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Review Paper

GENETIC APPROACHES FOR ENGINEERING BIOTIC STRESS RESISTANCE IN POTATO (*Solanum tuberosum* L.)

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ABSTRACT

Potato is one of the most important food crops in terms of annual production and food security worldwide. The crop is affected by several types of biotic stresses, e.g. insects, viruses, fungus, nematodes and weeds, which are the prominent limiting factors for its production. The conventional breeding methods in potato have been associated with limitations; none of the present day commercial cultivar has built-in resistance against biotic stresses. There is strong need for the development of new resistant potato varieties to cope against biotic stresses using non-classical approaches in combination with classical methods. The scientific literature suggests the contribution of modern biotechnological techniques for the development of transgenic potato lines resistant against insects and diseases. The present comprehensive review describes different genetic engineering approaches for the development of transgenic potatoes resistant to insects, weeds, nematodes, fungus and viruses by fellow researchers worldwide. It also gives an insight into modern technologies, e.g. RNAi and CRISPR-Cas9, which have emerged recently and can be implemented in the development of biotic stress resistant potato cultivars.

Key Words: Biotic stress; transgenic technology; crop productivity; genetic approaches.

INTRODUCTION

Potato was introduced to the old world in the sixteenth century and as per an estimate, it has contributed to 1/4th of the population growth between 1700-1900 (Nunn and Qian, 2011). Potato is the fourth largest crop in terms of annual production and the third largest food crop (Haverkort and Struik, 2015). Potato is an important crop in terms of being food for millions of people. The potato tubers are rich source of carbohydrate, vitamin C, B and potassium (Camire *et al*., 2009). In potato, introgression of agronomic traits conventionally is quite tedious due to the sexual barriers among the domesticated and wild varieties. Abiotic stresses such as chilling, salt, heat and drought stresses that pose threat to the potato crops have been widely studied and potato varieties tolerant to these stresses have been developed with the help of genetic engineering tools (Dangol *et al*., 2018). Similarly, there are plenty of studies that have been conducted to address the potato plants combating biotic stresses.

The insect pests and diseases pose a continuous threat to crop plants leading to 37% losses of the agricultural production world-wide (Gatehouse *et al*., 1992); likewise insect pest and disease losses on potato have been estimated to be 40% in a recent study (Beddington, 2010). A wide range of insect pests damage potato crop worldwide, mainly two pests, Potato tuber moth (PTM; *Phthorimaea operculella* Z) and Colorado potato beetle (CPB; *Leptinotarsa decemlineata* 'Say'). These pests are the most widespread insect pests of potato (Visser, 2005; Ferro *et al*., 1985, 1993). PTM damages crop in the field, storage affect the quality of the food as well as increases the risk of pathogen infection. As it attacks both the foliage and the tuber, potato yield is reduced tremendously (Capinera, 2001, Alyokhin *et al*., 2008). The losses in storage can reach up to 100% in warmer climates (Lagnaoui *et al*., 2001). Besides PTM, CPB is another serious pest of potatoes belonging to coleopteran insect order. Both the adults and larva of CPB feed on potato leaves, thus reducing yield significantly and can even kill plants. CPB is notorious pest for development of resistance to insecticides over short periods of time (Kamenova *et al*., 2008; Ahmed *et al*., 2017). Similarly, different species of nematodes are also very important pests of potato and cause significant losses in potato (Ali *et al*., 2015).

Potato crop is also severely affected by viruses, mainly *Potato virus Y* (PVY) being the most important from quarantine point of view. Glais *et al*. (2002) categorized these viruses into four groups (PVY ^{NTN}, PVY ^{O}, PVY^{N}, PVY^{N}W) based on their virulence and host responses. The host plant resistance is considered one of the significant strategies to control viruses (Khetarpal *et al*., 1998). Some of the studies have revealed the investigations and discovery of the resistance genes against PVY (almost in all known strains) in Europe, and further have been used in breeding

programme for varietal development (Solomon-Blackburn and Barker, 2001; Plaisted *et al*., 2001).

Another most important biotic stress is the fungus, specially *Phytophthora infestans*, which is a problem for agriculture since decades. It has resulted in severe epidemics in Europe in 1845 that led to potato famine in Ireland. Genetic engineering is being considered a promising solution to develop resistance against this fungus. Still the diverse genome of fungus, its population diversity and abundance are the main threats to the crop (Fry, 2008).

Weed management in potato crop has been challenging as weeds lead to the significant yield losses by interfering with crop production practices. Weeds make agricultural practices relatively difficult; the weeds also harbor pests leading to the spread of various crop diseases (Zimdahl, 2007). Weeds can potentially decrease the yield of potato crop, nearly 16%-76%, depending upon the intensity of the weeds present (Tripathi and Singh, 1989; Uremis *et al*., 2009). Recently a study by Jafari *et al*. (2013) reported a loss of at least 15% to over 40% of crop yield caused by weeds, showing an important area of improvement for plant breeders using modern day biotechnological tools.

The advancement in biotechnology and genetic engineering has resulted in many success stories for controlling the biotic stresses of potatoes. This review enlists the significant studies among those.

Insect resistant potatoes: The different types of insect pests incur significant losses to potatoes either directly (chewing or sucking leaves or feeding on tubers) or indirectly (serving as pathogen transmitter) (Vincent *et al*., 2013). Many breeding attempts have been made to develop insect resistant potatoes. Due to narrow genetic base of potato, conventional breeding strategies are usually inefficient (Douches *et al*., 1996). Biotechnology has assisted the classical breeding by providing alternatives for the improvement of potato in the area of insect resistance.

The insect resistant transgenic crops have already been commercialized and potato has no exception. The source of insecticidal genes has been derived from different origins, of bacterial (utilizing strains of *Bacillus thuringienesis* (*Bt*)), plants (proteinase inhibitors, amylase inhibitors and lectins) and other origins such as bean chitinase (BCH). These genes have been used to induce resistance against insect pests. Most importantly, different strains of *B. thuringienesis* produce a variety of δ-endotoxins or crystal proteins with specific host range. The various *Bt* endotoxin gene(s) encoding resistance against lepidopteran and coleopteran pests has been incorporated in potatoes as evident from scientific literature available (Amiri and Bakhsh, 2019, Ahmed *et al*., 2017, Mohammed *et al*., 2016; Mi *et al*., 2015; Veale *et al*., 2012; Kumar *et al*., 2010; Davidson *et al*., 2004; Chakrabarti *et al*., 2000; Perlak *et al*., 1993). There are also attempts to transfer genes of plant origin (proteinase inhibitors, amylose inhibitors and lectins) that encode resistance against these notorious insects (Table-1).

