

INSIGHT ON THE POSSIBILITY TO CONTROL LETHAL YELLOWING DISEASE (LYD) IN COCONUT PALMS USING CRISPR/Cas9 SYSTEM AND BIO-CONTROL (ENTOMOPATHOGENS) AMONG OTHER MEASURES

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ABSTRACT

Lethal yellowing disease (LYD) of coconut palms is caused by a wall-less prokaryote in the genus Phytoplasma. Its wall-less nature has made it difficult to culture in any artificial media and the low concentration in phloem of infected plants is a limitation to its in-vitro study. Ongoing research in breeding for resistant/tolerant coconut varieties is currently the major hope to curtail this deadly disease but these efforts are being outpaced by recent reports across regions that tell of increased incidence of this disease. Hence, the need to use integrated control measures or encourage trials on the use of novel environmental and eco-friendly techniques such as the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 Nuclease System or entomopathogens in controlling the problems of LYD among other control measures.

Keywords: *LYD, Phytoplasma, CRISPR/Cas9 system, Entomopathogens*

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) of the more than 1500 species in the Palm family of plants (Arecaceae), the Coconut palm is the best known. The coconut is known for its great versatility as seen in the many uses of its different parts. It is found throughout the tropics and subtropics (James, 1983) although its center of origin is not exactly known. The exceptionally wide distribution of coconuts today is due to the influence of humans, having been carried from place to place by explorers and immigrants. Fossilized coconuts have been found in New Zealand, and the trees have been cultivated for over 4,000 years in India. This leads most botanists to believe that the species originated somewhere around the Indian Ocean. It is a very important component in the global agricultural industry, supporting commerce and consumers worldwide.

The coconut palm's domestication are affected by array of diseases, including anthracnose, black scorch, bole rot, bud rot, dry basal rot, Ganoderma butt rot, leaf blight, leaf spot, nut fall, powdery mildew, root rot, thread blight, and lethal yellowing disease (LYD) among others.

LYD, the most deadly disease affecting the coconut palms, also affects at least 40 species in other Areceaceous genera throughout the world (Danyo, 2011). *C. nucifera* has been the main species investigated due to its economic importance in some countries. Various forms of LYD are caused by phytoplasmas- plant parasitic, wall-less, phloem-limited prokaryotes. Their wall-less nature make it difficult to culture them, limiting the in-vitro studies that can be performed. Studies on phytoplasmas have so far been in-situ, taking advantage of their presence in various hosts to perform various assays including bio-imaging, immunological and molecular procedure.

Unlike most other plant pathogens, phytoplasmas have unique characteristics in their ability to produce various effector proteins. Secreted AY-WB proteins (SAPs), the most studied effector proteins; it alters and modulate the morphology and physiology of infection susceptible plants in ways that sustain or augment the fitness of the vector. For example, the roles of two effector proteins [(SAP11 ad SAP54) in *Arabidopsis thaliana* Heynh.] infected with Aster Yellows phytoplasma strain Witches' Broom (AY-WB) have been demonstrated by Sugio *et al.* (2011) and Maclean *et al.* (2011) in the down-regulation of lipoxygenase (LOX) expression, jasmonate (JA) synthesis, an increase in insect vector progeny, formation of leaf-like and green flowers (phyllody and virescence) and increased formation of stems and branches (witches' broom).



Fig1. Decapitated coconut palm that had been infected with LYD



Fig2. A decapitated coconut palm with two others palms showing acute symptoms of LYD



Fig3. Coconut palm infected with LYD showing witch-broom symptom drooping of nut and blackening inflorescence



Fig4. Infected palm with LYD showing acute symptom with complete wilting



Fig5. Healthy coconut palm with fruits

TRANSMISSION OF PHYTOPLASMA RESPONSIBLE FOR LETHAL YELLOWING DISEASE (LYD)

Phytoplasma transmission occurs mainly as a movement either from plant to plant with the aid of its insect vectors or movement within plant using phloem from source to sink, as they are able to pass through sieve tube elements. In some rare cases grafting could be responsible for its movement between plants. Phytoplasmas are mainly spread between plants by insect vectors in the superfamilies; Membracoidea (leafhoppers), Fulgoroidea (planthoppers), and Psylloidea (jumping plant lice). When insect vectors feed upon infected plants, phytoplasmas enter the insect's body through the stylet, move through the intestine, and transcytose to the hemolymph (Christensen *et al.*, 2005). From there they proceed to colonize the salivary glands, a process that can take up to three weeks (Christensen *et al.*, 2005), this cycle allows systemic distribution within the vector as phytoplasmas are found in most major organs of an infected insect host.

