Phytophthora infestans* directly through interaction with the microbial cell surfaces followed by membrane permeabilization. Further, the inhibitors proved fungicidal against *F. solani* by targeting protease secreted by them.

Transgenic rice contain rice actin I gene+wound inducible PI-II promoter showed resistance against pink stem borer [73]. Potide-G, a small (5578.9 Da) antimicrobial peptide was isolated from tubers of *S. tuberosum* cv. Golden Valley was capable of suppressing the proteolytic activity of trypsin, chymotrypsin and papain effectively.

Kim *et al.*, [74] demonstrated that inhibitors from potato tubers strongly inhibited the growth of a wide variety of bacteria like *Staphylococcus aureus*, *Listeria monocytogenes*, *Clavibacter michiganense*, and *Escherichia coli*, and fungi such as *Candida albicans* and *Rhizoctonia solani*. Different results reported by Bar-Ziv *et al.*, [75] related with the synthesis and induction in tomatoes as a defence mechanism against many pathogens including Tomato yellow leaf curl virus (TYLCV). In the absence of pathogen exposure, the inhibitor level detected was minimal. Potide-G, a small (5578.9 Da) antimicrobial peptide, was isolated from potato tubers (*Solanum tuberosum* L. cv. Golden Valley). Sanchez-Serrano [76] reported potato proteinase inhibitor-II (PI-II) and its constitutive expression in tubers and floral buds by wounding the leaves. Further, proteomic evidence of the wound-healing mechanisms of PIs from potato tissue was proved by Chaves *et al.*, [77] using 2-D electrophoresis and further identified using MS/MS analysis.

#### Insecticidal activity

The insecticidal potential of PPIs was investigated as early as 1947, when Mickel and Standish observed the larvae of certain insects were unable to develop normally on soybean products. These inhibitors were proteins or peptides capable of inhibiting catalytic activities of proteases. They are grouped in to serine, cysteine, aspartic or metallo protease inhibitors. Diverse protease have been identified from the extracts of the digestive tracts of insects from many families, particularly those of *Lepidoptera* and many of these enzymes are inhibited by PIs. Broadway and Duffey [78] compared the effects of purified soybean birk trypsin inhibitor (SBTI) and potato inhibitor II (an inhibitor of trypsin and chymotrypsin) on the growth and digestive physiology of larvae of *Heliothis zea* and *Spodoptera exigua* and proved that growth of larvae was inhibited at various levels. Potato tubers possess an aspartic proteinase inhibitor, cathepsin D that shares considerable amino acid sequence identity with the trypsin inhibitor-SBTI from soybeans [79].

Dunse *et al.*, [80] studied molecular basis for the resistance of *Helicoverpa larvae* chymotrypsin to a potato type II proteinase inhibitor from *Nicotiana glauca*. Effect of PIs of *S. lycopersicum* against *Leptinotarsa decemlineata* on defences was evaluated by Seung and Gary [81]. The fourth-instar larvae were reared on the leaves of *S. lycopersicum* resulted in to the induction of transcripts of the proteinase inhibitors pin1 and pin2. Mosolov [63] reported that the Chymotrypsin PIs from *S. tuberosum* suppress the growth and development of the oomycete of *P. infestans*. Urwin *et al.*, [82] reported that transgenic *S. tuberosum* express proteinase inhibitors against *Globodera pallida* and there by offering resistance.

Hartl *et al.*, [83] identified four SPI from *S. nigrum* differ substantially in substrate specificity, accumulation patterns, and their insecticidal mechanisms. Hartl *et al.*, [54] reported high variations in constitutive levels of trypsin inhibitors expression among control and *S. nigrum* exposed to *Spodoptera exigua*. Intrinsic inhibitors from this plant may affect larval pest growth, indicating a potential natural defence mechanism against herbivore insects.

Remya *et al.*, [84] screened plant extracts containing protease inhibitors against gut proteases of *Spodoptera mauritia* larvae. 20% inhibition of gut protease activity of *Spodoptera mauritia* larvae were obtained by *S. melongena* and *S. lycopersicum*

Transgenic petunia, birch and lettuce of Potato PI-I and PI-II genes displayed resistance against insect pests. Transgenic tobacco with chymotrypsin inhibitor gene of potato enhances resistance against *Chrysothrips eriosoma* [73]. The protease inhibitor gene CpTi was successfully transferred producing transgenic tobacco with significant resistance against tobacco hornworm (*Manduca sexta*) [85]. Sane *et al.*, [86] studied the efficiency of transgenic tobacco plants expressing CpTi was tested against armyworm in feeding trails under laboratory conditions. Reduction to the extent of 50% was observed in the biomass of army worm larvae fed on transgenic leaves expressing 3-5 µg of CpTi/g of fresh leaves. Tomato plants over expressing JIP21 have been generated and showed resistance against larvae of the Lepidopteran Egyptian cotton worm [87].