The use of genetically engineered potatoes against PTM included *cryI* and *cryIIa* (Crickmore *et al*., 1998). The transgenic potato lines expressing native (*Bt884*) and truncated (*cry1Ab6*) under the control of TR2 promoter showed enhanced resistance against PTM (Jansens *et al*., 1995; Canedo *et al*., 1997). Potatoes with the Bt-*cry1Ia1* gene showed 100% mortality against potato tuber moth larvae (Mohammed *et al*., 2000). The introduction of *cry1Ac9* and *Bt-cry5* genes from *Bt* have also been reported to confer resistance against PTM in transgenic potatoes (Davidson *et al*., 2004; Veale *et al*., 2012). Very recently, a report from Amiri and Bakhsh (2019) revealed up to 100% resistance in some expressing *cry1Ac* transgenic potatoes lines against PTM and CPB (Figure-1).

Biotechnology and genetic engineering approaches have showed an improvement in potato crop against CPB (Kamenova *et al*., 2008). *Cry3a* insecticidal gene isolated from *Bacillus thuringiensis* sp. tenebrionis exhibits toxicity against CPB which is the most notorious insect of potatoes. There are evidences where transgenic potatoes expressing *cry3a* gene showed increased toxicity against CPB (Mi *et al*., 2015; Zhou *et al*., 2012; Krieg *et al*., 1983). Earlier, *cry3A* transcript levels were recorded as low in eukaryotes that was rackled by codon optimization that can enhance increased *cry3A* expression (Zhou *et al*., 2012). The codon optimized *cry3a* gene was introduced to Atlantic cultivar of potato that enhanced its resistance against CPB (Mi *et al*., 2015). Chakrabarti *et al*. (2000) reported the development of genetically engineered transgenic potatoes which showed resistance against *Helicoverpa armigera*.

Guo *et al.* (2016) conducted another study to develop selectable marker-free transgenic potatoes to avoid the use of SMG *nptII* which is being prohibited in China's commercial markets. They introduced *cry3A* and *npt II* genes simultaneously in potato plants by harbouring in different plasmids via *Agrobacterium* mediated transformation. Bioassay for resistance to CPB and self-crossing segregation was used to identify selectable marker-free transgenic plants expressing *cry3A*. These lines could be commercialized more easily (Guo *et al*., 2016).

In order to address biosafety concerns of using CaMV35S which is widely used to regulate genes in insect resistant potatoes, researchers have used tissue specific promoter to limit expression of foreign gene proteins in green parts of the plants (Meiyalaghan *et al*., 2004, 2006; Conner and Jacobs, 1999).

The use of tissue specific or light inducible promoter is being considered a more promising strategy in order to address public concerns on biosafety of

transgenic crops (Anayol *et al*., 2016). The use of light inducible promoter restricted *cry1Ab* expression to aerial parts of transgenic potatoes and resulted in enhanced resistance against PTM. Using *SN19* gene, under the control of constitutive (35S) and wound inducible promoter (AoPR1), Ahmed *et al*. (2017) were able to develop transgenic potato lines equally effective against CPB and PTM.

The expression of *cry1Ab* driven by light inducible promoter (PEPC) was confined to light exposed tissue of transgenic potatoes (Hagh *et al*., 2009). The absence of foreign proteins expression in potato tubers can lead to more acceptability and marketability of transgenic potatoes. Veale *et al*. (2012) used OCS3mas promoter to drive expression of *cryIIaI* gene in potato cultivar Mnandi to encode resistance against PTM, later on similar kind of results was obtained by Estrada *et al*. (2007).

Besides cry toxins from *Bacillus thuringiensis*, various genes from plants and other origins have been reported to encode resistance against insect pests (Reviewed in Bakhsh *et al*., 2015). Proteinase inhibitors are the ones that have been succeessfully used in transgenic plants with considerable inhibitory activity against insect digestive enzymes. The growth and development of larvae of tomato moth was significantly affected when total soluble protein up to 1% of transgenic potato leaves expressing Cowpea trypsin inhibitor (CpTI) was added in their artificial diet (Bell *et al*., 2001a).

There are scientific evidences of the use of plant lectins in transgenic plants for the control of insect pests (Bakhsh *et al*., 2015; Khabbazi *et al*., 2016). Though lectins show toxicity against coleopterans, lepidopterans and dipterans, there are few reports of the transgenic potatoes expressing plant lectins. Transgenic potatoes were developed by transformation of gene encoding snowdrop lectin (Galanthus nivalis agglutinin, GNA). Green house studies revleaed that the transgenic plants significantly reduced the level of pest damage caused by parasitic effect of phytophagous insect pest *Lacanobia oleracea* (Bell *et al*., 2001b).

Herbicide resistant potatoes: The potato crop yield is severely affected by weeds in terms of quantity and quality. Weeds affect directly by competing for light, moisture and nutrients and also serve as the host for different pests and diseases (Baldwin and Preston, 1999). Weeds are also harmful during the harvesting time as they cause mechanical damage to tuber and affect the efficiency of harvesting operations. Hutchinson *et al*. (2011) reported up to 30% yield reduction and tuber quality of potato crop because of season long competition of *Solanum sarachoides* with potato crop for water and other essential nutrients. Weeds serve as host for the transmission of plant viruses and hence hasten viral spread in agroecosystem (Norris and Kogan, 2005).

Genetic improvements in potato against herbicides have been reported. Figueira *et al*. (1994) evaluated the potato cultivar Mantiqueira for herbicide resistance using *A. tumefaciens* carrying plasmid pGV1040 harboring *bar* gene that encodes for enzyme phosphinothricin acetyltransferase (Jefferson *et al*., 1987; Bevan, 1984). Soto *et al.* (2007) described a relatively faster method of *A. tumefaciens* mediated genetic transformation using internodal explants segments of cultivar Désirée. The phosphinothricin (PPT, glufosinate ammonium) was used as a selection agent for the screening of transformants. The transformants exhibited higher level of resistance to PPT up to 500 mg/l with normal phenotype. Furthermore, these transgenic plants when sprayed with herbicide Finale® (Bayer Sciences) remained healthy and green, whereas non-transgenic plants died after a week of herbicide application.

Besides *PAT*/*BAR* gene, Monsanto introduced New Leaf™ Plus Russet Burbank potatoes expressing insect, potato leaf roll virus and glyphosate resistance traits. Largely due to poor sales and anti-GMO activism fueling public debate regarding the safety of biotech crops, it eventually led to problems in marketing NewLeaf™ potatoes used for processing (reviewed in Halterman *et al*., 2016). However, with the widespread approval and adoption of other biotech crops, there is a renewed interest in the development of biotech potato which has led to the arrival of biotech potatoes back in the market in 2015. Very recently, Bakhsh *et al.* (2020) engineered herbicide resistant trait in potatoes by incorporating *CP-4 EPSP* synthase gene in different cultivars i.e. Lady Olympia, Desiree, Agria and Granola. On application of glyphosate, transgenic lines exhibited tolerance compared to control.