Several researchers have considered whether the understory vegetation on coconut farms could serve as alternate LYD hosts. For example, Brown (2008) examined the understory vegetation on coconut farms and found that eight among the 50 plant species examined contained LYgroup (16SrIV) phytoplasma. Eziashi *et al.*, (2013) found weed species harboring or hosting insect vectors of phytoplasma responsible for LYD in coconut palms. However, weed species hosting insect vectors of LYD are not necessarily alternate host of phytoplasma as reported. For insects and weeds to be considered host species of any in the LYD group phytoplasmas, they must contain the respective 16SRNA.



Fig6. Planthopper (*Flugoridea*)
(Source: Eziashi *et al.*, 2014)



Fig6. Jumping plant louse (*Psyllidea*)
(Source: www.lib.ncsu.edu/specialcollections/digital/.../jumpingplantlice)

CONTROL APPROACHES TO LETHAL YELLOWING DISEASE (LYD) OF COCONUT PALMS

Several control measures have been suggested in order to curtail the menace of the LYD in coconut palms. Some control measures are practicable while others might not be. For example, Weintraub, 2007, highlighted four (4) ways for the control of insect vectors of phytoplasma and the pathogen phytoplasma; they include; “traditional control, symbiont control, plant lectins, and systemic acquired resistance”.

Traditional vector control methods are insufficient to control the disease (Weintraub and Beanland, 2006). The most reliable means of controlling vectors is by covering the crop with insect-proof screening. Screening for example, is the only method to attain excellent vector control; however, its applicability is so severely limited due to the logistics of large scale agriculture in major crops – sugar cane, corn, rice, fruit trees, grapes, coconut – that its use cannot even be contemplated. On the other hand, conventional insecticides, even when frequently used (Wally *et al.*, 2004), will not control the appearance of disease because pathogen transmission occurs faster than insecticides can act, and there is often a constant influx of new vectors from surrounding habitats. At best, use of insecticides might help control vector populations, and thus reduce intra-crop transmission (Weintraub, 2007).

Another potential powerful tool for controlling pathogen transmission is through the manipulation of symbiotic bacteria, it can be said to be “symbiotic control” strategy. Many arthropods carry a diverse assembly of symbiotic microorganisms that are maternally inherited and which have major effects on their hosts. These bacteria can be genetically modified to prevent the transmission of pathogens; arthropods containing these transformed bacteria are called paratransgenic (Weintraub, 2007).

As previously stated, phytoplasma vectors feed specifically in phloem cells, obtaining nutrition from free amino acids and sugars. As such, the activity of carbohydrate binding plant lectins, which would directly affect vector nutrition and/or be toxic, has been examined as a means of

controlling vectors. These lectins are usually inserted into the target plant by *Agrobacterium rolC* (from *A. rhizogenes*), specific for expression of the lectins and stability in the phloem (Saha *et al.*, 2006). There are two plant lectins that have shown efficacy in vectors: snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) (Nagadhara *et al.*, 2004) and a 25-kDa homodimeric lectin, *Allium sativum* leaf lectin (ASAL) (Dutta *et al.*, 2005). The mechanism of GNA is complex and not fully understood: it is not degraded by midgut proteases; it binds to D-mannose in the midgut of insects and is transported across the epithelial barrier to the circulatory system (Fitches *et al.*, 2001). In bioassay, feeding on GNA rice caused 90% mortality in *Sogatella furcifera* (Nagadhara *et al.*, 2004) and 29% and 53% mortality in *Nephotettix virescens* Distant and *Nilaparvata lugens* Stål, respectively (Foissac *et al.*, 2000). ASAL has a high degree of sequence similarity to GNA (Majumder *et al.*, 2004); however, it may decrease the permeability of the gut membrane and seems to be effective at much lower levels than GNA (Biandyopadhyay *et al.*, 2001). A major receptor of GNA in the phytoplasma vector *N. lugens* is 26 kDa ferritin, thus ASAL may also be involved in iron homeostasis (Du *et al.*, 2000). One of the primary functions of spider venom is to paralyze prey; often these toxins are polypeptides that target the nervous system of the host. Some of these polypeptides bind to specific receptors and can affect the neuronal ion channels, neuronal receptors or presynaptic membrane proteins. Since GNA is able to cross the intestinal epithelium, it has the potential to transmit peptides fused to it into the hemolymph. In a novel application of this idea, Down *et al.* (2006) demonstrated the insecticidal effects of spider venom (SFI1) on the planthopper, *N. lugens*. Although the SFI1/GNA fusion product and smaller levels of GNA was found in the hemolymph, the mechanism of toxicity is not known. Possibly the fusion protein was cleaved, allowing the SFI1 toxin to act.

