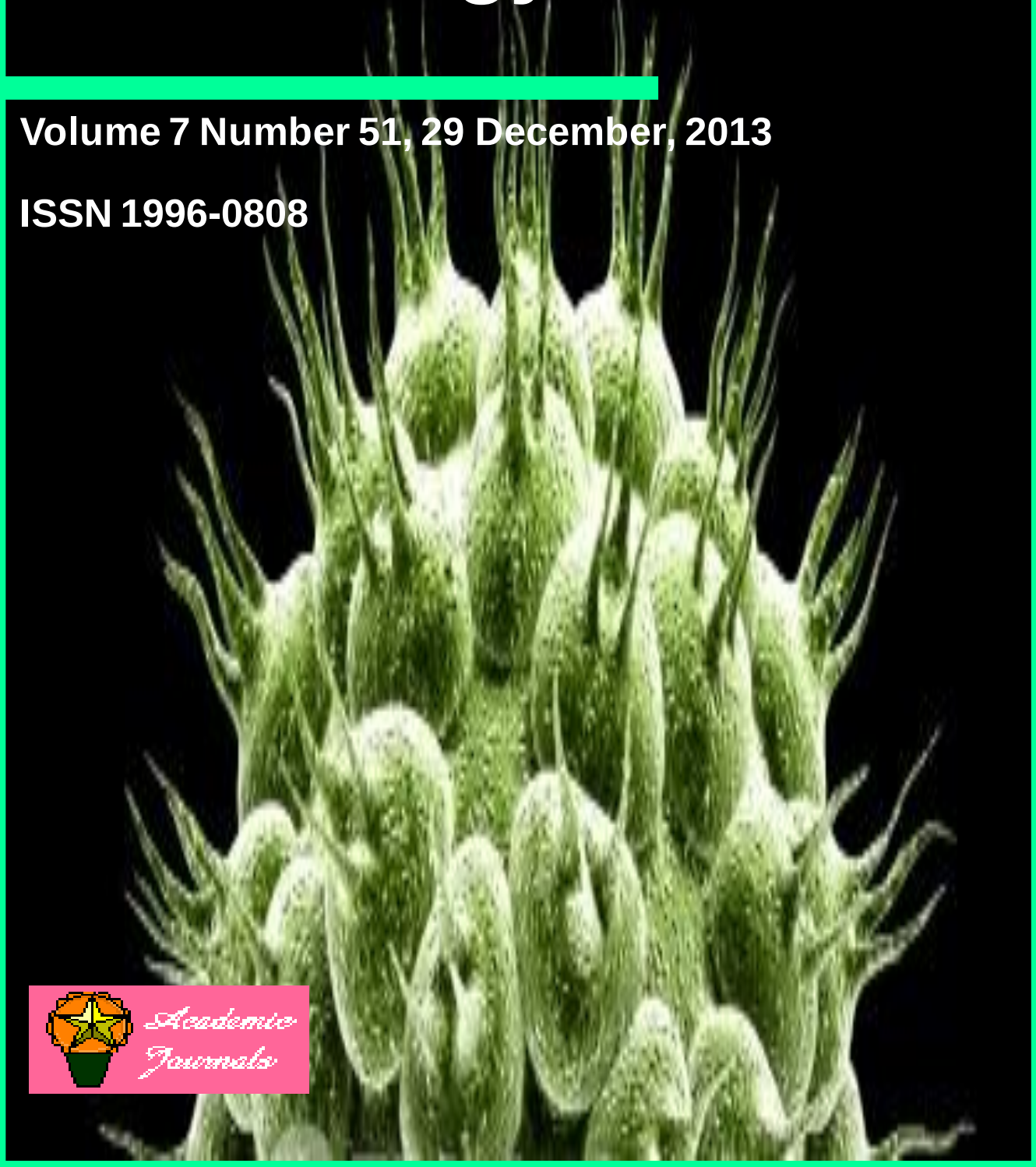
African Journal of Microbiology Research

Volume 7 Number 51, 29 December, 2013

ISSN 1996-0808



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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Microbiology Research

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FOURTH ANNUAL REVIEW ISSUE

academicJournals

Vol. 7(51), pp. 5758-5765, 29 December, 2013
DOI: 10.5897/AJMR2013.5366
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Which bee honey components contribute to its antimicrobial activity? A review

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Accepted 12 December, 2013

The antimicrobial activity of bee honey is one of its most studied biological properties, with an extensive spectrum of activity against medically important microorganisms. The specificity of this activity depends on the components of the honey, which vary according to its floral and entomological origin. The action mechanisms of the most transcendental compounds for honey antimicrobial activity; acidity, osmolarity and hydrogen peroxide, are reported. Recent studies have demonstrated the existence of other compounds contributing to the antimicrobial activity of honey such as, enzymes, antimicrobial peptides, phenolic compounds and Methylglyoxal. This article describes both the most important therapeutic properties and the components attributing antimicrobial activity to bee honey, as well as the microorganisms which this effect has been evaluated and their respective minimum inhibitory concentration (MIC). In conclusion, it has been demonstrated that the antimicrobial activity and other biological activities of bee honey such as antitumor, anti-inflammatory, antioxidant and antiviral properties are conferred by a group of components intrinsic to honey and it depends of the botanical, geographical and entomological origin of the honey.

Key words: Bee honey, antibacterial activity, minimum inhibitory concentration (MIC).

INTRODUCTION

Honey is a natural substance produced by bees from the nectar of flowers or other parts of a plant. This substance has been used as a human food source since ancient times in many different cultures due to its high nutritional content. This characteristic derives from a diversity of compounds such as, carbohydrates, organic acids, proteins and polyphenolic compounds. Honey also contains other compounds, in lesser quantity, such as, free amino acids, minerals and vitamins (Bogdanov et al., 2008). This composition depends on the type of bee and the region in which it is produced. At present, one of the most commercialized types of honey is produced by the *Apis mellifera* bee due to its high production rate, level of technification and for the properties attributed to this food source, which is considered to be a functional food of therapeutic use, given its antioxidant and antimicrobial properties (Cooper et al., 2002; Sherlock et al., 2010; Cooper and Jenkins, 2012).

Honey is well known in many parts of the world as an important agent in the treatment of infectious diseases (Mandal et al., 2010). The use of this food source in traditional medicine has been practiced since the dawn of humanity and is considered to be one of the most effective traditional medicines in the treatment of a number of human diseases (DebMandal and Mandal, 2011; Maniy-Loh et al., 2011; Ajibola et al., 2012; Alvarez-Suarez et al., 2013). This article describes the therapeutic properties of honey, with an emphasis on antimicrobial activity, and each one of the factors reportedas significant in this activity, such as,

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osmolarity, pH and acidity, and compounds of protein and phenolic origin will be discussed.

Therapeutic properties of bee honey

From pre-Hispanic times, bee honey has been considered as a remedy for the treatment of infectious diseases, and today, medical science has "rediscovered" the use of this natural food source for humans as an alternative when modern therapeutic agents do not fulfill their expectations. The first documented reference to the use of honey can be found in Sumerian writings, dating from 2100 to 2000 BC, in which its use as medication and ointment is described. At present, a number of scientific studies on the medicinal properties of honey have reported its use in the treatment of wounds or stomach ulcers, and have demonstrated the antimicrobial activity exhibited by Manuka honey against pathogenic bacteria such as, *Staphylococcus aureus* and *Helicobacter pylori* (French et al., 2005).

It has been reported that the ingestion of one daily dose of honey improves gastrointestinal health due to its prebiotic function originating from a number of oligosaccharides, which are beneficial to certain probiotic bacteria found in the human intestinal tract, as is the case of bifidobacteria (Bogdanov et al., 2008).

Honey is also known to have a beneficial effect on cardiovascular health; a daily ingestion of 75 g decreases the number of triacylglycerides and low density lipoproteins (LDL), and slightly increases high density lipoproteins (HDL) causing a reduction in the risk of cardiac diseases (Bogdanov et al., 2008). Besides these uses, recent reports have mentioned its antimutagenic, antitumor and anti-inflammatory activity (Orsolic et al., 2004; Al-Waili et al., 2005; Fernandez-Cabezudo et al., 2013). Jaganathan and Mandal (2009) demonstrated that honey has the capacity to induce apoptosis in colon cancer cells, and for this reason, these authors propose honey as an anticancer agent of natural origin. Abubakar et al. (2012) reviewed the molecular mechanisms of the anti-leukemic activity of several honey phenolic compounds and strongly recommended that more studies should be conducted to determine the potential role of honey in both chemoprevention and chemotherapy in leukemia. Recently, Fernández-Cabezudo et al. (2013) demonstrated the anti-proliferative effect of Manuka honey on three cancer cell lines.

Histological studies in animals have shown that honey has an anti-inflammatory effect, demonstrated by a reduction in the number of inflammatory cells in wounded tissue. These studies also revealed that honey has a calming effect when applied to wounds (Gupta et al., 1992; Postures et al., 1997; Subramanian, 1993). Chepulis and Francis (2012) assessed the anti-inflammatory activity by measuring inhibition of neutrophil TNF-α secretion indicating that the cyclodextrin-based complexes of Manuka honey may potentiate the anti-inflammatory activity of honey. Candiracci et al. (2012b) reported that Honey Flavonoid Extracts significantly inhibited the release of pro-inflammatory cytokines such as TNF-α and IL-1β

Studies carried out on animals and humans have demonstrated that honey stimulates angiogenesis and tissue regeneration, due to a reduction of turgency as a result of the application of the honey, which increase its oxygenation. Moreover, the high osmolarity of honey promotes the elimination of fluid from the wound. This outflow of lymph contains dissolved nutrients which also provide nutrition for tissue regeneration (Schmidt et al., 1993; Kaufman et al., 1985; Mcinerney, 1990).

Vit et al. (2004) reported that the honey from stingless bees is used as a coadjutant in the treatment of stomach ulcers, gastritis and other stomach problems. Moreover, one of the medicinal characteristics attributed to stingless bee honey is its capacity to inhibit growth of bacteria, fungi and yeasts of the *Candida* genus and other microorganisms that are pathogenic for humans. Shahzad and Cohrs (2012) showed that the honey has significant *in vitro* anti-varicella zoster virus activity and the nematicidal activity of natural honey using *C. elegans* as the model system was clearly demonstrated by Sajid and Azim (2012). The Mayans also used the honey from Melipona bees as an antiseptic agent and as a remedy for respiratory tract infections such as: laryngitis, sinusitis and bronchitis (Carrillo, 1990). Other therapeutic uses attributed to this type of honey are: in the treatment of cataracts and ocular growth, as an adjuvant in the treatment of infectious and traumatic conjuntivites, ocular ulcers and red or bloodshot eyes (Molan, 1992).