Phytophthora and verticillium resistant potatoes: The devastation caused as much as 5.2 billion Euros annually and globally in the potato tuber yield loss due to the late blight disease of potatoes is caused by a heterothallic oomycete *Phytophthora infestans.* The colossal damage in the tuber yield, leaves and stems with outright failure of the crops is observed in infected plants (Bradeen *et al*., 2009; Haverkort *et al*., 2009; Hirut *et al*., 2017). The use of fungicides has been widely popular in the developed world in combating the late blight disease of potatoes. However, the application of fungicides could pose a potential threat to the environment including its dangers on human health. Similarly, screening of resistant, local and exotic potato germplasms, against different diseases could also be helpful to find resistance sources (Shehroz *et al*., 2018). Introduction of resistant (*R*) genes could bring about ameliorated plant performance and the application of fungicides would not be inevitable. Similarly, the traditional early planting, exterminating inoculum source and the adoption of cultivars which are naturally resistant are different approaches been adopted

to combat potato late blight disease (Hirut *et al*., 2017; Halterman *et al*., 2008).

Majority of the *R* genes have been deemed to be robust to mitigate the repercussions imposed by the plant pathogens such as *P. infestans*. The R proteins secreted by *R* genes identify the pathogenic avirulence effectors for the instigation of the immune response triggered by these effector molecules. Stably transforming the plants with the aid of single *R* gene would bring about an upshot of reduced probability of the plant being radically amended in its physiology as well as its genetic structure under favorable conditions (Halterman *et al*., 2008). On the other hand, the introgression of *R1* to *R11* series from *Solanum demissum* in potatoes have been found tarnished as each of the gene in this gene series was specific for a particular race with its function limited to only certain pathogens, thereby circumventing the recognition by the plant. In addition, the generation of hypersensitive reaction (HR) may impose severe selective pressure on the pathogenic populace. Nevertheless, adoption of *R* genes conferring resistance against late blight disease from the wild counterparts of cultivated potatoes could prove to be promising. For example, *Rpi-blb3*, *RB* and *Rpi-blb2* genes introgressed from *Solanum bulbocastanum* are devoid of pathogen race specificity (Bradeen *et al*., 2009).

Superior, Dard Red Norland, Russet Burbank and Katahdin cultivars of potato have been engineered with *RB* gene under the control of its native promoter and terminator (Halterman *et al*., 2008). All the transgenic cultivars with *RB* gene exhibited ameliorated resistance against the late blight disease compared to the non-transformed cultivars (Figure-2). However, the increased resistance in tubers of transgenics wasn't observed in the field trial for yield performed for two years as compared to the ameliorated foliar resistance in the transgenics, with no major effect in the size of the tuber or the yield in the transgenics. Similar transformation of the same gene with similar gene construct from *S. bulbocastanum* genotype PT29 was done in the aforementioned potato genotypes. With a field trial of 2-year replication, it was observed that the resistance against the disease was effectively developed without any application of the fungicides. The transgenic plants with approximately 15 copies of the *RB* genes displayed its elevated transcript levels with ameliorated resistance against the late blight disease.

Abreha *et al*. (2015) transformed cv. "Desiree" potato susceptible to the late blight disease with *Rpi-blb1* gene isolated from *S. bulbocastanum* including the downstream as well as upstream regulatory elements native to this gene via *Agrobacterium* transformation. It was observed that in the cv. "Desiree" potatoes on 5 days post-inoculation of *P. infestans*, the plants exhibited the symptoms related to the late blight disease, whereas the transgenics did not. Haesaert *et al*. (2015) transformed susceptible cv. "Desiree" cultivars with single/multiple *R* genes: marker free transformation of *Rpi-vnt1.1* (obtained from *S. venturii*) and kanamycin marker assisted transformation of *Rpi-sto1* (obtained from *S. stoloniferum*) and *Rpi-sto1*:*Rpi-vnt1.1*:*Rpi-blb3*. *Rpi-blb3* was obtained from *S. bulbocastanum*. The different *R* genes developed different level of resistances in the transformed potatoes than the non-transformed ones with those transformed with gene stacking of multiple *R* genes performed the best, followed by *Rpi-vnt1.1* and *Rpi-sto1* events.

The *Rpi-blb2* gene isolated from *S. bulbocastanum* (wild potatoes in Mexico) was transformed to cv. "Desiree" via agro-infection and it conferred high resistance against the late blight disease in the transgenics with high dose inoculation of two isolates of *P. infestans* to the entire transgenic plant, demonstrated by the unsuccessful reinoculation event of *P. infestans* in the transgenic potatoes (Orbegozo *et al*., 2016). With the use of the *oxalate oxidase 4* gene (*Osoxo4*) isolated from rice overexpressed and transformed in *S. tuberosum* L. cvr Chipsona 3 via *Agrobacterium* mediated transformation under the regulation of 35S promoter, elevated activity of the enzyme oxalate oxidase was demonstrated in the transgenic potatoes with higher level of reactive oxygen H_2O_2. Ameliorated resistance against the late blight disease was reported in the transgenic lines. The genes responsible for plant defense (phenylalanine ammonia lyase and anionic peroxidase) were found to be highly transcribed after the inoculation of the pathogen (Ghosh *et al*., 2016) (Table-2).

The use of *R* genes has the possibility of accruing novel strains of pathogenic *P. infestans* population. Hence, the loss of function of plants' susceptible (*S*) genes that are utilized by the pathogens during colonizing and infecting the host plants have been utilized via RNAi transformation to ameliorate resistance against the late blight disease in potato. Eleven *S* genes from *Arabidopsis thaliana* have been utilized to silence orthologous genes in cv. "Desiree" and resulted in replete resistance against, or plummeted susceptibility, to the late blight disease (Sun *et al*., 2016).

The use of non-*Agrobacterium* strain for the purpose of transformation of potato for developing late blight resistance has also been described. *Ensifer adhaerens* OV14 has been used to transform *RB* gene isolated from *S. bulbocastanum* to the internodal tissue of the potato var. Maris Peer. The results demonstrated similar event as was found in *Agrobacterium* based transformation such as enhanced resistance against the potato late blight disease in *Ensifer*-transformed potatoes and similar transcriptional differences relative to low (2 copy number) and high (five copy number) copy number of *R* genes as in *Agrobacterium*-transformed potato lines. However, the use of gene stacking of *R* genes is yet to be

demonstrated via this novel approach (Wendt *et al*., 2012).