Lastly, in system acquired resistant (SAR), plants can activate defense mechanisms when challenged by either an arthropod or pathogen. This response can also be elicited by a number of chemicals (Sticher *et al.*, 1997). *Colladonus montanus* (Van Duzee) is an efficient vector of X-disease in fruit trees, and can also efficiently transmit the phytoplasma to *Arabidopsis thaliana* Columbia under laboratory conditions. Infected

A. thaliana is stunted and produces fewer or no siliques. Treatment with benzothiadiazole (BTH) protected plants from phytoplasma; ~ 74% of non-treated control 172 plants became infected, as compared to only 35% of the plants protected with 4.8 mM BTH a week prior to leafhopper feeding (Bressan and Purcell, 2005). The mechanism for this effect is not clear: the plant phloem could have been morphologically modified to prevented phytoplasma from establishing or replicating, but the BTH could also have elicited production of a substance inhibiting vector feeding, hence inhibiting transmission. Fewer leafhoppers survived on BTH-treated *A. thaliana* than on non-treated plants.

The above control measures as stated by Weintraub, 2007, focus majorly on the insect vectors with little control on the phytoplasma. In order to reduce the disease incidence and the possibly of preventing further outbreak, a cost effective integrated control approach has to address concerns relevant to the following;

- ✓ Deadly phytoplasma responsible for LYD in coconut,
- ✓ Insect vectors of phytoplasma,
- ✓ Coconut palms
- ✓ Alternate potential weed hosts.

Breeding for disease resistant coconut palms to LYD is currently the only major hope to address the problems of LYD but if measures are not taken to address the causal agent phytoplasma, insect vectors and potential alternate hosts there might be possibility to future outbreak and

reoccurrence of this disease, hence the need to use integrated control measures to address the problems of LYD in coconut and other plants of economic importance. For example regular removal and burning of weeds, coconut palm logs, diseased coconut palms and pruned coconut fronds in plantations from coconut plantations (Eziashi *et al.*, 2013) would reduce exposure to the phytoplasma and its insect vectors, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 Nuclease (CRISPR/Cas9) system, if extended to LYD, might handle the deadly phytoplasma by knocking-out its DNA from either insect vectors or host plants. Lastly, trials on the use of entomopathogenic fungi (bio-control) might assist in insect vector elimination. This control approaches if successful might in the long run address the problems of Lethal yellowing disease and prevent the possibility of future outbreak.

CRISPR/Cas9 SYSTEM

Increasing success in gene editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has increased their usage relative to other genome engineering methods like zinc finger nucleases (ZFNs) or transcription-activator-like effector nucleases (TALENs) because this method is inexpensive, efficient, and precise, with high-throughput.

Three types of CRISPR mechanisms have been identified, of which type II mechanism is the most studied (further details see: <https://www.neb.com/tools-and-resources/feature-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology> and Alex *et al.*, 2014). In this case, CRISPR regions of the bacterial genome contain DNA that originated from previously invading bacteriophage (bacterial virus). In bacteria, CRISPR DNA is transcribed into RNA which is complementary to the bacteriophage DNA. When this RNA teams up with Cas9, it acts as a guide to recognize the bacteriophage DNA when it invades again. When the RNA-enzyme complex finds its target bacteriophage DNA, it binds by complementary base pairing and the nuclease enzyme cuts the DNA, preventing the virus from replicating. Scientists have taken advantage of this mechanism to create custom made CRISPR-style guide RNA that teamed up with custom Cas9 in order to target a gene for disruption, removal or replacement with another gene of benefit introduced at the same time.