Antimicrobial activity of honey

The use of honey as a traditional remedy in the treatment of a diversity of diseases and ailments produced by microorganisms dates back to ancient times, and this has inspired a number of studies (Molan, 1992). Numerous investigations have been carried out on Manuka honey, which has shown to be an efficient antimicrobial agent against several human pathogens, such as, *Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium,* and *S. aureus* (Lusby et al., 2005; Visavadia, et al., 2006; Sherlock et al., 2010; Schneider et al., 2012). Certain studies have also demonstrated that honey is effective against methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococcus sp* (VRE) (Allen et al., 2000; Kingsley, 2001; Jenkins et al. 2012). Other scientific studies have revealed the antimicrobial potential of honey against coagulase-negative strains of clinical importance (due to its high incidence in hospitals) of the *Staphylococci* genus, principally *S. aureus* (Cooper et al., 2002; French et al., 2005; Bizerra et al., 2012; Cooper and Jenkins, 2012). Recently, the potential of manuka

honey in the topical treatment of wounds containing *Staphylococcus pyogenes* has been reported (Maddocks et al., 2012). These studies have demonstrated the antimicrobial effectiveness of honey against medically important organisms, thus this food source for humans can be considered an antibacterial agent of natural origin.

In general, studies on the antimicrobial capacity of honey focus on microorganisms which cause infections in humans. Microbial diseases which are susceptible to treatment with honey include: urinary tract infections (*Proteus* spp., *P. aeruginosa*), cholera (*Vibrio cholerae*), nosocomial infections (*S. aureus*), septicemia (*E. coli*), bloodstream infections (*S. maltophilia*), infections in burn wounds (*Micrococcus luteus, Cellulosimicrobium cellulans, Listonella anguillarum*), gastritis and gastric neoplasias (*H. pylori*) and tuberculosis (*Mycobacterium tuberculosis*). The antifungal activity of honey has been evaluated against several *Candida* species, *Aspergillus niger*, *Penicillium chrisogenum*, etc. (Candiracci et al., 2012a; Kuncic et al., 2012). The qualitative method most used for the evaluation of antimicrobial activity is that of disk diffusion; however, the macrodilution method is also frequently used, as it is possible to calculate the minimum inhibitory concentration (MIC) which reflects the quantity necessary for microbial growth inhibition. However, as the information provided by *in vitro* studies is not yet sufficient, it is not possible to affirm that this food source is a substitute for current medications, nevertheless, it can be used as a coadjutant.

The origin of honey and its antimicrobial activity

A number of reports are currently available on the extensive variety of honeys from the *Apis mellifera* (honey bee) which present antimicrobial activity. One of the most well-known honeys is the Manuka (*Leptospermum scoparium*), with an inhibitory effect against 60 bacterial species, including aerobic and anaerobic, Gram-positive and Gram-negative (DebMandal and Mandal, 2011). Tan et al. (2009) reported the broad spectrum of activity against enteric bacteria presented by Tualang honey in Malaysia; they also observed that it could be used effectively to alleviate different kinds of wounds. Similarly, Badawy et al. (2004) found that honey from Egyptian clover (*Trifolium alexandrinum*) was capable of inhibiting *S. typhimurium* and *E. coli* O157:H7. Many honeys of other floral origins have been reported with antimicrobial activity, as in the case of the honeys from Erica (*Erica arborea*); Canola (*Brassica napus*); Castaña (*Castanea sativa*); Abeto (*Pinacease abies*); Acacia (*Fabaceae acacia*), and from multi-floral origin. The latter depends on the region in which the bee is found; Brazil, Ethiopia, New Zealand, to mention but a few (Amiot et al., 1989; Soler et al., 1995; Miorin et al., 2003; Mulu et al., 2004; French et al., 2005). More recently, Alzahrani et al., 2012 reported that the differences among honey samples in terms of antibacterial and antioxidant activity could be attributed to the natural variations in floral sources of nectar and the different locations.

In addition, the entomological origin of the honey is also an important differential factor since its composition can confer therapeutic properties. Apart from the *Apis mellifera*, the most important bees whose honey is used for these purposes, include several species of stingless bees, such as: *Friesiomelita nigra, Melipona solani, Melipona quadrifasciata, Trigona australis, Trigona nigerrima, Trigona sivestri Trigona fulviventris, Trigona carbonaria, Trigona biroi, Nannotrigona perilampoides, Scaptotrigona pectoralis, Scaptotrigona bipunctata, Scaptotrigona mexicana, Tetragonisca angustula* and *Melipona beecheii*, which were greatly appreciated in the Mayan culture for their therapeutic and antimicrobial properties (Carrillo, 1990; Ramos-Elorduy and Moreno, 2002).

Minimum inhibitory concentration of bee honey

The minimum inhibitory concentration of several types of honey against different pathogenic microbial strains has been determined by many authors. Table 1 shows the MICs against a number of medically important micro-organisms.

As can be seen in Table 1, the quantity of honey used to inhibit the different microorganisms presented did not exceed 40%, confirming the effectiveness of honey against different strains of microorganisms. Moreover, a wide range of MICs was observed for the honeys from different floral origins. At the same time it is possible to observe the low values of MICs (2.5 to 16%) for the Meliponinae bee tribes (stingless bees) against bacterial strains, in comparison with the MICs (25 to 40%) presented by the Apini tribe, excepting with the exception of the Manuka and pasture honeys whose MIC values are between 3.4 and 3.6%, respectively. This would indicate that the botanical and entomological origin is a determining factor in the antimicrobial potential of honey. Another point to consider among the different types of microorganisms evaluated is the broad spectrum this food source has shown not only in evaluations with bacteria resistant to antibiotics, but also with yeasts of the Candida species, among other eukaryotic microorganisms. For this reason, antimicrobial activity is one of the most important characteristics of this food source, and is generated by several factors which, in conjunc-tion, inhibit microbial growth. Up until a few years ago, it was believed that osmotic pressure, generated by the high concentration of sugars present in honey, was the only factor conferring antimicrobial activity. In recent years, particularly in *Apis* honey, the presence of other components having an influence on antimicrobial activity has been reported. These factors include: acidity

Table 1. Minimum inhibitory concentration of different types of honey against medically important microorganisms.

Type of honey	MIC range (%)	Microorganism	Reference
Apis mellifera Manuka (*Leptospermum scoparium*)	3.4 - 20	*S. pyogenes, Staphylococci, MRSA, S. aureus, S. maltophilia, A. baumannii, E. coli P. aeruginosa, S. typhi*	DebMandal and Mandal (2011)
Apis mellífera pasture	3.6 ± 0.7	Coagulase-negative *Staphylococci*	French et al. (2005)
Apis mellifera Egyptian clover (*Trifolium alexandrinum*)	35 - 40	*A. schubertii, H. paraphrohaemlyticus, M. luteus, C.cellulans, L. anguillarum. baumannii*	Badawy et al. (2004)
Apis mellifera (different flora)	2.5 - 50	*E. coli, P. aeruginosa, S. aureus, E. faecalis, C. albicas*	Kuncic et al. (2012) Alzahrani et al. (2012)
Apis dorsata Tualang (*Koompassia excelsa*)	8.75 - 25	*S. pyogenes, Staphylococci, MRSA, S. aureus, S. maltophilia, A. baumannii, E. coli y P. aeruginosa, S. typhi, E. cloacae*	Tan et al. (2009)
Apis dorsata Nilgiri	25, 35 and 40	*S. aureus, P. aeruginosa and E. coli*	Rajeswari et al. (2010)
Melipona beecheii flora unknown	4 - 5	*S. aureus, P. aeruginosa*, *E. coli*	Chan-Rodríguez et al. (2012)
Tetragonisca angustula flora unknown	2.5 - 10	*Bacillus cereus, Saccharomyces cerevisiae*	Dardón and Enriquez (2008)
Stingless bees flora unknown	4 - 16	*gram-positive and gram-negative Bacteria, Candida spp*	Boorn et al. (2010)
Stingless bees flora unknown	32	*C. albicans and C. glabrata*	Boorn et al. (2010)

(gluconic acid), hydrogen peroxide, phenolic compounds (flavonoids), enzymes (Glucose oxidase, proteases and amylases), proteins and antimicrobial peptides.

Chemical properties of bee honey and it is antimicrobial activity (osmolarity and acidity)

The osmolarity of honey is a result of its high concentration of sugars, and it is the most well-known antimicrobial factor of this food source. Eighty four per cent of solids in honey is a mixture of sugars, mainly glucose and fructose and, together with the low humidity content, this leads to a_w (water activity) values between 0.56 and 0.62, values which impede the growth of almost any microorganism with the exception of certain osmophilic yeasts and bacteria. It is the opinion of several authors that the sugar concentration of honey is the only factor responsible for its antibacterial effect (Seymour and West, 1951; White et al., 1963; Keast-Butler, 1980; Bose, 1982; Chirife et al., 1983; Tovey, 1991). However, the results of some scientific studies have clearly demonstrated that the antibacterial activity of honey is not just a result of water elimination in bacteria caused by osmolarity. Cooper et al. (2002) compared the activity of Manuka honey with that of a synthetic honey (a solution of sugars in the same proportions typically found in honey) against MRSA (methicillin-resistant *S. aureus*), and VRE (vancomycin resistant *Enterococcus*). The results showed that 30 and 28.75% of artificial honey, respectively, was required to inhibit growth in these two pathogens.

On the other hand, the acidic pH of honey is due to the presence of organic acids; therefore, the pH can also be considered an important antimicrobial factor. The main organic acid present in honey is gluconic acid, a product of the glucose oxidase reaction (Estrada et al., 2005). Because the pH of honey ranges between 3 and 3.5 it provides an acidic environment unfavorable for bacterial growth, as the majority of these microorganisms prefer neutral or slightly alkaline environments (Molan, 2001). Some studies have reported minimum inhibitory concentrations with higher pH levels than those presented by undiluted honey. Pothmann (1950) measured the pH of the medium in which the minimum inhibitory concentration of honey (4.5%) was being evaluated against *Corynebacterium diphtheriae*, and obtained a pH of 6.2, demonstrating that acidity does not contribute to the inhi-

bition of *C. diphtheriae*. Although these observations appear to indicate that the acidity of honey has no importance, it does not mean that acidity plays no role in the antibacterial activity of honey. Under experimental conditions, in particular with strongly diluted honey, the culture medium used tends to neutralize the acidity of the honey, thereby eliminating any inhibitory action; however, when honey is applied directly on a wound or ulcer, the bacteria can come into contact with the honey and thus acidity could be an important factor (Molan, 1992).