A group of antimicrobial peptides, magainins, obtained from the African clawed frog (*Xenopus laevis*) on its skin secretions have suppressed activity against broad spectrum of pathogens including fungi, bacteria and viruses. The gene obtained from this frog was synthetically constructed (*MSI-99m*) to express in potatoes with codon optimization for this plant organism and transformed in cv. "Desiree" potatoes via *Agrobacterium* transformation (strain GV3101) using freeze thaw method. The transgenics exhibited ameliorated resistance against *P. infestans* and *Ralstonia solanacearum* (Hong *et al*., 2013).

Roman *et al*. (2017) showed that resistance could be produced against *P. infestans.* Gene for gene interaction has been reported for *Avr* avirulance gene of *P. infestans* and *R* gene of potatoes. They did a comparative study of genes by taking *Avr-Vnt1* gene from two isolates (POX067 and POX109) of Ec-1 lineage of *P. infestans* and transgenic potatoes were developed by transformation of Rpi-vnt1.1 (obtained from *Solanum Venturii*) in potato cv. "Desiree". EC-1 lineage was reported for being virulent on Rpi-Vnt1.1 transgenic plants. In their research, five transgenic plants were reported which showed resistance to both isolates of *P. infestans.* The expression of *Rpi-vnti1.1* became steady after 5 days of inoculation after a two-fold increase initially. Steady expression of *R* gene in resistance transgenic events became reason for resistance to isolates expressing very low level of *Avr* gene. The authors claimed that although the transgenic potato plants showed resistance to isolates of even Ec-1 lineage but pathogen population is an important factor. Presence of virulent isolates although in low frequency can diminish the *R* gene-mediated reistance.

Besides P. *infestans*, potatoes are also severely affected by either of the two species of *Verticillium* species (*Verticillium dahliae* or *Verticillium alboatrum*). Reduction in tuber size, stem-end discoloration and tuber quality for processing in potatoes are observed. To cope with this problem, antifungal protein and their expression in plants has been reported (Shah, 1997). Plant defensins bind to fungal cells and inhibit their growth by permeating fungal cell membranes (Thevissen *et al*., 1999). Gao *et al*. (2000) established antifungal activity of alfafa antifungal protein gene *alfAFP*, isolated from *Medicago sativa* in potatoes. Results revealed that transgenic potaotes expressing *alfAFP* show enhanced resistance against *Verticillium dahlia* in green house conditions. Further three transgenic lines exhibited better performace in field conditions when evaluated for their efficacy against *V. dahlia.*

In other study by Gianessi *et al*. (2002), Russet Burbank cultivar was transformed with *alfAFP* using *Agrobacterium*. Preliminary results revealved enhanced resistannce to *Verticillium* wilt compared to the control plants. The expression of *alfAFP* was observed in all parts of the plants including roots that are initial infection sites of pathogen. Later on, transgenic plants were challenged against this fungus by cultivating in *Verticillium* infested soil compared to the control in Oregan and Wisconsin. Transgenic plants expressing plant defesins showed greater resistance to *Verticillium* compared to non-transgenic plants with six-fold reduction in fungal levels.

Potatoes with virus resistance: Potato crop is infected by variety of virus, i.e. Potato virus Y (PVY), Potato virus X (PVX), Potato virus S (PVS), Potato leafroll virus (PLRV), Potato virus M (PVM). Many attempts to engineer virus resistance in potatoes from fellow researchers have been documented.

PVY-specific short hairpin RNA (shRNA) has been used in potatoes to induce resistance against PVY. The recombinant binary vector contained shRNA driven by 35S CaMV promoter that can target conserved region of PVY coat protein. Potato cv. "Cardinal" was transformed with this construct via *Agrobacterium.* 0.05-22% reduction of coat protein mRNA of PVY was recorded compared to the control plants. Based on the results of RNA silencing approach, Tabassum *et al*. (2016) concluded that this methodology can remarkably change the strategy of plant defense against viral infection (Tabassum *et al*., 2016).

A broad spectrum resistance against different strains (PVY^{O}, $PVY^{N:O}$ and PVY^{NTN}) of PVY has been reported in transgenic potatoes by the modification of *eIF4E* gene (Arcibal *et al*., 2016). When transgenic plants were challenged against PVY by inoculating, not a single viral detection was recorded in inoculated leaves, emerging leaves and sprouting tubers. *eIF4E* gene variant *Eva1* originated from *S. chacoense*, *S. demissum*, and *S. etuberosum*. When overexpressed under the control of 35S promoter in potato variety "Russet Burbank" slowed down the symptom of PVY infection (Duan *et al*., 2012).

Romano *et al*. (2001) developed potatoes resistant against two Brazilian PVY strains by engineering with coat protein (*CP*) gene of PVY^{O}. Dusi *et al*. (2009) investigated the resistance in these transgenic lines for three consecutive years under the field condition. The transgenic plants were strictly monitored and evaluated against PVY by DAS-ELISA. After three years, no any infection of PVY was observed in 1P clone whearse 63P clone had just 1% of infection compared to 90% infection of PVY in non transformed plants.

CP based chimeric gene derived from strains of PVY (PVY^{O}, PVY^{C} and PVY^{N}) and PVS, was cloned in marker-free vector to develop dsRNA that was transformed to potato cv. "Zihuabai". The transgenic

potato lines exhibited immunity against both PVX and PVY (Bai *et al.*, 2009).

Orbegozo *et al*. (2016) reported development of transgenic potatoes cv. "Desiree" resistant against Potato leafroll virus (PLRV) using self-excisable Cre-LoxP in vector that contained inverted repeats of *CP* gene under the control of heat inducible promoter and *nptII* as selection marker. The transgenic plants obtained were marker-free. Nickel *et al*. (2008) reported PLRV resistant transgenic potatoes cv. "Gala" that expressed coding sequence of single chain variable fragment *ScFVP1-1* under the control of constitutive promoter 35S promoter. Chung *et al*. (2013) transformed potatoes cv. Vales Sovereign using *Agrobacterium* harboring inverted repeated of dsRNA (designed from 200 bp tandem sequence of capsid protein genes of PVY, PLRV and PVA) interspread by an intronic sequence. When tested against aphid transmitted viruses, transgenic lines showed 100% resistance against PVA and PVY^{o} and 72-96% resistance against PLRV.

The particle sequences of PVX *ORF2* gene, *PVY-Hc-Pro* (Helper Competent Proteinase) gene and PLRV Coat protein gene (*CP*) were cloned in expression vector under the control of 35S and were transformed in Potato cv. Desiree and Kuroda (Arif *et al.*, 2012). 20% of the transgenic lines exhibited resistance against PVX, PVY and PLRV.