A current notable example using the CRISPR/Cas9 system in gene editing includes the introgression of parasite-resistance genes into mosquito populations, thereby modifying the ability of the vector to transmit the pathogens (Gantz *et al.*, 2015). This research work could be extended in editing of genes of other vector borne diseases such as the elimination of phytoplasma DNA from its insect vectors and host plants as a way of limiting the pathogen transmission. To develop such genetic editing control method, it is necessary to first identify the pathogen in the target vector and host plant whose characteristics appear promising; this technique can compromise transmission: by reducing vector competence, by expressing a gene product that could kill the pathogen, or by creating physical competition for space that the pathogen would normally occupy.

Genome editing can also be used in molecular plant breeding by allowing the introduction of precise and predictable modifications directly in an elite background, and the CRISPR/Cas9 system is particularly beneficial because multiple traits can be modified simultaneously. Non-homologous end joining (NHEJ)-mediated gene knockouts are the simplest form of targeted modification, and these could be used e.g., to eliminate genes that negatively affect food quality,

to confer susceptibility to pathogens or to divert metabolic flux away from valuable end-products. For example, Wang *et al.*, (2014b) used both TALEN and CRISPR/Cas9 technologies to target the genes of the mildew-resistance locus (MLO) in wheat and successfully knocked out all three MLO homoalleles, generating plants resistant to powdery mildew disease. Precise nucleotide exchanges using oligonucleotide donor sequences could be used to modify the regulatory sequences upstream of genes that determine agricultural performance therefore improving crop yields. The insertion of large sequences by NHEJ or homologous recombination (HR) would allow the introduction of transgenes at defined loci that promote high-level transcription and do not interfere with the activity of endogenous genes. Site-specific nucleases also allow targeted molecular trait stacking, i.e., the addition of several genes in close vicinity to an existing transgenic locus. This makes it feasible to introduce multiple traits into crops with a low risk of segregation, which is difficult to achieve by classical breeding or even conventional genetic engineering (Ainley *et al.*, 2013). Once stacking has been achieved, the entire array of transgenes can be mobilized into other germplasm by crossing because it behaves as a single locus. It is possible to achieve these aims using site-specific recombination, but targeted integration using programmable nucleases combined with precise NHEJ or HR does not leave behind any footprints associated with the integration method, such as loxP or attB sequences. Although the European regulatory framework for genetically modified crops focuses on the process and not the product (hence two identical plants produced by conventional mutagenesis and genetic engineering would be regulated differently under the current guidelines), there is hope and confidence that plants altered by the excision of a few nucleotides using genome editing tools such as CRISPR/Cas9 would not be classified as genetically modified organisms (Hartung and Schiemann, 2014; Li *et al.*, 2012; Podevin *et al.*, 2013). There are several ways to create transgene-free mutated plants using programmable nucleases, including the transient expression of the nuclease components using agro-infiltration or viral vectors, the delivery of the components directly as functional gRNA and Cas9 protein or the incorporation of the gRNA and Cas9 transgenes on a separate chromosome to the targeted locus so that they can be removed by segregation. Although the specificity of the CRISPR/Cas9 technology remains to be investigated in detail, it is already clear that the frequency of off-target mutations is well below that caused by chemical and physical mutagenesis techniques (Podevin *et al.*, 2013).

BIO-CONTROL (ENTOMOPATHOGENS) MEASURES

Bio-control measures involve the use of predators, parasitoids, pathogens or any biological agent to suppress, reduce, eliminate and manage insect pest populations. Entomopathogens refers to microorganisms (fungi, bacteria, viruses, nematodes) that can destroy or kill insect pests. Biological control of pests is primarily an exercise in applied ecology, requiring an understanding of population dynamics, predator-prey interactions, and intra- and interspecific competition. However, the relevance and application of evolutionary principles to biological control have received rather less attention (but see Roderick and Navajas 2003; Hufbauer and Roderick 2005). Evolution and biological control are most commonly discussed in the context of classical biological control, in which long-term pest suppression results from the introduction and establishment of a natural enemy collected from the native range of an exotic pest (Hufbauer and Roderick 2005). However, microevolution can also influence other forms of biological pest control. Insect pathogens have a long history of use in biological control: in particular, bacteria [mainly various strains of *Bacillus thuringiensis* (Bt)], entomopathogenic fungi (e.g., *Beauveria*