Chemical composition of bee honey and its antimicrobial activity

Hydrogen peroxide and methylglyoxal

In 1962, a report was published to the effect that hydrogen peroxide could be the substance responsible for the antibacterial activity of honey (Aococx, 1962) and, even though subsequent reports have described the presence of other antimicrobial compounds in honey, some authors still consider hydrogen peroxide to be the principal antimicrobial factor (DebMandal and Mandal, 2011; Oelchlaegel et al., 2012). Hydrogen peroxide is a product of the reaction of glucose oxidase, as glucose is degraded.

This enzyme is found in the salivary glands of bees and continues its activity even when the honey is diluted; in fact it has been reported that the efficiency of the reaction actually increases with the dilution of the honey. Several authors have reported that the antibacterial activity of honey depends exclusively on H_2O_2, and argue that when this compound is eliminated through the application of catalase, honey loses its antimicrobial activity (Kwakman and Zaat, 2012). Brudznyski (2006), in a study with Canadian honeys, concluded that there is a strong correlation between antibacterial activity and the H_20_2 content, and that this could be a biomarker for the therapeutic potential of honey.

Although some authors have reported that the action mechanism of hydrogen peroxide is not clear, Cantoni et al. (1989) stated that the antibacterial action of hydrogen peroxide is due to two forms of action: its toxicity for the hydroxyl radical, which is formed by the reaction with the divalent iron ion (Fenton reaction), and the production of the superoxide anion (O_2^-) which causes damage to the DNA.

The methylglyoxal (MGO), which forms nonenzymatically from nectar-derived dihydroxyacetone (DHA), has been reported as an antimicrobial component in honey. Majtan et al. (2012), Al-Habsi and Niranjan (2012) and Holt et al. (2012) reported that MGO is the major antibacterial component in Manuka Honey and studies that correlate the MGO and Hydrogen peroxide levels with the antimicrobial activity were evaluated by Lu et al. (2013). However, an additional and synergistic mode mode activity of Manuka honey has been reported (Packer et al., 2012).

Enzymes and antimicrobial peptides

Several reports have demonstrated the existence of compounds of protein origin which confer antimicrobial activity to honey. Glucose oxidase is the enzyme responsible for the production of gluconic acid and hydrogen peroxide from glucose. White et al. (1963) reported an increase in the activity of the glucose oxidase present in honey when it was diluted to 50%, leading to a subsequent increase in the quantity of gluconic acid and hydrogen peroxide which inhibited the growth of *S. aureus*.

Other authors have proposed that the concen-tration of glucose oxidase is an important factor for the activity of honey (Cooper et al., 2002; White et al., 1963). Although glucose oxidase does not interact directly with the microorganisms in antimicrobial activity, this enzyme is recognized as an antimicrobial agent given the fact that its products contribute directly to antimicrobial activity.

Lysozyme has also been reported as an antimicrobial agent in honey, this enzyme hydrolyzes the β-1,4 unions between the residues of N-acetylmuramic acid and N-acetyl-D-glucosamine in the peptidoglycan of the bacterial wall. Mohrig and Messner (1968) evaluated the lysozyme content in 71 honey samples, obtaining the presence of this hydrolytic enzyme in all the samples evaluated. They also reported a reduction in the concentration of the enzyme in relation to the age of the honey. However, other studies indicate an absence of lysozyme in honey (Bogdanov, 1984).

More recently, Kwakman et al. (2010) reported that enzymes are not the only contributors to antimicrobial activity in honey; there are also other molecules of protein origin, such as the antimicrobial peptides, as in the case of bee defensin-1, also known as royalisi, this peptide was previously identified in bee hemolymph (Casteels-Josson et al., 1994), in the head and thoracic section of the bee (Klaudiny et al., 2005) and in royal jelly (the main food of queen bee larvae). It has been reported that bee defensin-1 shows a powerful activity against gram-positive bacteria, including *B. subtilis, S. aureus, and Paenibacillus larvae* (Kwakman et al., 2010; Bachanova et al., 2002). Although the mechanism of action has not been described with clarity, this peptide can now be considered an antimicrobial agent of honey (Kwakman et al., 2011). Also it has been reported that MRJP1 (Major Royal Jelly Protein 1) is a multifunctional protein that acts as a precursor of short antimicrobial peptides jelleines (Majtan et al., 2012)

The protein content and its relationship to antimicrobial activity in honey have not yet been fully studied; reports indicate that the protein content may vary according to the geographical region and entomological origin of the

honey (Bogdanov, 2008).

Phenolic compounds and flavonoids

Several authors have studied the phenolic and flavonoid content of honey in order to determine the presence of antimicrobial activity (Alvarez-Suarez et al., 2009; Alvarez-Suarez et al. 2010). It was found that, although antimicrobial activity in most honeys has been attributed to hydrogen peroxide, the persistence of antimicrobial activity, even after the elimination of hydrogen peroxide, suggests that this activity may be related to different phenolic compounds with antimicrobial activity (D'Arcy, 2005). Cooper et al. (2002) reported that phytochemicals such as flavonoids, aromatic acids and phenolic antioxidants are recognized for their capacity to inhibit a wide range of Gram-positive and gram-negative bacteria.

In this way, 33 flavonoids have been identified in bee products, of which 11 have been found in the nectar of certain flowers, 9 in pollen and 25 in propolis. In addition, more than 70 phenolic compounds have been identified both in honey and in propolis (D'Arcy, 2005).

Montenegro et al. (2009) reported that phenolic extracts from mono-flower honeys of *Quillaja saponaria* were capable of inhibiting *in vitro* growth of *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* STH 2370, *S. aureus* (ATCC 25923) and *Streptococcus* β hemolitic, for which the presence of chlorogenic acid, aesculetin, caffeic acid, syringic acid, rutin, scopoletin, p-coumaric acid, vanillic acid and salicylic acid, quercetin and naringenin was determined. Similarly, Aljadi and Yusoff (2003) also reported that phenolic extracts obtained from the honeys of coconut and gelam (*Melaleuca cajuputi*), showed antimicrobial activities against *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), methicillin resistant *S. aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA), with the presence of gallic acid, caffeic acid and benzoic acid reported for both extracts. However, according to Weston et al. (1999) although the majority of phenolic compounds in Manuka honey may have antimicrobial activity, they can have no significant influence, either individually or collectively, on antimicrobial influence not related to the peroxide in Manuka honey, and essentially, this activity is associated with the fraction of carbohydrates. In part, this discrepancy can be explained by reports to the effect that the type of solvent used in the extraction of phenolic compounds may lead to different results in the antimicrobial activity of the phenolic extracts obtained (Aljadi and Yusoff, 2003, Schneider et al., 2012)

Recently, Candiracci et al. (2012a) tested the antifungal effect of flavonolic extracts from honey against *Candida albicans,* and found that the formation of germination tubes and hyphae in the yeast was delayed when flavonoid extracts from honey were added. The main flavonoids identified in these extracts were: luteolin, quercetin, apigenin, kaemferol, acacetin, tamarixetin, chrysin and galangin.

Despite these findings, few studies have been carried out on the mechanism of the antibacterial activity of flavonoids; however, there are indications that different compounds of this class of phytochemicals may target diverse functions and components in bacterial cells (Cushnie and Lamb, 2005; Alvarez et al., 2008; Alvarez-Suarez et al., 2010).

In this sense, it has been suggested that certain flavonoids may act by inhibiting the function of the cytoplasmic membrane or through the inhibition of the activities of DNA gyrase and the carrier protein β-hydroxyacyl-acyl deshidratase (Paiva et al., 2010). Furthermore, quercetin has also been found to neutralize the distribution of internal loads of pores formed by proteins denominated porines which are present in gram-negative microorganisms, thereby giving other flavonoids access to the interior of the cell (Alvárez et al., 2008).

Conclusions

The number of scientific publications demonstrating the antimicrobial properties of bee honey highlights this food source as a promising antimicrobial agent against microbial infections of medical interest. Furthermore, to date, there have been no reports documenting microbial resistance to honey (Dixon, 2003). This antimicrobial property is the result of a number of factors present in honey such as, osmolarity, acidity, hydrogen peroxide, methylglyoxal phenolic compounds and protein compounds, which makes it difficult for microorganisms to acquire resistance to this food source. However, there is still some disagreement as to whether osmolarity and acidity can be considered antimicrobial factors of honey, due to the fact that *in vitro* studies use diluted honey with a neutral pH and low osmolarity. The enormous variety of honeys reported to have antimicrobial activity and the diversity of phenolic compounds found in this food source, indicate that floral origin plays an important role in its antimicrobial activity. Similarly, the entomological origin is a key factor in the diversity of antimicrobial properties to be found in different honeys, since honey from stingless bee has been reported such as medical honey. Furthermore, several recent reports about other biological activities of honey have been showing that it, or its compounds, has antitumor, anti - inflam m atory, antioxidant and antiviral properties. There can be no doubt, therefore, that honey must be recognized as a promising medication of natural origin.

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academicJournals

Vol. 7(51), pp. 5766-5770, 29 December, 2013
DOI: 10.5897/AJMR2013.6521
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Molecular response of Mexican lime tree to "*Candidatus Phytoplasma aurantifolia*" infection: An overview

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Accepted 27 December, 2013

"*Candidatus Phytoplasma aurantifolia*" is the causative agent of witches' broom disease in the Mexican lime tree (*Citrus aurantifolia* L.), and is responsible for major tree losses in Southern Iran and Oman. The pathogen is strictly biotrophic, and thus is completely dependent on living host cells for its survival. The molecular basis of compatibility and disease development in this system is poorly understood. Transcryptomic analysis of the susceptible lime cultivar at the representative symptoms stage showed a number of candidate genes that might be involved in the interaction of Mexican lime trees with "*Ca P. aurantifolia*". These included the genes for modifier of *snc1*, autophagy protein 5, formin, importin B3, transducin, L-asparaginase, glycerophosphoryl diester phosphodiesterase, and RNA polymerase b. In contrast, genes involved in basal metabolism like a proline-rich protein, ubiquitin-protein ligase, phosphatidyl glycerol specific phospholipase C-like, and serine/threonine-protein kinase. Proteomic analysis results reveal proteins that were involved in oxidative stress defense, photosynthesis, metabolism, and the stress response, regulate in infected trees. For the moment these results should help to identify genes that could be targeted to increase plant resistance and inhibit the growth and reproduction of the pathogen.