The different molecular approaches have been used to encode resistance against aforementioned virus. The genes encoding for coat protein, non structural proteins, antisense RNAs and ribozymes have been used and reported as protective strategy to resistance infection from respective viruses. Most importantly among these, coat protein methodology has been widely used to encode resistance against viruses (Doreste *et al.*, 2002). To investigate cross protection, experiment was conducted in tobacco by transforming it with *CP* gene. Delayed or reduced symptoms were observed in plants when infected with viral strain. Several examples of pathogen derived resistance have been successfully reported (Turner *et al.*, 1987; Powell *et al.*, 1986). Later on, a study from Hemenway *et al*. (1988) reported that transgenic plants expressing *CP* gene exhibited resistance against PVX (Table-3). *CP* gene mediated strategy was also reported by Doreste *et al*. (2002) to induce resistance against PVX infection. 16 clones out of 20 clones successfully showed protection against PVX, whereas four resistant clones of cv. "Desiree" were further screened by repeated field experiments.

Nematode resistance in potato: Plant parasitic nematodes (PPNs) are obligate biotrophic parasites which are responsible for lethal crop damage and severe yield reductions. Various economically important genera parasitize various crop plants. Three most economically prominent nematodes such as cyst, root lesion and root-knot are the genera of the plant parasitic nematodes (PPNs) and Heteroderidae family. The management of plant parasitic nematodes has been a big challenge for the agricultural scientists and farming community. Transgenic plants harboring nematode resistance genes have established its practical implications in the field of plant nematology. The control of nematodes below the threshold level is very important for agricultural sustainability and food security (Ali *et al*., 2017). Several case studies have shown the pre-eminence of genetic engineering approaches to induce nematode resistance in potato.

Van der vossen *et al*. (2000) discussed about the isolation of *Gpa2* gene in potato that confers resistance against potato cyst nematode (PCN), *Globodera pallida*. The molecular analysis of *Gpa2* gene showed about 88% homology (amino-acid identity) between Gpa2 and Rx1 proteins. The latter contributes to PVX resistance in potato. In this case, nematode and virus resistance cascades share common components, thus possessing a potential to confer resistance to distinct plant pathogens in potato. Nematode resistance in plants can be achieved through transgenic expression of resistance proteins that also induces the expression of pathogenesis related (PR) proteins. The potato roots expressing *Hero A* gene (salicylic acid (SA)-dependent PR genes) confer resistance to potato cyst nematodes (PCN) and is considered as a hallmark for the cultivar resistance against PCN (Uehara *et al*., 2010). The endo-parasitic RCN (root cyst nematode) *Globodera rostochiensis* causes considerable damage in potato cultivation. The *Gro1* resistance locus to *G. rostochiensis* was found on potato chromosome VII which is co-localized with a resistance gene like DNA marker. The constitutive expression of *Gro1* has increased resistance in potato, against *G. rostochiensis* pathotype Ro1 (Paal *et al*., 2004). Similar study was conducted by Bakker *et al*. (2004) who revealed that *H1* gene confers resistance to *Globodera rostochiensis* in potato.

Chemo-disruptive peptides are considered as another critical option to curtail the plant parasitic nematodes invasion into the roots of crop plants. Nematodes use AChE (acetylcholinesterase) and/or nicotinic acetylcholine receptors for the proper functioning of its nervous system. The normal functioning of these receptors can be inhibited through binding with peptides such as ACHE-1-7.1 and LEV-1-7.1 (Winter *et al*., 2002). Both peptides have been reported in potato and causes chemo-disruption of J2 nematodes by blocking their reaction to chemical signal at very minute concentrations of up to 1 nm. Transgenic potato plants expressing a secreted peptide that inhibited nematode AChE eventually led to disorientation of invading nematode (*G. pallida*) and caused 52% reduction in the number of female nematodes (Liu *et al*., 2005; Lilley *et al*., 2011).

The application of plant-delivered RNAi to silence essential nematode genes has recently emerged as a potentially valuable resistance strategy (Fuller *et al*., 2008). The possibility of engineering nematode resistance by the *in-planta* production of dsRNA to target essential nematode genes has been recognized since the first demonstration of RNAi in plant-parasitic nematodes (Urwin *et al*., 2002; Atkinson *et al*., 2003). Huang *et al*. (2006) showed that ingestion of 16D10 dsRNA *in vitro* silenced the target parasitism gene in root knot nematode and resulted in reduced nematode infectivity. In potato, 65–68% reduction in the number of egg masses was obtained using RNAi approach to silence *Mc16D10L* gene that confers resistance against nematode specie *Meloidogyne chitwoodi* (Dinh *et al*., 2014; Banerjee *et al*., 2017).

Application of recent technologies against biotic stress: RNA interference (RNAi) is the ability of double-stranded RNA (dsRNA) to inhibit homologous gene expression at the transcriptional or post transcriptional level. The specificity is sequence-based and depends on the sequence of one strand of the dsRNA corresponding to a part or all of a specific gene transcript. These RNAs include micro RNAs and small interfering RNAs, both of which use RNA-induced silencing complexes (RISC) also known as ribonucleoprotein for regulation of target gene repression (Kamthan *et al*., 2015; Hussain *et al*., 2019). RNAi has proved its significance in functional genomic research and proved itself as a potential strategy in crop improvement for the control of insect pests and diseases.

RNAi strategeies have been implemented in various crops for improvement of their characteristics. It has also been used for developement of transgenic crops resistant to biotic stresses, e.g. *Arabidopsis* (Navarro *et al*., 2006); rice (Jiang *et al*., 2009; Yara *et al*., 2007); soybean (Peltier *et al*., 2009); wheat (Xin *et al*., 2010); cassava (Vanderschuren *et al*., 2009); tomato (Schwind *et al*., 2009); tobacco (Kamthan *et al*., 2015).

A construct consisting of GFP marker gene along with hairpin RNA was developed and tested in cv. “Desiree” potato. After 72 hours of post-inoculation of *P. infestans* in transgenic leaf, a 55-fold plummet in the intensity of signal associated with GFP expression was observed as compared to the wild-type. The study pointed out that the RNA interference technology used in the potato can target the pathogen transcript following its processing. The study tested for *PiPEC*, *PiCESA2* and *PiGPB1* genes which are crucial in *P. infestans* infection process, along with PiGAPDH involved in the maintenance of cell (Jahan *et al*., 2015).