bassiana and *Metarhizium* species), numerous species of *baculovirus*, and also entomopathogenic nematodes (e.g., *Steinernema* and *Heterorhabditis* species) (Lacey and Kaya 2007). Entomopathogens are distinguished from other means of insect biological pest control primarily by their methods of application. For the most part, insect pathogens are applied using an inundative release strategy. That is, they are applied in large numbers onto intermediate to high densities of pest populations, with the expectation of 'immediate' pest control. Immediate means that pest suppression does not rely on the long term reproduction or establishment of the pathogen, although in reality it can take several days for the pathogens to replicate and kill their host (Cory and Franklin, 2012). It is also likely that with some pathogens and with some target groups, effective control relies on secondary cycling of the pathogen [e.g., locusts (Thomas *et al.*, 1995) and forest Lepidoptera (Woods and Elkinton 1987)], although mechanistic studies of the role of secondary transmission are rarely carried out in the pest control context. Insect pathogens have also been used successfully as classical biological control agents, with a limited number of introductions resulting in long-term pest suppression, although this approach has tended to be more restricted to pests of forest or plantation crops (Hajek *et al.*, 2007).

Research over decades has demonstrated that microbial control of insects has played an integral part in insect control as way of controlling vector populations, and thus reduces inter-crop/intra-crop transmission. Microbial control is of importance when other methods have been found less effective especially when the toxic substances release from chemical insecticides pose serious threat to humans and the environment. Detailed study on entomopathogens nature and their biology will in future guarantee effective control of insects and probably in integration with other control agents. In addition to other benefits of entomopathogens, they can become established in a pest population or its habitat (host and alternate host) and provide control during subsequent pest generations or seasons (Canan, 2013).

Although the use of insect pathogens have mostly be useful and demonstrated in crawling insects (at larvae, pupae or adult stage) with few cases shown in flying insects. Among the few cases, a notable example is the use of an insect-pathogenic fungus (*Metarhizium anisopliae*) for control of the adult African malaria vector *Anopheles gambiae* (Scholte *et al.*, 2008). The use of *M. anisopliae* to control termites and other agricultural insect pests is currently ongoing in Nigeria. A better understanding of the biology and ecology of insect vectors of LYD and the possible microbial ecology may lead us to new pathogens or new means of interfering with vital process on possible application of control. By no means, it is a quick route to implementation of novel and economically viable insect vectors of LYD control methods, but ongoing microbial survey efforts are gradually providing the necessary foundation.

CONCLUSION

If the threats pose by lethal yellowing disease (LYD) persist, coconuts may become a rarity. Breeding for LYD resistant/tolerant coconut progenies is not a guarantee of success, as continued exposure to mutants of phytoplasma might render them susceptible again. Hence, continued improvements are needed in the use of integrated management system or the development of new methods to combat LYD to ensure sustainable, profitable and efficient coconut farming in the world.