Key words: *Candidatus phytoplasma aurantifolia*, proteomix, transcryptomix.

INTRODUCTION

Witches' broom disease, which affects Mexican lime trees (*Citrus aurantifolia* L.), is caused by an obligate biotrophic plant pathogen, "*Candidatus Phytoplasma aurantifolia*". Phytoplasmas are prokaryotes that inhabit the phloem and are transmitted by phloem-sucking insects (Cimerman et al., 2006 and Hanboonsong et al., 2002, Zamharir, 2011b). This is a devastating disease that results in significant economic losses. The disease was first reported in the Northern coastal plain of the Sultanate of Oman, and since then, it has extended throughout the region (Bove et al., 1993). The disease has also affected Mexican lime trees in Southern Iran since approximately ten years (Zamharir et al., 2011a and Taheri et al., 2011). In the field, affected trees present with witches' brooms, which are shoot structures that are characterized by their compactness and their very small, pale green leaves. Witches' brooms display many thin secondary shoots with shortened internodes; these shoots develop from axillary buds that normally stay dormant. In the advanced stages of the disease, the leaves become dry and many witches' brooms appear. Finally, the tree collapses within four or five years after infection. The witches' broom structures lack flowers or fruits and normal shoots in infected plants produce fruits

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that are reduced in size (Bove et al., 1988). Interference with hormonal balance by ‘‘*Ca. P. aurantifolia*’’ has also been correlated with a series of morphological changes in infected plants, such as virescence (petals are green), phyllody (transformation of the floral organs in leaves), proliferation (development of leaves from floral organs), formation of witches' brooms from lateral buds, and floral malformations and abortions.

In addition, alterations in flowering and vegetative cycles (flowering in winter and anticipation of vegetative growth), modification of the internodes, and production of small and malformed fruits have been observed in phytoplasma-infected plants (Zamharir et al., 2011a; Taheri et al., 2011; Chang, 1998 and Lee et al., 2000). Our knowledge about the molecular mechanisms that are involved in ‘‘*Ca. P. aurantifolia’’* pathogenicity and the symptoms evoked in host plants is limited. Recent Studies by different molecular methods have been help to identify genes and proteins involved in Mexican lime tree response to ‘‘*Ca. P. aurantifolia*’’ infection (Zamharir et al., 2011a, Taheri et al., 2011).

The present paper identifies a number of candidate genes and proteins that might be involved in the interaction of Mexican lime trees with “*Ca P. aurantifolia*”. These results should help to elucidate the molecular basis of the infection process.

HISTOPATHOLOGY AND METABOLOMIC

The deposition of callous in the sieve plates, accumulation of starch in chloroplasts and disorganization of chloroplasts, alterations in cell wall thickness, and accumulation of polyphenols have been reported in plants infected with phytoplasma (Mardi et al., 2011). Phloem necrosis has also been observed as a symptom of diseases caused by phytoplasma.

Changes in metabolism and secondary metabolites, including reductions in chlorophyll a, chlorophyll b, and in the total chlorophyll content, have been reported (Zamharir et al., 2011a, Taheri et al., 2011). In addition, the content of carotenoids in leaves of lime plants decreases in several weeks after inoculation (Taheri et al., 2011).

TRANSCRYPTOMIC ANALYSIS

Comparative transcriptomic analysis of healthy Mexican lime trees and those infected by “*Ca. P. aurantifolia*” shows transcriptional changes that affected the expression of several genes related to physiological functions that would affect most leaves in infected tissues. Infection with “*Ca. P. aurantifolia*” causes wide-spread gene repression in Mexican lime trees (Zamharir et al., 2011a).

Several genes that were modulated in Mexican lime trees by infection with “*Ca. P. aurantifolia*” were related to defense, cell walls, and response to stress. The expression of autophagy protein 5 was repressed. Autophagy is a survival mechanism that protects cells against unfavourable environmental conditions, such as microbial pathogen infection, oxidative stress, nutrient starvation, and aggregation of damaged proteins (Kwon and Park, 2008).

It has been shown that carbohydrate starvation induces the expression of autophagy genes (Rose et al., 2006) and stimulates the formation of reactive oxidative species (ROS) in plants (Kwon and Park, 2008). It is likely that the accumulation of carbohydrate reduces the expression of autophagy genes in the host and limits the burst of ROS burst (hypersensitivity reaction). These effects might result in reduced host resistance to phytoplasma and create a suitable condition for phytoplasma survival in the host (Zamharir et al., 2011a).

It has been shown a cell wall hydroxyl proline-rich protein transcript was induced in lime response to “*Ca. P. aurantifolia*”. Proline-rich proteins are among the major structural proteins of plant cell walls. Environmental stresses can alter the composition of the plant cell wall markedly (Lamb et al., 1989). The induction of the hydroxyl proline-rich protein might reflect a defense mechanism of Mexican lime tree in response to phytoplasma infection (Zamharir et al., 2011a).

Another induced transcript contained a lysine domain that is found in several enzymes that are involved in degradation of the bacterial cell wall (Bateman et al., 2000). The role of this gene in the response of Mexican lime trees to the pathogens remains to be determined (Zamharir et al., 2011a).

Two of repressed genes were identified as a modifier of *snc1* (*MOS1*). Plant resistance (R) genes encode immune receptors that recognize pathogens directly or indirectly and activate defense responses (Jones and Dang, 2006). The expression levels of R genes have to be regulated tightly due to costs to the fitness of plants that are associated with maintaining R-protein mediated resistance. Recently, it has been reported that MOS1 regulates the expression of SNC1 which encodes a TIR-NB-LRR-type of R protein in Arabidopsis. It has been shown that mos1 mutations reduce the expression of endogenous *snc1*, which results in the repression of constitutive resistance responses that are mediated by *snc1* (Li et al., 2006). It is likely that down-regulation of Mexican lime tree MOS1 in response to the pathogen reflects a reduction in plant resistance responses to phytoplasma infection (Zamharir et al., 2011a).

Lipid-derived molecules act as signals in plant pathogen interactions, and the roles of jasmonic acid and related oxylipins that are produced from membrane-derived fatty acids through beta-oxidation, are particularly important (Shah, 2006). During infection, low level defense responses can be activated in susceptible plants (Lin et al., 2007; Polesani et al., 2008). Therefore, it is likely that well-established “*Ca. P. aurantifolia*” infections

involve the up-regulation of genes that encode components of the lipid metabolism pathway, such as phosphatidyl glycerol specific phospholipase C-like. This enzyme regulates the phosphatidylglycerol content via a phospholipase C-type degradation mechanism (Simockova et al., 2008). Another gene involved in lipid metabolisms, glycerophosphoryl diester phosphodiesterase was repressed during the infection. This enzyme has both phosphoric diester hydrolase and glycerophosphodiester phosphodiesterase activity and is involved in the metabolism of glycerol and lipids (Tommassen et al., 1999).

Among finding transcripts several were related to metabolism. These were genes that encoded ribosomal proteins and enzymes involved in protein degradation. The expression of ubiquitin-protein ligases and a 50 S ribosomal protein L15 were repressed, whereas another 50 S ribosomal protein L15 was induced. This suggests that the infection results in a general induction of protein turnover, which could reflect an adaptive response in the plants to remove misfolded proteins that have accumulated as a result of stress (Zamharir et al., 2011a).

Few modulated genes had signal transduction and/or gene regulation functions. They corresponded two transducin family protein that were repressed by infection and a serine/threonine protein kinases that was induced during infection (Zamharir et al., 2011a). Serine/threonine protein kinases are a group of enzymes that catalyze the phosphorylation of serine or threonine residues in proteins, with ATP or other nucleotides acting as phosphate donors. The phosphorylation of proteins on serine, threonine, or tyrosine residues is an important biochemical mechanism to regulate the activity of enzymes and is used in many cellular processes (Romeis, 2001).

Among down-regulated proteins, some were identified as members of the transducin family and contained WD40 domain. This domain is found in several eukaryotic proteins that with wide variety of functions, which include adaptor/regulatory modules in signal transduction, together with proteins involved in pre-mRNA processing, and cytoskeleton assembly (Lee et al., 2006). It is unclear how these changes contribute to the response of Mexican lime tree to infection (Zamharir et al., 2011a).

PROTEOMIC ANALYSIS

Proteomic analysis shows that some proteins were less abundant in infected lime plants by *Ca. P. aurantifolia* than in healthy plants, and some others proteins were more abundant in infected plants than in healthy plants. These proteins were involved in stress response, metabolism, growth and development, signal transduction, photosynthesis, cell cycle, and cell wall organization (Monavarfeshani at al., 2013).

It has been distinguished oxidative scavenging enzymes is downregulated in Mexican lime trees in response to pathogen ‘‘*Ca. P. aurantifolia*’’ (Taheri et al., 2011). The downregulation of ROS scavenging enzymes has also been reported in resistant rice plants during bacterial leaf blight infection (Kottapalli et al., 2007) and cucumber response to *Pseudoperonospora cubensis* (Li et al., 2011). It is likely that downregulation of these proteins in Mexican lime trees in response to pathogen ‘‘*Ca. P. aurantifolia*’’ contributes to the accumulation of ROS, which in turn induces a hypersensitive response in the plant (Taheri et al., 2011).

The proteomic analysis provided evidence for the downregulation of photosynthetic proteins including two oxygen-evolving enhancer proteins 1, two ribulose-1,5 bisphosphate carboxylase activases and ribulose-1,5 bisphosphate carboxylase/oxygenase (Taheri et al., 2011). These results are consistent with the previous reports that environmental stresses inhibit the expression of genes that encode photosynthetic proteins (Li et al., 2011, Seki et al., 2002 and Wu, et al., 2010). The differential expression and degradation of photosynthetic proteins have also been revealed by the proteomic analysis of the response of mulberry to phytoplasma (Ji, et al., 2009). Scharte et al. have suggested that photosynthesis must be switched off to initiate respiration and other processes that are required for plant defence against pathogen (Scharte et al., 2005).