Hameed *et al*. (2017) developed transgenic cv. “Desiree” potatoes that expressed amalgamated viral coat protein CDS from PVS, PVY and PVX under the control of 35S promoter, that engendered dsRNAs (hairpin loop structure) from the expression cassette (Ec1/p5941). Almost one hundred percent resistance was found for all PVS, PVY and PVX infections in the transgenic lines, which indicated stable immunity against viruses in potatoes using this technology. Recently, report of transgenic potato lines expressing hairpin RNAi construct of molting-associated *EcR* gene (associated with highly specific molting) exhibited enhanced resistance against CPB that is notorious insect pests of potato crop worldwide (Figure-3). These promising results reveal the functionality of robustness of RNAi applications for the control of insect pests as effective pest management strategy (Hussain *et al*., 2019). The nuclease degradation of dsRNA in the gut lumen of *Leptinotarsa decemlineata*, a CPB, has been thought to be the reason in the responsiveness of orally delivered dsRNA in different insect species. Two such nuclease genes have been investigated and have been implicated to the ameliorated protection of potato plants (Spit *et al*., 2017).

The utility of the chloroplast, which is devoid of RNAi mechanism, of potatoes to generate dsRNAs to target *Shrub* and *β*-actin has been performed. Long dsRNAs can be accreted in the chloroplast. When the CPBs were allowed to feed on the transgenic potato leaves, the mortality generated was 100 percent (Vogel *et al*., 2019; Zhang *et al*., 2015). Artificial microRNAs (amiRNAs) have been used for more promising gene silencing at the posttranscriptional level in the plants. The *Avr3a* gene in *P. infestans*, that is related with host cell virulence as well as the inhibition of hypersensitive cell death, has been investigated in the potato varieties “Kufri Pukharaj” and “Kufri Khyati” with the help of five *Avr3a* amiRNA gene constructs that can target *Avr3a* gene of *P.infestans* in five different regions. It was shown in the study that the *P. infestans Avr3a* gene was effectively silenced and led to either a decrease in the virulence or the death of the invading pathogen (Thakur *et al*., 2015).

Concerning other biotic stresses in potatoes, RNAi technology has already been used for the development of transgenic potatoes resistant to PVY) (Missiou *et al*., 2004) and late blight (Sun *et al*., 2016). The technology can be implemented for the development of transgenic potatoes resistant against all types of biotic stresses.

In recent years, a novel genome editing technique has been emerged, i.e. Clustered Regularly Interspaced short palindromic repeats (CRISPR) associated cas9/sgRNA system. It has been derived from bacterial immune system and is an easy, inexpensive and user-friendly genome editing technique, which has revolutionized the molecular biology research. CRISPR is being used in plant genetic engineering research in studying function of genes (Khatodia *et al*., 2016).

CRISPR-Cas9 has been used to produce transgenic potatoes resistant to beet sever curly top virus (BSCTV). It was proved that sgRNA-cas9 constructs

inhibited virus accumulation in transgenic plants and introduced mutations in targeted sequences. It was also shown that overexpression of sgRNA and Cas9 in transgenic *Arabidopsis* and *N. benthamiana* made them highly virus resistant (Ji *et al*., 2015).

The research done by Wang *et al*. (2015) confirmed that the CRISPR/Cas9 system can be successfully used for obtaining monoallelic and biallelic homozygous mutations in transgenic potatoes. They emphasized that this technology can be used for studying function of different uncharacterized genes in potatoes. Targeted mutageneises can be achieved in potatoes (both diploid and tetraploid) by using CRISPR/Cas9. *Agrobacterium* mediated transformation was used to develop transgenic potatoes in which *Acetolactate Synthase1* (*StALS1*) gene was targeted. Stable events were obtained with targeted mutations in cases of both sgRNA and T-DNA. The targeted mutation in primary events was being carried through clonal generations and the Cas9-free progeny was obtained in the germline (Butler *et al*., 2015).

CRISPR-Cas9 was used for complete knockout of *GBSS* gene function in tetraploid potato through transient transfection and regeneration from isolated protoplasts. In 2% of generated lines, mutation in all of the four alleles in a single transfection was reported. Phenotypic analysis was used to confirm the full knockout of GBSS enzyme activity in transgenic lines. Significant production of amylase by GBSS enzyme activity was shown to be sufficient by activity of remaining one wild type allele (Andersson *et al*., 2017).

Attempts have been made to establish the CRISPR/Cas9 system in *Phytophthora insfestans*, the oomycete which is a causal agent for potato and tomato late blight diseases, after the success in oomycete *P. sojae* (oomycete pathogen of soybean). The study targeted 3 genes: *Avr1*, *PiTubA2*, *PiAP5* genes in *P. infestans*. However, even with RNP delivery system and the same construct that was being used for the targeting *P. sojae*, the study failed to observe any transformants with target gene mutagenized. The authors pinpoint the failure to be due to inactivity of Cas9. *P. infestans* are incubated at 18 °C, whereas other *Phytophthora* spp. that gained success is incubated at 25 °C, since the isoform of Cas9 being used was human optimized and isolated from *Streptococcus pyogenes* which is active at 37 °C. This means that the SpCas9 activity declines at declining temperatures. The authors recommend in focusing at systematic scrutiny of factors that limit the competence of the system (Fang and Tyler, 2016).

Cas13 enzymes in CRISPR system have been implicated, expressed transiently as well as transformed stably, in resistance against viruses such as Turnip Mosaic Virus *Nicotiana benthamiana* (Aman *et al*., 2018). In the same way, potato crops can adopt the use of CRISPR/Cas13a system in generating virus resistant gene edited crops that can target RNA viruses such as PVX, PVY, etc. which pillage potato crops.

CRISPR-Cas9 and other CRISPR systems are being used in the development of biotic tolerance in model plants. It has also been successfully implemented in potatoes for inducing mutations to develop gene edited plants. So it would be quite promising to see CRISPR systems for use in development of biotic stress resistant transgenic potatoes. A more comprehensive review on application of various gene editing technologies in potato can be read for further information (Dangol *et al*., 2019).

Table 1. Salient examples of transgenic potatoes against targeted insect pest.