REFERENCES

- Ainley, W. M., Sastry-Dent, L., Welter, M. E., Murray, M. G., Zeitler, B., Amora, R., *et al.* 2013. Trait stacking via targeted genome editing. *Plant Biotechnol J* 11:1126–34.
- Biandyopadhyat, S., Roy, A. and Das, S. 2001. Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity.- *Plant Science*, 161 (5): 1025-1033.
- Bressan A. And Purcell A. H. 2005.- Effect of benzothiadiazole on transmission of X-disease phytoplasma by the vector *Colladonus montanus* to *Arabidopsis thaliana*, a new experimental host plant. *Plant Disease*, 89 (1): 1121-1124.
- Brown, S. E., Been, B. O., and McLaughlin, W. A. 2007. First report of the lethal yellowing group phytoplasmas in the weeds *Emilia fosbergii* and *Synedrella nodiflora* in Jamaica. *New Disease Reports*.
- Canan, U. 2013. Microorganisms in Biological Pest Control — A Review (Bacterial Toxin Application and Effect of Environmental Factors). *Current Progress in Biological Research*. 13: 287-317
- Carraro, L., Loi, N., Favali, M. A. and Favali, M. A. 1991. "Transmission characteristics of the clover phyllody agent by dodder". *J. Phytopathol* 133: 15–22.
- Christensen, N., Axelsen, K., Nicolaisen, M. and Schulz, A. 2005. "Phytoplasmas and their interactions with their hosts". *Trends in Plant Sciences* 10 (11): 526–535.
- Cory, J. S. and Franklin, M. T. 2012. Evolution and the microbial control of insects. Blackwell Publishing Ltd 5: 455–469.
- Danyo, G. 2011. Review of scientific research into Cape St. Paul Wilt Disease (CSPWD) of coconut in Ghana. *African Journal of Agricultural Research* 6(45): 67-4578.
- Dollet, M., Quaiocoe, R. and Pilet, F. 2008. Overview of the Lethal Yellowing Type Disease of Coconut: Diversity, Variability, and Diagnosis. Proceedings of the International Workshop on Lethal Yellowing Disease of Coconut, Accra, Ghana, June, 2008, p. 8.
- Down, R. E., Fitches, E. C., Wiles, D. P., Corti, P., Bell, H. A., Gatehouse, G. A. and Edwards, J. P., 2006. Insecticidal spider venom toxin fused to snowdrop lectin is toxic to the peachpotato aphid, *Myzus persicae* (Hemiptera: Aphididae) and the rice brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Pest Management Science*, 62 (1): 77-85.
- Du, J. P., Foissac, X., Carss, A., Gatehouse, A. M. R. and Gatehouse, J. A., 2000. Ferritin acts as the most abundant binding protein for snowdrop lectin in the midgut of rice brown planthoppers (*Nilaparvata lugens*). *Insect Biochemistry and Molecular Biology*, 30 (4): 297-305.
- Dutta, I., Saha, P., Majumer, P., Sarkar, A., Chakraborti, D., Banerjee, S. and Das, S. 2005. The efficacy of a novel insecticidal protein, *Allium sativum* leaf lectin (ASAL), against homopteran insects monitored in transgenic tobacco.- *Plant Biotechnology Journal*, 3 (6): 601-611.
- Eziashi, E. and Omamor, I. 2010. Lethal yellowing disease of the coconut palms (*cocos nucifera* L.): An overview of the crises. *African Journal of Biotechnology* 9(54): 9122-9127.
- Eziashi E.I., Omamor, I.B., Aisuen, N.O., Aisagbonhi, C.I., Airede, C.E., Ikuenobe, C.E., Oruade-Dimaro, E.A., Odewale, J.O. and Osagie, I.J. 2014. Screening Potentials Weed Species

- as Alternate Hosts of Insect Vectors of the Lethal Yellowing Disease (LYD) of Coconut Palms (*Cocos nucifera* L.) in Nigeria. *British Journal of Applied Science and Technology* 3(1): 123-130.
- Fitches, E., Woodhouse, S. D., Edwards, J. P. and Gatehouse, J. A. 2001. *In vitro* and *in vivo* binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*, Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action. *Journal of Insect Physiology*, 47 (7): 777-787.
- Foissac, X., Loc, N., Christou, P., Gaehouse, A. M. R. and Gatehouse, J. A. 2000. Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). *Journal of Insect Physiology*, 46 (4): 573-583.
- Gil, A. and Rotem, S. 2016. CRISPR–Cas adaptation: insights into the mechanism of action. *Nature*.
- Hajek, A. E., McManus, M. L. and JrDelalibera, I. 2007. A review of introductions of pathogens and nematodes for classical biological control of insects and mites. *Biological Control* 41:1–13.
- Hartung, F. and Schiemann, J. 2014. Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. *Plant J* 78:742–52.
- Hufbauer, R. A. and Roderick, G. K. 2005. Microevolution in biological control: mechanisms, patterns, and processes. *Biological Control* 35:227–239.
- James, A. Duke, Handbook of Energy Crops, unpublished 1983. "Cocos nucifera L.". Purdue University, NewCROP, the New Crop Resource Online Program. Retrieved 4th June 2015.
- Lacey, L. A. and Siegel, J. P. 2000. "Safety and Ecotoxicology of Entomopathogenic Bacteria", in *Entomopatgenic Bacteria: From Laboratory to Field Application*.
- Lacey, L. A. and Kaya, H. K. 2007. Field Manual of Techniques in Invertebrate Pathology, 2nd edn. Springer, The Netherlands.
- Li, T., Liu, B., Spalding, M. H., Weeks, D. P. and Yang, B. 2012. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat Biotechnol* 30:390–2.
- MacLean, A. M., Sugio, A., Makarova, O. V., Findlay, K. C., Grieve, V. M., Toth, R., Majumder, P., Banerjee, S. and Das, S. 2004. Identification of receptors responsible for binding of the mannose specific lectin to the gut epithelial membrane of the target insects.- *Glycoconjugate Journal*, 11 (3): 194-200.
- Nagadhara, D., Ramesh, S., Pasalu I. C., Rao, Y. K., Sarma, N. P., Reddy, V. D. and Rao K. V. 2004. Transgenic rice plants expressing the snowdrop lectin (gna) exhibit high-level resis173 tance to the whitebacked planthopper (*Sogetella furcifera*). *Theoretical and Applied Genetics*, 109 (7): 1399-1405.
- Nicolaisen, M. and Hogenhout, S. A. 2011. Phytoplasma Effector SAP54 Induces Indeterminate Leaf-Like Flower Development in Arabidopsis Plants. *American Society of Plant Biologists* 157: 831-841.
- Nicholson, G. M. 2007. Fighting the global pest problem: Preface to the special Toxicon issue on insecticidal toxins and their potential for insect pest control, *Toxicon*, 49: 413–422.
- Podevin, N., Davies, H. V., Hartung, F., Nogue, F. and Casacuberta, J. M. 2013. Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. *Trends Biotechnol* 31:375–83.