Pathogenesis-related protein (PR)-10 was identified as one of the upregulated proteins (Taheri et al., 2011). Induction of this protein is consistent with a previous report of the expression of the PR-5 gene in grapevine and *Chrysanthemum carinatum* in response to phytoplasma infection (Margaria and Palmano, 2011; Zhong. and Shen, 2004).

Upregulation of PR-10 gene expression has been demonstrated in a wide variety of plant species after infection by pathogens, including infection of *Capsicum annuum*, *Cronartium ribicola* on *Pinus monticola*, *Pseudomonas syringae* pv. *pisi* on *Vitis vinifera*, *Magnaporthe grisea*, and *Acidovorax avenae* on rice (Taheri et al., 2011). The PR-10 family is one of the most important among 17 groups of PR proteins (Yan, et al., 2008). PR-10 is typically intracellular and it has been reported to have various functions, including antimicrobial activity, *in vitro* ribonuclease activity, and enzymatic activity in plant secondary metabolism (Liu and Ekramoddoullah et al., 2006). These functions implicate PR-10 in plant defence against pathogen attack (Taheri et al., 2011).

In addition miraculinlike proteins and three homologues of it upregulate during infectious process of lime trees by ‘‘*Ca. P. aurantifolia*’’(Taheri et al., 2011). Miraculins are highly glycosylated proteins that belong to a family of protease inhibitors. The specific function of miraculin-like proteins in the stress response has not yet been elucidated. However, upregulation of these proteins has been reported in compatible pathogen–plant interactions, in

including some that are caused by fungi or treatment with methyl jasmonate (MeJA) (Tsukuda et al., 2006) in *Citrus sinensis* leaves infested by the leafhopper *Homalodisca oagulate* (Mozoruk et al., 2006) and in citrus leaves in response to the spotted spider mite *Tetranychus urticae* (Maserti, et al., 2011). The different responses of various isoforms of miraculin-like proteins suggest that a complex regulatory network modulates their expression patterns. The upregulation of miraculin-like might provide insight into the defence mechanism of the Mexican lime tree against the pathogen ‘‘Ca. *P. aurantifolia”* (Taheri et al., 2011).

Several differentially expressed proteins were involved in protein translation and fate. These included the 40S ribosomal protein S12, a copper chaperone and ubiquitin-conjugating enzyme 1 which were upregulated, and three heat shock proteins and a putative GroES chaperonin, which were downregulated. The downregulation of heat shock proteins, which act to maintain the structural and functional integrity of damaged proteins, and upregulation of ubiquitin-conjugating enzyme, which is involved in important cellular mechanisms that target abnormal or short-lived proteins for degradation, could reflect an adaptive response in plants to remove misfolded proteins that have accumulated as a result of phytoplasma infection (Taheri et al., 2011).

Overall, these results suggest that proteomic changes in response to infection by phytoplasmas might support phytoplasma nutrition by promoting alterations in the host's sugar metabolism, cell wall biosynthesis, and expression of defense-related proteins. Regulation of defense-related pathways suggests that defense compounds are induced in interactions with susceptible as well as resistant hosts, with the main differences between the two interactions being the speed and intensity of the response ((Monavarfeshani at al., 2013).

COMPARISON OF TRANSCRIPTOME AND PROTEOME OF THE MEXICAN LIME TREE INFECTED BY PHYTOPLASMA

Comparison of the responses of the transcriptome and proteome of the Mexican lime tree to phytoplasma infection shows that different sets of modulated proteins were identified by these two approaches. Although the expression of ubiquitin-protein ligase was decreased at the mRNA level, we found that the level of ubiquitin-protein ligase protein increased in response to phyto-plasma infection (Zamharir et al., 2011a).

qRT-PCR analysis also showed a similar expression pattern at mRNA and protein levels and there was little correlation between the changes in mRNA and protein expression levels under stress conditions relative to normal conditions (Taheri et al., 2011). This poor correlation between transcriptomic and proteomic results confirmed that mRNA levels do not necessarily correlate with protein levels. Discrepancies between the expression levels of mRNA and those of their corres-ponding proteins have also been shown elsewhere. The lack of correspondence between transcript and protein levels might have been due to the fact that mRNA levels usually peak before protein levels increase. Post-transcriptional and post-translational modifications and different rates of degradation of mRNA and protein could also contribute to the discrepancies (Taheri et al., 2011; Zamharir et al., 2011a).

CONCLUSION

We believe that analysis of the expression of genes, proteins and metabolites involved in the interaction of Mexican lime trees with “*Ca. P. aurantifolia*” allowed several novel genes to be identified from Mexican lime trees, because a significant proportion of the TDFs and proteins are not currently represented in citrus databases. Researches show that infection resulted in the down-regulation of Mexican lime tress transcripts and proteins in all major functional categories. However, certain genes and proteins required for plant pathogen interactions were modulated positively during infection at the symptomatic stage. These results will serve as a basis to address new questions and design new experiments to elucidate the biology of plant-phytoplasma interactions and the associated re-programming of the host metabolism. They might also pave the way to identify genes and proteins that can be targeted to elevate plant resistance or inhibit the growth and reproduction of the pathogen. However, further research is required to elucidate the roles of these genes and proteins in the susceptibility/resistance of Mexican lime tress to “*Ca. P. aurantifolia*”, and to determine how strategies might be developed to incorporate these genes into molecular breeding programs (Taheri et al., 2011 and Zamharir et al., 2011a).

There are several immediate extensions that will increase our understanding of plant response to pathogen and may result in applications enhancing plant resistance. These extensions include using mass spectrometry to identify proteins that remain unidentified from the differentially expressed proteins reported before, examining highly responsive proteins and transcripts such as ascorbate peroxidase 2, Cu/Zn superoxide dismutase, miraculin-like proteins, and annexin p35 in other tissues and different time points and understanding whether the regulated proteins reflect a direct effect of the interaction with the phytoplasma or a secondary effect of the development of symptoms. It is also interesting to determine whether the observed protein and transcripts changes in response to pathogens are reflections of changes in gene and protein expression or post-translational modifications. Future studies are required to understand the role of the regulated genes

and proteins.

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Vol. 7(51), pp. 5771-5780, 29 December, 2013
DOI: 10.5897/AJMR2013.6305
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Bluetongue: Virus proteins and recent diagnostic approaches

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Accepted 27 December, 2013

Bluetongue (BT) is an infectious and non-contagious arthropod borne viral disease of domestic and wild ruminants namely sheep, goat, cattle, camels, llamas, deer and antelopes. It is characterized by high fever, catarrhal inflammation of the buccal and nasal mucous membranes, and inflammation of the tongue, intestine and sensitive laminae of the foot. It is caused by Blue tongue virus. Bluetongue virus is a member of the genus *Orbivirus* in the family Reoviridae. Its genome consists of ten double-stranded (ds) RNA segments coding for seven structural proteins (VP1-VP7) and four non-structural proteins (NS1-NS3 or NS3A, and NS4). At present, 26 serotypes have been reported throughout the world. Bluetongue can be diagnosed from several kinds of samples. For virus isolation, embryonated eggs, 9 to 12 days old, at intravenous inoculation is best however, cell lines like KC line, the insect cell line C6-36 derived from Aedes spp., Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) lines can be used. For antigen identification sandwich enzyme linked immunosorbent assay (ELISA), serotype specific reverse transcriptase Polymerase chain reaction (RT-PCR), Serogroup-specific RT-PCR, Real time quantitative PCR, Sequencing and phylogenetic analysis (targeting conserved genome segments), Restriction enzyme profile analysis (REPA), Molecular probes, RNA polyacrylamide gel electrophoresis (RNA-PAGE) are now available in an increasing number of laboratories for the identification of Bluetongue virus (BTV). For antibody identification, it is possible to use blocking ELISA, competition ELISA, indirect ELISA, agar-gel immunodiffusion test (AGID), Complement-fixation test (CFT) and Haemagglutination inhibition test (HI), serum or virus neutralisation test (VNT/SNT), Immunofluorescence, Immunoperoxidase, and Dot immunobinding assays (DIA).

Key words: Antibody, antigen, bluetongue, bluetongue virus, diagnosis, structure, molecular technique.

INTRODUCTION

Bluetongue (BT) is an infectious and non-contagious arthropod borne viral disease of domestic and wild ruminants namely sheep, goat, cattle, camels, llamas, deer and antelopes. BT primarily affects sheep and deer with frank clinical symptoms, but subclinical disease occurs in cattle and goat. In 2006, serotype 8 has been reported to be implicated in causing clinical signs in cattle in UK (Maclachlan, 2011). It is characterized by high fever, catarrhal inflammation of the buccal and nasal mucous membranes, and inflammation of the tongue, intestine and sensitive laminae of the foot. Bluetongue has been known in South Africa for over a 100 years and endemic in wild ruminants since antiquity (Maclachlan et al., 2009, Maclachlan, 2011; Sperlova and Zendulkova, 2011; Maan et al., 2012a; Bitew et al., 2013).

BT is endemic in an extensive band that includes

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includes tropical, subtropical, and temperate regions of the world between latitudes of approximately 40° North and 35° South that is, America, Africa, Australia and Asia where vectors (*Culicoides* sp.) are present (Maclachlan, 2011; Bitew et al., 2013). BT is a disease of ruminants in temperate zones. However, clinical disease is reported in tropical and subtropical areas of the world when non-native breeds of ruminants are introduced in virus endemic area (Sperlova and Zendulkova, 2011). The European outbreaks of BT due to BTV-8 in 2006 have dramatically changed the geographic and ecological episystems around the world (Maclachlan, 2011; Maan et al., 2008). Due to segmented nature of virus, it often undergoes mutation by the process of drift and shift (reassortment of BTV gene segments).

The economic losses due to bluetongue is around 3 billion US$ per year in the world (Sperlova and Zendulkova, 2011). The direct losses are death, abortions, weight loss and reduced milk and meat productions and indirect losses are export restrictions of live animals, semen and foetal calf serum (Bitew et al., 2013).

At present 26 serotypes have been reported throughout the world (Maan et al., 2012 a, b ,c) with recent additions of the 25th serotype ("Toggenburg orbivirus") from Switzerland in goat and 26th serotype from Kuwait in sheep and goat (Hofmann et al., 2008; Maan et al., 2011; Maan et al., 2012c; Bitew et al., 2013). There is only low level of cross-protection among the BT virus serotypes and making vaccination strategies and control programmes a daunting task (Hofmann et al., 2008; Eschbaumer et al., 2009; Bitew et al., 2013).