Gene used	Target Insects	Crop	References
Crystal proteins			
cry1Ab	*Helicoverpa armigera* & *Phthorimaea operculella*	Potato	Jansens *et al*., 1995
cry1Ia1, cry 1Ac9 & Bt-cry 5	*Phthorimaea operculella*)	Potato	Davidson *et al*., 2004; Veale *et al*., 2012; Mohammed *et al*., 2016
cry3A	*Leptinotarsa decemlineata*	Potato* and Tobacco	Perlak *et al*., 1993; Coombs *et al*., 2002; Mi *et al*., 2015
cry1Ac	*Leptinotarsa decemlineata*	Potato	Amiri and Bakhsh, 2019
Proteinase inhibitors			
c-II (soybean serine-proteinase inhibitor)	Lepidoptera	Potato	Marchetti *et al*., 2000
cpTI (cowpea trypsin inhibitor)	Lepidoptera	Potato	Burgess and Gatehouse, 1997
PI-IV (soybean serine-proteinase inhibitor)	Lepidoptera	Potato	Marchetti *et al*., 2000
Soybean Kunitz trypsin inhibitor (KTi3, SKTI)	Lepidoptera	Potato	Marchetti *et al*., 2000
Lectins			
Snowdrop lectin (GNA)	Homoptera, Lepidoptera	Potato*, sweet potato, rice,	Gatehouse *et al*., 1996; Gatehouse *et al*., 1997; Michiels *et al*., 2010; Aasen

		sugarcane and tomato	and Hågvar, 2012
Pea lectin (p-lec)	Homoptera, Lepidoptera	Potato* and Tobacco	Gatehouse *et al*., 1996
Others			
Bean chitinase (BCH)	Homoptera Lepidoptera	Potato	Gatehouse *et al*., 1996

Table 2. Salient examples of transgenic potatoes expressing genes against *Veticillim* and *Phytophthota*

Gene used	Target Fungus	Crop	References
alfAFP (defensin)	*Verticillium dahlia* (Wilt)	Potato	Gao *et al*., 2000
R2-like genes	*Phytophthora infestans* (Late Blight)	Potato	Plich *et al*., 2015

Table 3. Transgenic potatoes with resistance to different viruses

Gene Used	Target	Crop	References
Coat Protein gene	Potato virus X (PVX) and PVS	Potato and Tobacco	Doreste *et al*., 2002; Hemenway *et al*., 1988
RNA polymerase (RdRp) gene	Potato virus X	Potato and Tobacco	Bai *et al*., 2009; Mueller *et al*., 1995

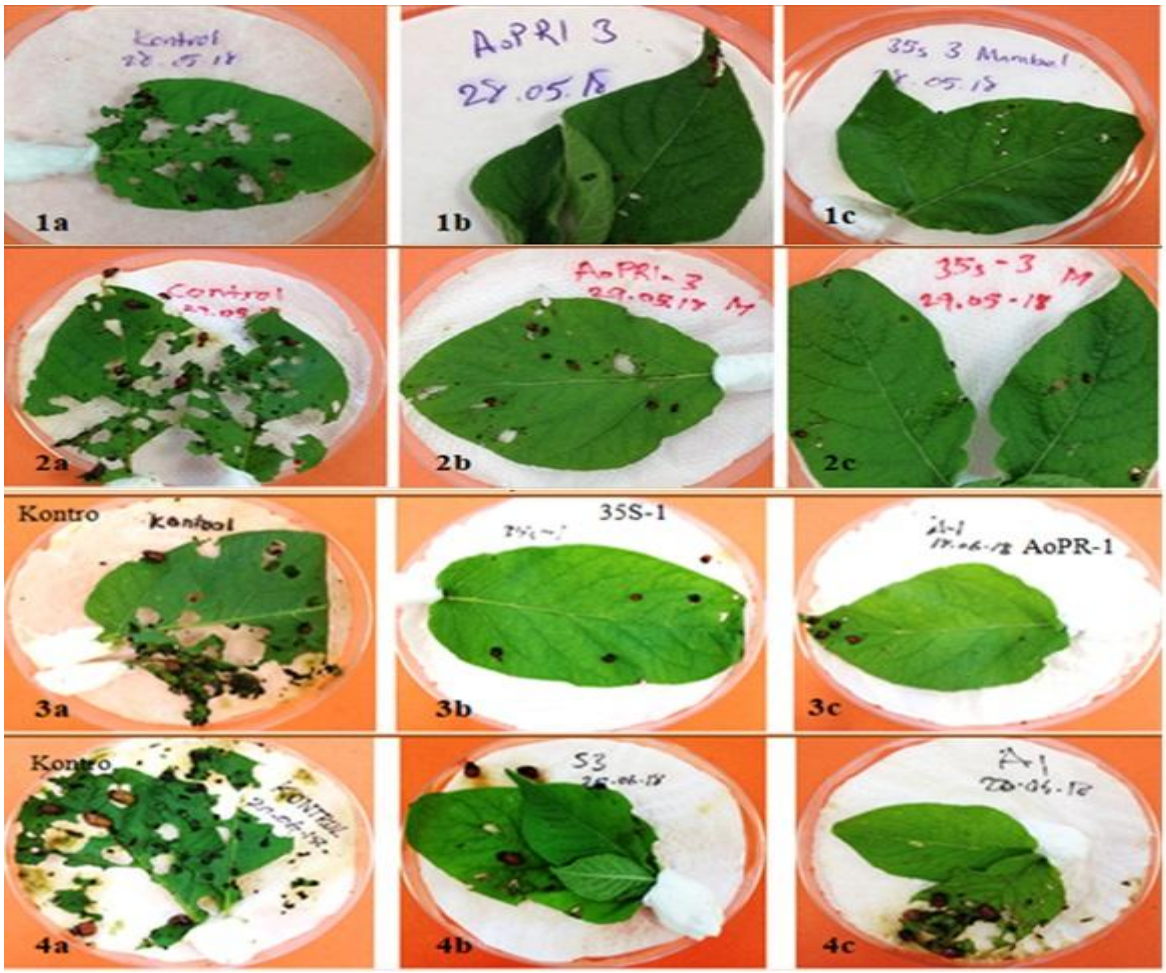

Figure 1. The insect resistant transgenic potatoes (cv. Marabel) expressing *cry1Ac* insecticidal gene showed higher mortality rates of 1st, 2nd, 3rd and 4th instars of CPB larvae. 1-4 a represents control plants whereas 1-4b and c represent transgenic plants. The picture has been taken from one of the articles of corresponding author to establish the efficiency of insect resistant potato lines against targeted insect pests (Amiri and Bakhsh, 2019).