- Roderick, G. K. and Navajas, M. 2003. Genes in new environments: genetics and evolution in biological control. *Nature Reviews Genetics* 4:889–899.
- Saha, P., Dasgupta, I. and Das, S. 2006. A novel approach for developing resistance in rice against phloem limited viruses by antagonizing the phloem feeding hemipteran vectors. *Plant Molecular Biology*, 62 (4-5): 735-752.
- Scholte, E. J., Knols, B. G. J. and Takken, W. 2008. An entomopathogenic fungus (*Metarhizium anisopliae*) for control of the adult African malaria vector *Anopheles gambiae*. *Entomologische Berichten* 68 (1): 21-26
- Sticher, L., Mauch-Mani, B. and Métraux J. P. 1997. Systemic acquired resistance. *Annual Review of Phytopathology*, 35: 235-270.
- Sugio, A., Kingdom, H. N., MacLean, A. M., Grieve, V. M. and Hogenhout, S. A. 2011. Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. 108: 48-57.
- Sugio, A., MacLean, A. M., Kingdom, H. N., Grieve, V. M., Manimekalai, R. and Hogenhout S. A. 2011. Diverse targets of phytoplasma effectors: from plant development to defense against insects. *Annu Rev Phytopathol.* 49: 175–195.
- Thomas, M. B., Wood, S. N. and Lomer. C. J. 1995. Biological control of locusts and grasshoppers using a fungal pathogen – the importance of secondary cycling. *Proceedings of the Royal Society Series B* 259:265–270.
- Wally, O., Daayf F., Khadhair, A. H., Adam, L., Elliott, B., Shinnars-Carnelley, T., Iranpour, M., Northover, P. and Keyworth, S. 2004. Incidence and molecular detection of yellows-type disease in carrots, associated with leafhoppers in southern Manitoba, Canada. - *Canadian Journal of Plant Pathology* 26 (4): 498-505.
- Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu J., Gao, C, *et al.* 2014. Simultaneous editing of three homoeoalleles in hexaploid breadwheat confers heritable resistance to powderymildew. *Nat Biotechnol* b; 32:947–51.
- Weintraub, P. G. 2007. Insect vectors of phytoplasmas and their control – an update: *Bulletin of Insectology* 60 (2): 169-173.
- Weintraub, P. G. and Beanland, L. 2006. Insect vectors of phytoplasmas. *Annual Review of Entomology* 51: 91-111.
- Woods, S. A. and Elkinton, J. S. 1987. Bimodal patterns of mortality from nuclear polyhedrosis virus in gypsy moth (*Lymantria dispar*) populations. *Journal of Invertebrate Pathology* 50:151–157.