BT is multiple species disease to the OIE, World Organisation for Animal Health (OIE, 2009; Maclachlan, 2010; 2011,) and to veterinary authorities in many countries (Eschbaumer et al., 2009). BTV is almost exclusively spread by *Culicoides* spp. biting midges (*Diptera*) and occurs worldwide. All the serotypes can cause bluetongue disease (BT), a non-contagious hemorrhagic disease of domestic and wild ruminants and camelids with no known zoonotic potential (Eschbaumer et al., 2009).

Although a galaxy of serological and molecular diagnostic tools are available for the prompt, reliable and precise detection and characterization of BTV strains/-serotypes and large number immunoprorphylactic agents have been developed for the control of the disease however, it is still endemic in many countries with substantial economic losses. Further, X-ray crystallography and cryo-electro-microscopy studies showed the minute details of the molecular structure of the bluetongue virus as well as different proteins coded by the different RNA segments of BTV (Schwartz-Cornil et al., 2008).

This review would provide the structural details of the BTV and the diagnostic tools developed over the times for the sensitive detection and molecular characterization of BTV for the benefit of the scientific fraternity, researchers, scientists and academicians involved in the bluetongue virus research.

THE BLUETONGUE VIRUS

BTV is the etiological agent of BT, an insect transmitted disease of ruminants. Bluetongue virus with closely related species African Horse Sickness virus (AHSV) and Epizootic Hemorrhagic Disease virus (EHDV) belongs to the genus Orbivirus (comprising at least 20 species overall) in the family Reoviridae (Eschbaumer et al., 2009; Maan et al., 2012a). The virions have a diameter of 90 nm. Bluetongue virus is having density 1.337 g/cm^3 and relative molar mass of about 10.8×10^7, 12% of which is genomic RNA (Eschbaumer et al., 2009; Schwartz-Cornil et al., 2008). The virus is a non enveloped with a genome of approximately 19.2 kbp and composed of ten linear double-stranded RNA (dsRNA), containing 57% AU and 43% GC, with conserved 5' and 3' terminal sequences (GUUAAA at 5', and ACUUAC at 3' ends of the positive strand) (Schwartz-Cornil et al., 2008). The genome segments are numbered 1-10 (Large: L1-3; medium: M4-6; small: S7-10) in the order of decreasing size on agarose gels (Bhanuprakash et al., 2009).

Ten (10) dsRNA segments are packaged within a triple layered icosahedral protein capsid (90 nm in diameter) (Maan et al., 2012 a, b, c, d, e). The genome encodes seven structural and four non-structural proteins. Each segment contains one open reading frame flanked by non-coding regions. The open reading frame on segment 10 encodes two proteins by alternate translation initiation (Eschbaumer et al., 2009) (Figure 1). The outer layer of BTV particle is composed of two structural proteins (60 trimers of VP2) and 120 trimers of VP5). The intermediate layer consists of the major immunodominant VP7 structural protein organized in 260 trimers. VP7 forms the outer layer of the transcriptionally active virus 'core' (Eschbaumer et al., 2009) (Figure 1). The subcore consists of the 12 decamers of the VP3 protein, one centered on each of the five fold axes of the icosahedral particle structure. The 120 molecules of VP3 houses the viral genome segments and three minor proteins involved in transcription and replication, namely the RNA-dependent RNA polymerase (VP1), the RNA capping enzyme (VP4) and the dsRNA helicase (VP6) (Eschbaumer et al., 2009; Maan et al., 2012 a, b, c, d, e) (Figure 1). Non-structural proteins (NS1, NS2, NS3, NS3A and NS4) probably participate in the control of BTV replication, maturation and export from the infected cell. Unlike most single stranded RNA (ssRNA) viruses, the orbiviruses are genetically and antigenically stable throughout infection; point mutations do not appear to arise *in vivo*, at least at the high frequency noted with

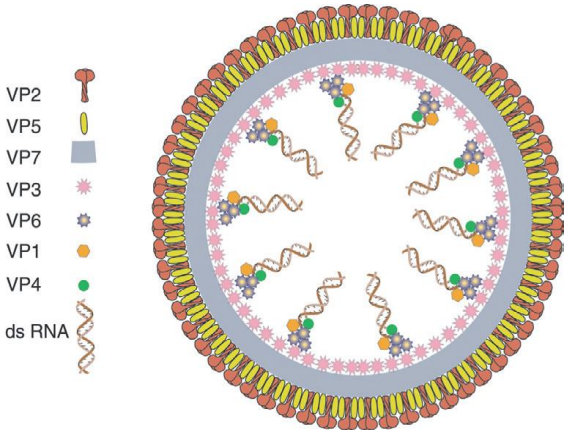

Figure 1. Representative scheme of BTV structural proteins and dsRNA segments [kindly taken from Schwartz-Cornil et al., (2008)].

many non-segmented ssRNA viruses (Ratinier et al., 2011) (Table 1).

There are 26 serotypes of BTV (Maan et al., 2012c) which are distinguished by epitopes on the outer capsid protein VP2 although VP5 also can influence neutralization through its conformational influence on VP2. The L2 gene which encodes VP2 is the only serotype specific BTV gene with a considerable variation amongst the different serotypes. The two outer capsid proteins VP2 and VP5 are responsible for virus entry and establishment of virus infection within the host cells, the core proteins as well as three non-structural proteins are less variable and responsible for replication of the viral genome. This genetic diversity of BTV is consequence of both drift (point mutation) and shift (reassortment of BTV gene segments) (Maan et al., 2012a, b, d, e).

Structural proteins

Outer shell protein

Outer shell composed of two structural proteins VP2 and VP5. The trimers of the VP2 form 'triskelion' motifs (three interlocked spirals) on the outer layer (Schwartz-Cornil et al., 2008). VP2 is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies (Dahiya et al., 2004; Schwartz-Cornil et al., 2008). Recombinant VP2 has a strong affinity for glycolphorin A, a sialoglycoprotein component of erythrocytes, an interaction that could be involved in BTV binding to erythrocytes. Furthermore, VP2 and glycophorin can inhibit BTV attachment to susceptible cells, suggesting that the BTV receptor involves VP2 interaction with a cell surface glycoprotein (Dahiya et al., 2004; Schwartz-Cornil et al., 2008). Inside the cell, VP2 associates to vimentin, which allows the proper sub-cellular localization of the protein and the interaction of mature BTV particles to intermediate filaments (Bhattacharya et al., 2007). Disruption of the VP2/vimentin interaction by pharmacological inhibitors leads to blockade of the virus egress (Bhattacharya et al., 2007).

VP2 is the major determinant of BTV serotype, with a minor role for VP5. Phylogenetic comparisons of VP2 from the 24 reference strains (Dahiya et al., 2004; Maan et al., 2007; Maan et al., 2012f) show a perfect correlation between sequence variation in genome segment 2 (Seg-2), coding for VP2, and determining BTV serotype. Sequences of seg-2 from the 24 BTV types cluster as ten distinct evolutionary lineages, identified as nucleotypes A-J. The inter-serotype VP2 nucleotide sequences varied from 29% (BTV-8 and BTV-18) to 59% (BTV-16 and BTV-22). Sequencing and phylogenetic comparisons of VP2 gene also revealed significant variations between strains of the same serotype that were derived from different geographical areas, with a maximum of 30% nucleotide sequence variation within the same serotype (Maan et al., 2007). These geographical variations define eastern and western VP2 topotypes within individual serotypes (Figure 2).

Oligonucleotide primers can be designed targeting Seg-2 that can be used in RT-PCR assays to facilitate typing of BTV field isolates and vaccine virus of each serotype and topotype (Mertens et al., 2007). Despite the overall sequence variability, some features of VP2 appeared to be conserved across serotypes, including the hydrophobicity profile, charge distribution and the position of certain cysteine residues (Maan et al., 2007).

In contrast to VP2, VP5 is significantly more conserved but shows some degree of variations that reflects the geographic origin (Singh, 2005). Trimers of VP5 form the globular motifs of the outer layer of the BTV virus particle (Nason et al., 2004). VP5 has recently been shown to be a membrane penetration protein that mediates release of viral particles from endosomal compartments into the cytoplasm. Analysis of the VP5 sequence using secondary structure prediction algorithms indicates that this protein is predominantly α-helical, with an amphipathic helical domain at the N terminus followed by a coiled domain, thus sharing structural features with class I fusion proteins of enveloped viruses (Nason et al., 2004). Furthermore, VP5 undergoes pH-dependent conformational changes that allow membrane fusion and syncytium formation (Forzan et al., 2004). The syncytium formation by VP5 is inhibited in the presence of VP2 when expressed in a membrane-anchored form.

The major core proteins

VP3 and to a lesser extent VP7 are conserved proteins, hydrophobic in nature and are forming major core protien

Table 1. Bluetongue virus genome segments and proteins.

Segment	Size (nt)	Encoded protein	Location (number of copies per virion), proposed function	Protein size* (weight)
1	3954	VP1	Within the core (12), RNA-dependent RNA polymerase	1302 aa (150 kDa)
2	2926	VP2	Outer capsid (180), type-specific structural protein	961 aa (111 kDa)
3	2772	VP3	Inner (sub-core) capsid (120), scaffold for VP7 layer	901 aa (103 kDa)
4	2011	VP4	Within the core (24), RNA capping enzyme	644 aa (75 kDa)
5	1770	NS1	Non-structural protein (0), forms tubules of unknown function in host cells	552 aa (64 kDa)
6	1639	VP5	Outer capsid (360), structural protein, co-determinant of virus serotype	526 aa (59 kDa)
7	1156	VP7	Core capsid (780), group-specific structural protein	349 aa (39 kDa)
8	1123	NS2	Non-structural phosphoprotein (0), forms viral inclusion bodies in host cells	354 aa (41 kDa)
9	1046	VP6	Within the core (72), RNA helicase	329 aa (36 kDa)
10	822	NS3	Non-structural glycoprotein (0), membrane protein, aids virus release from host cells	229 aa (26 kDa)
		NS3A	Expressed by alternate translation initiation	216 aa (24 kDa)

*Size (amino acids, aa) and weight (Dalton, Da) data are for the European reference isolate of BTV-8. (Kindly taken from Eschbaumer et al., 2009).