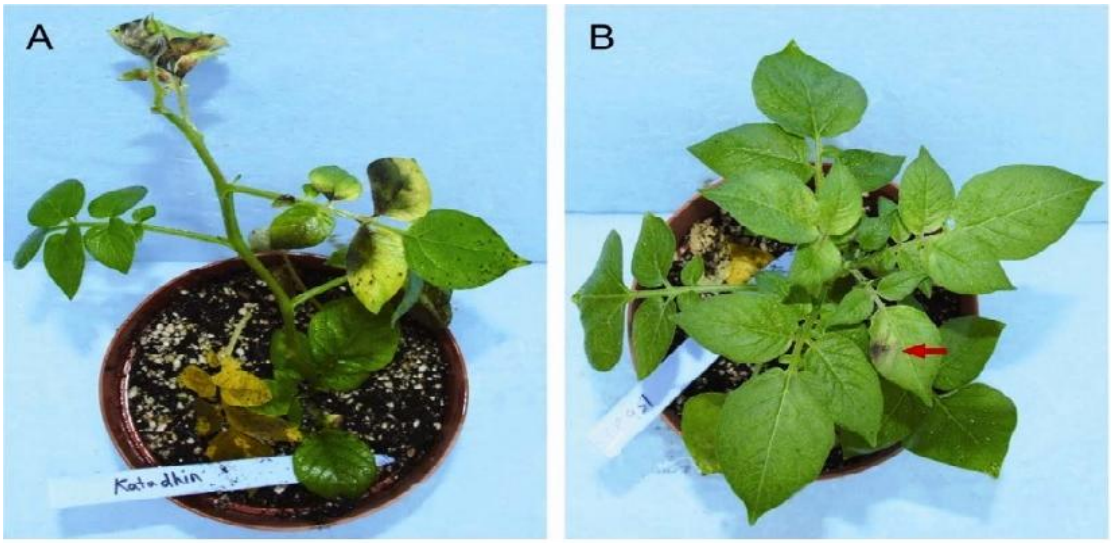

Figure 2. The transgenic potatoes expressing *RB* genes from *Solanum bulbocastanum* that encode resistance against late blight. A. Non transformed control plant, B. RB-transgenic plants inoculated with strain of *Phytophtora infestans* at concentration of 75,000 sporangia/mL. The picture has been taken from Halterman *et al*. (2008) after permission.

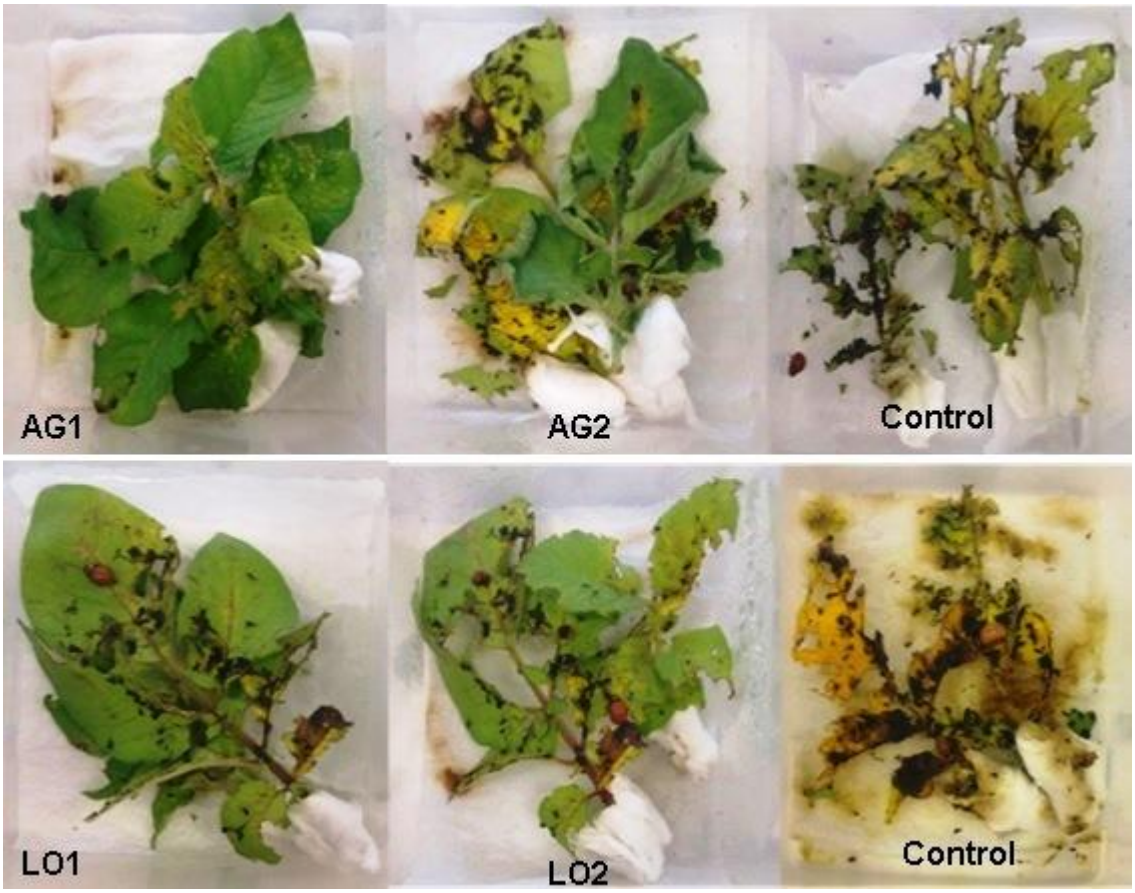

Figure 3. The leaf biotoxicity assay of CPB larvae feeding on T_0 transformants of Agria (AG) and Lady Olympia (LO) expressing hairpin RNAi construct of molting-associated *EcR* gene. Data was recorded after 72 hours of feeding. The figure has been used from one of the published articles of corresponding author to show how RNAi approach can be effective against insect pests (Hussain *et al*., 2019).

Conclusion: Undoubtedly, conventional breeding played a pivotol role in potato improvement; however, recent biotechnological advances furnished the scientific community with the knowledge of excision of gene (s) from one source/organism and to transfer it to an unrelated source/organism. The major challenges to potato breeders are losses incurred due to insect pests, weeds, viral diseases and other abiotic stresses. The farm productivity of agricultural crops worldwide has been severely affected by insect pests. The genes *cry3A* and *cry1Ac* have been successfully introduced in potato to combat these pests. The commercialization of insect-resistant crops expressing *Bt* genes has been outstanding in terms of crop productivity and economic benefits to the farming community. However, it is important to note here that almost all commercialized insect-resistant crops contain genes from *B. thuringiensis*. Though insect resistant potatoes expressing *Cry3a* were introduced earlier and later on removed from the market, however, keeping in view the need and demand, innate potatoes have been introduced in the market. Besides that, there are also reports of the incorporation of genes in potato from other origins (lectins, proteinase inhibitors, etc.). Weeds, viral and fungal diseases incur significant crop yield losses. As scientific data is evident of the introduction of traits resistant to these biotic stresses, there is a dire need to utilize these lines/germplasm in breeding programme to develop resistant cultivars for future.

Modern day technologies like RNAi and CRISPR systems seem promising alternate options for sustainable resistance against crop pests. Using such modern tools, there is a possibility of eliminating the traits that are involved in negative regulation of quality and yield parameters. Besides that, genes of interest can also be introduced in close proximity to specific loci that may remain linked throughout the generations. The use of Cas13 enzymes recently also hold promising for targeting the viruses that are detrimental to the potato yield. All these technologies are quite promising in that we can speed up the current breeding programs of potatoes to develop better resistance, yield and nutritional value.

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