Enzyme threonine deaminase (TD), of *S. lycopersicum* serves dual role in isoleucine (Ile) biosynthesis in plants and Thr degradation in the insect midguts of *Manduca sexta*, where they destroy these nutritionally important amino acids, ultimately resulting in decreased larval growth [88]. Egyptian cotton worm larvae fed on transgenic tomato plants overexpressing JIP21 (strong chymotrypsin inhibitor belonging to the Ser proteinase inhibitors) showed an increase in mortality and delay in growth when compared with larvae fed on wild-type plants. These larvae belong to the Lepidoptera group with digestive enzymes belongs to Ser proteases [39] (table 2).

**Table 2: Solanum protease inhibitors active against insect pests**

Inhibitor type	Crop	Pest	Reference
Tomato inhibitor I and II	Tobacco	<i>M. sexta</i>	Johnson <i>et al.</i> , [89]
<i>Nicotiana glauca</i> protease inhibitor (PI)	Tobacco	<i>Helicoverpa punctigera</i>	Heath <i>et al.</i> , [90]
Potato inhibitor II		<i>Heliothis zea</i>	Broadway and Duffey [79]
		<i>Spodoptera exigua</i>	
<i>Nicotiana glauca</i> protease inhibitor (PI)	Peas	<i>Plutella xylostella</i>	Charity <i>et al.</i> , [91]

PP (potato protein) may be an alternative to medicated feed with antibiotics because it showed antimicrobial activity against coliform bacteria and also improve the performance of weanling pigs [92].

### Antiherbivore defenses

Trypsin Protease Inhibitors (TPIs) expression studies revealed the evidence for plant defence function of TPIs. In *Nicotiana attenuata*, manipulation of its endogenous trypsin PIs production demonstrates their antiherbivore effects. [93]

Ali *et al.*, [94] revealed a novel mechanism for the epigenetic basis of HIPV-mediated habituation by evaluating the recalled expression of Bowman-Birk trypsin inhibitor gene. Moreover, in the promoter region of trypsin inhibitor gene, the methylation sites were found to be demethylated by the HIPV treatments.

### Antioxidant (AOX)

Plant biomolecules display significant levels of antioxidants that can play a potential role in RBC membrane protection and reduce oxidative damages in the cells [95]. *S. aculeatissimum* PI exhibited significant IC<sub>50</sub> values for most of the AOX assays i.e., DPPH radical scavenging, reducing power, metal chelating ability, ABTS and OH. radical scavenging activity were comparable with the synthetic antioxidants like ascorbate and BHT. Free radicals scavenging activity of PI may be through the H-atom abstraction from the free hydroxyl group [96]. Mayasa *et al.*, [97] evaluated antioxidant potential of *S. tuberosum* protease inhibitors. The partially purified PI using ammonium sulfate precipitation was analysed in terms of proteolysis assay using casein as substrate and antioxidant activity by DPPH and H<sub>2</sub>O<sub>2</sub> scavenging.

### Antiviral

Molecular investigations on viral proteases reveals their prominent role in cleavage of viral polyprotein precursors and also in catalyzing the processing of the structural proteins necessary for the assembly and morphogenesis of virus particles [98]. Studies on *in vitro* antiviral activity of *S. nigrum* by Javed *et al.*, [99] suggest that *S. nigrum* extract in combination with interferon is a potential drug to cure Hepatitis C Virus infection.

### Phylogenetic analysis

Faiza *et al.*, [100] carried out an *in vitro* and *in silico* characterization of *S. lycopersicum* wound-inducible proteinase inhibitor-II gene. *In silico* insight of the phylogenetic evaluation revealed that 30 PIs from diverse plants share a common mother stock of evolutionary origin. Molecular and phylogenetic analysis of the wound-inducible PI-I gene was attempted for the 7 direct ancestors of tomato such as *Lycopersicon esculentum*: *L. pennellii*, *L. chilense*, *L. hirsutum*, *L. parviflorum*, *L. peruvianum* var. *humifusum*, *L. cheesmanii*, and *L. Peruvianum* [101].

Due to the zebra chip disease, caused by *Candidatus liberibacter solanacearum* (CLso), a decreased protease inhibitor content was observed in matured potato that results in an enhanced serine protease activity and altered protein profiles. Patatin, serine, aspartine and cystatin type protease inhibitors were either absent or greatly reduced in ZC affected tubers [102].

Weeda *et al.*, [103] analysed correlative changes in proteases and protease inhibitors during mobilisation of protein in potato seed tubers. They reported that catabolism of protease inhibitors may facilitate protein mobilisation from seed tubers.

### CONCLUSION

The diverse PI genes from *Solanum* species and their combination products are targeted at different pharmaceutical and agricultural levels. These include protease inhibitor genes and also lectins, α-amylase inhibitors, or other plant genes encoding insecticidal or anticancer potentiality. These researches minimize the use of synthetic drugs in near future by effectively complementing plant derived products. Recombinant PIs may also be an alternative way to protect plants from pathogens. Currently, screening gene pools without taxonomic constraint can help identify novel lead molecule for multipurpose needs. Exploring insecticidal proteins involved in host plant defense can lead to effective control of pest. This strategy

is an output of co-expression of numerous factors, each of which could be custom engineered by directed molecular evolution to maximize its effectiveness against implementing integrated pest management programs.

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#### CONFLICTS OF INTERESTS

Declared none

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