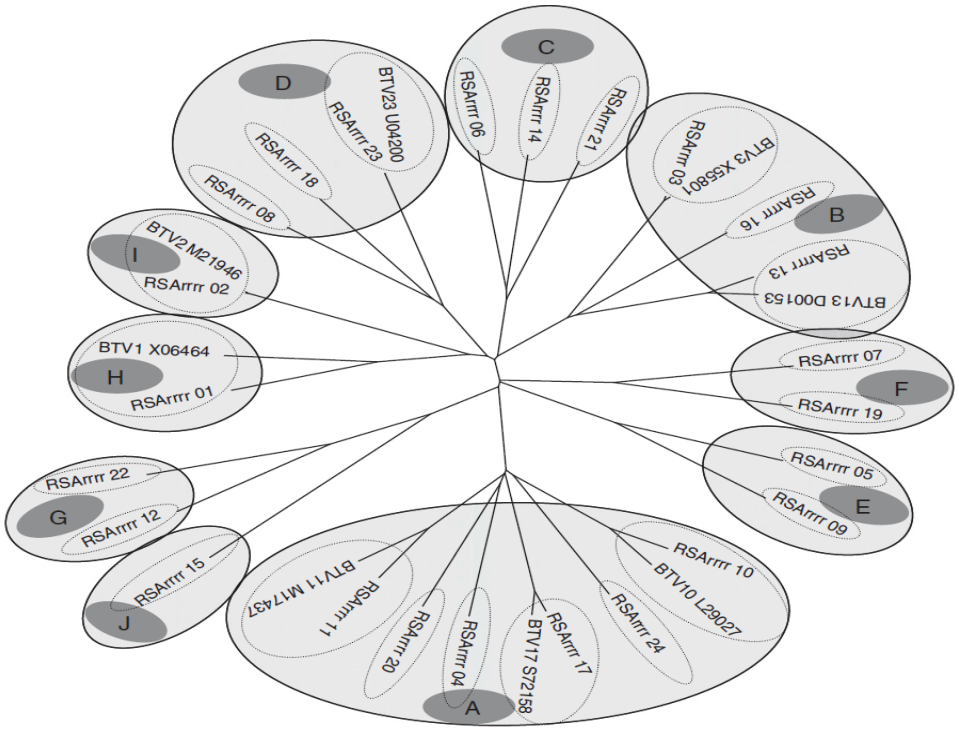

Figure 2. Unrooted neighbour-joining tree showing relationships between nucleotide sequences of Seg-2 from the 24 BTV types (Maan et al., 2007).

(Schwartz-Cornil et al., 2008). They play an important role in the structural integrity of the virus core. They express group-specific antigenic determinants defining several distinct phylogenetic groups (Anthony et al., 2007). Importantly, cores are poorly infectious or even non-infectious in different mammalian cells but they are at least 100 fold more infectious for adult *Culicoides* midges or a *Culicoides* cell line (KC cells) (Schwartz-Cornil et al., 2008). VP7 can mediate attachment and penetration of insect cells in the absence of either VP2 or

VP5, a process that may involve an arginine-glycine-aspartate (RGD) tripeptide motif present at amino acid residues 168 to 170 on the outermost surface of the VP7 trimers on the BTV core. VP7 can bind to glycosaminoglycans, although it appears likely that other specific receptors are also involved in cell attachment and penetration. The VP3/VP7 complex protects the viral dsRNA genome from intracellular surveillance, thus preventing activation of type I interferon (IFN) production via cytoplasmic sensors such as cytosolic helicases, or interactions with dicer and RNA silencing mechanisms(Schwartz-Cornil et al., 2008).

Minor core proteins

Minor core proteins also called transcription complex comprises VP1, VP4 and VP6.VP1 is present in a low molar ratio (approximately 12 copies per particle) within the virion (Schwartz-Cornil et al., 2008). VP1 can extend RNA synthesis from oligo (A) primers and acts as the BTV replicase that synthesizes dsRNA from a viral plus-strand RNA template (Boyce, 2004). VP1 has an optimal activity at 27 to 37°C, allowing efficient replication in both insect and mammalian cells. The early BTV mRNAs are capped. The cap (methylguanosine connected to the first nucleoside) stabilizes the mRNA and allows efficient translation. In cells, capping requires the action of four distinct enzymes. In BTV, all four reactions are catalyzed by the single VP4 protein, whose crystal structure shows an elongated modular architecture that provides a scaffold for an assemblage of active sites (Sutton et al., 2007). The VP6 protein has ATP binding activity and displays RNA-dependent ATPase and helicase functions. It unwinds duplexes of dsRNA and could assist mRNA synthesis from the genomic dsRNA template.

Non structural proteins (NSP)

The two larger BTV non structural proteins, NS1 and NS2, are the first and second most highly expressed proteins in infected cells whereas the two closely related minor proteins NS3 and NS3A are barely detectable in mammalian cells. However, NS3 and NS3a are synthesized in much larger amounts in insect cells, suggesting that their role may be primarily related to BTV replication and dissemination within the insect vector (Schwartz-Cornil et al., 2008). NS4 has been recently identified as additional to non structural proteins (Ratinier et al., 2011).

NS1

Electron microscopic analysis of thin sections of BTV-infected cells have revealed a large number of virus-specific tubules (52.3 nm diameter and 1000 nm long) composed of multimers of the NS1 protein, a striking intracellular morphological feature of BTV infection. NS1 has a role in BTV cytopathogenesis (Owens et al., 2004).

NS2

NS2 is the major constituent of the viral inclusion bodies (VIB) seen in infected cells mainly in the vicinity of the nucleus. NS2 binds to viral ssRNA and hydrolyses nucleotide triphosphates to nucleotide monophosphates (Schwartz-Cornil et al., 2008). These two properties imply that NS2 might be involved in some way in selection and condensation of the BTV ssRNA segments prior to genome encapsidation. NS2 expression in cells is sufficient for formation of inclusion bodies and it recruits VP3, suggesting that NS2 is a key player in virus replication and core assembly (Kar et al., 2007).

NS3

NS3 and its shorter form, NS3A, which lacks the N-terminal 13 amino acids of NS3, are the only membrane proteins encoded by orbiviruses. Interestingly, NS3 and NS3A appear to be associated with smooth intracellular membranes, although they are also present at the plasma membrane. NS3 functions as a viroporin, facilitating virus release by inducing membrane permeabilization (Han and Harty, 2004). NS3 allow BTV particles to leave host cells by a budding mechanism similarly to retroviruses. This budding mechanism might be involved in BTV egress from insect cells in which BTV does not induce significant cytopathic effect, whereas the viroporin mechanism would be more prominent in mammalian cells (Schwartz-Cornil et al., 2008).

NS4

It has been recently identified that BTV expresses a fourth non-structural protein (NS4) encoded by an open reading frame in segment 9 overlapping the open reading frame encoding VP6. NS4 is 77-79 amino acid residues in length and highly conserved among several BTV serotypes/strains (Ratinier et al., 2011).

RECENT DIAGNOSTIC APPROACHES

A preliminary diagnosis based on clinical signs, post-mortem findings and epidemiological assessment should be confirmed by laboratory examination (Sperlova and Zendulkova, 2011). Samples to be examined in the labo-

ratory should include non-coagulated blood (use of EDTA or heparin is preferred), serum, post-mortem tissue samples such as spleen, lymph nodes, lungs, liver, bone marrow heart and skeletal muscles. In addition, brain of the aborted foetus can be collected (Sperlova and Zendulkova, 2011). For transport, serum samples should be frozen at -20°C and the other samples should be kept on ice. The blood samples can be stored at +4 °C for a long time; isolated blood cells in 10% dimethyl sulphoxide require storage at a temperature of -70°C (Sperlova and Zendulkova, 2011).

Bluetongue virus isolation

BTV can be isolated from blood, semen and various other tissue samples including liver, spleen, brain, lymph nodes and mucosal epithelium. Bluetongue virus can be propagated in embryonated chicken eggs (ECE), cell cultures or in sheep. Embryonated eggs, 9 to 12 days old are inoculated with the materials by intravenous route for BTV isolation. This method is 100- 1000 fold more sensitive than yolk sac inoculation (Dadhich, 2004; Sperlova and Zendulkova, 2011; Biswas et al., 2010), but needs technical skills and experience. The material obtained from ECE can either be further propagated in cell culture or directly examined using molecular methods (PCR or *in situ* hybridisation) (Dadhich, 2004; Sperlova and Zendulkova, 2011).

Bluetongue virus can also be isolated in cell lines of different animal origin. Cell lines of insect origin include the KC line derived from *Culicoides sonorensis* cells or the C6/36 line from *Aedes albopictus* (AA) cells. The mammalian cell lines for BTV isolation like BHK-21, calf pulmonary artery endothelium (CPAE) or Vero cell lines can also be used (Mecham, 2006). The cytopathic effect produced by BTV is observed only on cell lines of mammalian origin at 3 to 5 days after inoculation and appears as foci of rounded and refractile cells. If Cytopathic effect (CPE) doesn't appear, a second passage is made in cell culture. The isolation of virus in cell culture is usually preceded by its passage in ECE which are more susceptible to BTV than cell lines (Sperlova and Zendulkova, 2011; Biswas et al., 2010). Sheep can provide a sensitive and reliable system for BTV isolation; however, today they are used only occasionally, for example, in cases when a sample contains a very low virus titre (Sperlova and Zendulkova, 2011). The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by antigen-capture ELISA, immunofluorescence, immunoperoxidase, SNT or VNTs.

Antigen identification

Sandwich ELISAs have been described for the detection of BTV antigens in infected cell cultures or adult Culicoides midges. Although antigen ELISAs are specific, they are insensitive requiring relatively large amounts of antigen (equivalent to ≥ 2.5 - 3.0 $\log_{10}$ infectious units of virus) to give a positive result and consequently are rarely used as a front line test for the detection of BTV (Batten et al., 2008). In addition to ELISA, molecular assay can be used to detect and identify the viral RNA of BTV or related viruses. A direct identification of BTV in blood or tissue samples is possible with use of the reverse transcription-polymerase chain reaction (RT-PCR) method that allows for serotyping and can detect BTV RNA in samples as late as 6 months after infection (Sperlova and Zendulkova, 2011). A quantitative assessment of RNA in a sample is possible by real time-RT-PCR (Shaw et al., 2007; Toussaint et al., 2007; Vanbinst et al., 2010; De Leeuw et al., 2013). RNA polyacrylamide gel electrophoresis (PAGE) has been used as a diagnostic tool for the identification BTV 10 segments. RNA PAGE has also been used to identify different genotypes of the same serotype, as well as to indicate different serotypes of BTV. Group specific non-radio-labelled probes, based on the NS1 and VP3 genes, have been developed in India for detection of BTV in clinical specimens or infected cell cultures. Serogroup-specific RT-PCR, sequencing, restriction enzyme profile analysis (REPA) and phylogenetic analyses (targeting conserved genome segments) are now available in an increasing number of laboratories for the identification of BTV. Serotype-specific RT-PCR assays (targeting genome segments 2 or 6) have also been used to identify different BTV serotypes (Maan et al., 2007, 2008; Mertens et al., 2007).

Reverse transcription PCR (RT-PCR)

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis. Results to date indicate that RT- PCR (particularly nested or real-time assays) techniques may be used, not only to detect the presence of viral nucleic acid (starting from 1-3 days of pi), but also to 'serogroup' orbiviruses and provide information on the serotype and possible geographic source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least 3 to 4 weeks to generate information on serogroup and serotype and yield no data on the possible geographic origin of the isolated virus (Dadhich, 2004). Oligonucleotide primers used to date have been derived from RNA 7 (VP7 gene), RNA 6 (NS1 gene), RNA 3 (VP3 gene) and RNA 2 (VP2 gene). The size of the amplified transcripts is usually small (in the order of several hundred nucleotides) but can also be a full-length gene. Primers derived from the highly conserved genes, such

as VP3, VP6, VP7, NS1 and NS3, may be used for serogrouping (i.e. they will react with all members of the BT serogroup) and topotyping (that is, they will react with BTV isolates from the same geographic area). NS1 is currently recommended as an RT-PCR target by the OIE (OIE, 2009). Primers whose sequence was determined from VP2 gene sequences provide information on virus serotype (Dadhich, 2004). Two major geographic groups of BTVs have been identified and have been designated as 'eastern' and 'western' topotypes. The eastern includes viruses from Australia and the Middle/Far East, and the western includes Africa and the Americas, respectively (Maan et al., 2008; Maan et al., 2012a, b, c, d, e).

The RT- PCR requires agar gel electrophoresis to show the amplification of the target sequence, which severely limits the speed of testing. The RT-PCR assay involves three separate procedures (Maan et al., 2008). In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanates (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available. The reagents provided with the kit are numbered and their use is indicated in the protocol. Again, Trizol is useful for the extraction of viral nucleic acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription (RT) to generate cDNA, which is amplified by PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the PCR product by electrophoresis (Dadhich, 2004; Maan et al., 2008; Maan et al., 2012).

Real time RT- PCR (qPCR)

Real-time RT-PCR is a sensitive method that can be used for the laboratory detection of viral RNA. Several types of real-time RT-PCR exist, most being based on either SYBR (where an intercalating molecule fluoresces upon binding to double stranded DNA) or on fluorescence resonance energy transfer (FRET) (for example, TaqMan and molecular beacon assays) (Yin et al., 2010). In the FRET-based assays, a fluorescent 'probe-oligonucleotide' binds specifically to the region between the two primers. A positive signal is generated when the probe is degraded by the polymerase as it synthesizes new complimentary DNA strands. The results of real-time RT-PCR assays are expressed as a cycle-threshold (Ct) value. This represents the number of amplification cycles that are required under standard test conditions to cross a certain threshold level of fluorescence and higher Ct values therefore indicate that smaller amounts of the target gene are present in the test sample than do lower Ct values. Blood samples taken from an infected animal at the peak of viraemia may give Ct values of <20, whereas a low level viraemia could still be detectable with a Ct value >35. Negative control samples should not achieve a positive Ct value. Standardization of the assay, described by Shaw et al. (2007) and using a dilution series of viral RNA indicated that a change of 3 Ct units was approximately equivalent to a 10-fold dilution of the sample.

There are two published real-time assays that have been shown to detect all 26 serotypes (Shaw et al., 2007; Toussaint et al., 2007). Of the two, the assay of Shaw et al. (2007) has been tested against more serotypes and topotypes to date. So far the real-time RT-PCR assays have not been validated to the level required by the OIE, although ring trials have been conducted (Batten et al., 2008). Vanbinst et al. (2010) developed a duplex real-time RT-PCR for the detection of bluetongue virus in bovine semen. De Leeuw et al. (2013) also reported Bluetongue virus RNA detection by real-time RT-PCR in post-vaccination samples from cattle.

Antibody identification

Serogroup-specific antibodies against BTV can be detected by a blocking ELISAs, competitive ELISAs and dot immunobinding assays (DIA) test targeted to the VP7 protein. This is a rapid method permitting determination of serum or plasma antibody as early as the 6^{th} day of post-infection (PI) (Mars et al., 2010; Kramps et al., 2008; Batten et al., 2008). Again an indirect ELISA based on VP 7 protein has been developed at Indian veterinary research institute (IVRI), Mukteswar (Chand et al., 2009). In addition, serogroup-specific antibodies can be identified by an agar-gel immunodiffusion test (AGID), a complement-fixation test and a haemagglutination-inhibition test (Sperlova and Zendulkova, 2011). Agar gel immuno-diffusion (AGID) tests, historically, have been widely used for the detection of group-specific antibodies against BTV. The AGID test relies on the availability of purified soluble antigens, derived from BTV-infected cell cultures and positive control serum from hyper-immunised animals. However, AGID may produce cross-reactions with other orbiviruses like African Horse Sickness virus (AHSV) and Epizootic Hemorrhagic Disease virus (EHDV) (Sperlova and Zendulkova, 2011).

Complement fixation tests (CFT) have been used to identify BTV or to detect a rise in BTV-specific antibody titre following infection. These assays that primarily detect early antibodies, IgM, depend on inhibition of the complement-mediated lysis of activated erythrocytes by BTV antigen/antibody complexes that can also fix the available complement. However, they may only be effective for a relatively short period of time following infection and have largely been superseded by the use of

the ELISA. There are several ELISA techniques recommended for the detection of humoral antibody response to BTV but the blocking and competition are the best methods. The current edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines (2009) cites the competition ELISA as a prescribed test for the detection of BTV-specific antibodies (OIE, 2009). A new indirect ELISA for the detection of BTV-specific antibodies in bulk milk (Kramps et al., 2008) and other samples (Chand et al., 2009; Gandhale et al., 2010) is reported to be robust, specific and sensitive. The SNT or VNT has the highest specificity and sensitivity of all the tests, but is also most expensive and time-consuming. Most of the antibodies that neutralize intact BTV particles are specific for VP2 although VP5 can also influence the specificity of the reaction, probably through its interactions with VP2 (Hamblin, 2004; Batten et al., 2008; Batten et al., 2013). Immunofluorescence, immunoperoxidase, and dot immunobinding assays (DIA) have been widely used for BTV antibody detection.

Serogrouping of BTV

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between BT and epizootic haemorrhagic disease (EHD) viruses raises the possibility that an isolate of EHD virus could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific monoclonal antibody (MAb) can be used (Gandhale et al., 2010; Sperlova and Zendulkova, 2011). A number of laboratories have generated such serogroup-specific reagents. Commonly used methods for the identification of virus to serogroup level are immonofluorescence, antigen-capture enzyme-linked immunosorbent assay (ELISA), immunospot test and indirect peroxidase/antiperoxidase identification (Sperlova and Zendulkova, 2011), but they are rarely used (Dadhich, 2004).

Serotyping of BTV

The serotype of each BTV strain is determined by the specificity of reactions between components of the outer capsid (proteins VP2 and VP5) of the virus particle and neutralizing antibodies that are generated during infection of the mammalian host (Maan et al., 2007). These reactions can be analysed and measured using a variety of micro-titre, plaque reduction or other neutralisation assays (SNT, VNT). SNT can be used to detect neutralizing antibodies that are specific for each BTV serotype in diagnostic serum samples. SNT is highly sensitive and is usually specific for each BTV serotype although circulation of more than one serotype in a region, leading to sequential infections with different serotypes, is likely to cause cross-reactions with multiple additional serotypes. VNT can be used to identify the serotype of BTV isolates. Alternative serotyping methods have used small filter-paper discs soaked in serotype-specific neutralizing antisera which are placed on an agar overlay to create a zone of protection in lawns of tissue culture cells that are challenged with the test virus isolate (OIE, 2009). More recently serotyping can be made RT-PCR using serotype specific oligonuclotide primers. Primers have been developed by Maan et al. (2012).

Detection of BTV in Culicoides midges

The detection of BTV in field collected populations of adult Culicoides biting midges is most commonly attempted in areas where outbreaks are occurring or from endemic regions during periods of intense transmission. The techniques used are, with a few exceptions, the same as those used for detection of the virus in ruminants. The detection of BTV in Culicoides is most often carried out using ECE, followed by passage (often blind) in cell culture, to isolate the virus from pools of parous midges. As with samples from ruminants, the isolation of BTV is not always successful and virus strains which fail to grow under these conditions will remain undetectable. In these cases, other techniques (particularly RT-PCR) can be used to identify the presence of viral RNA in the pooled insects providing evidence that the virus itself is present (Veronesi et al., 2009).

Differential diagnosis

The clinical signs of bluetongue can easily be mistaken for those of other ruminant diseases such as orf (contagious pustular dermatitis), foot and mouth disease, acute photo sensitization, acute haemonchosis (with depression and submandibular oedema), facial eczema, *Oestrus ovis* infestation, pneumonia, plant poisoning, salmonellosis, sheep pox, Peste des Petits Ruminants (PPR) (Williamson et al., 2008), malignant catarrhal fever, pododermatitis, rinderpest, infectious bovine rhinotracheitis, bovine viral diarrhoea, bovine popular stomatitis, bovine herpes mamilitis and epizootic haemorrhagic disease of deer (Mehlhorn et al., 2008; Williamson et al., 2008; Savini et al., 2011).

ACKNOWLEDGEMENT

This work was supported by the All India Network

Programme on Bluetongue Disease, Indian Council of Agricultural Research (ICAR), New Delhi, India. The authors would like to acknowledge the Director, Indian Veterinary Research Institute for providing the facilities for conduct of the study.

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Vol. 7(51), pp. 5781-5788, 29 December, 2013
DOI: 10.5897/AJMR2013.6461
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Influence of phytosiderophore on iron and zinc uptake and rhizospheric microbial activity

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Accepted 12 December, 2013

Micronutrients play a vital role in crop production and sustainable crop yield. High crop yield varieties make soil micronutrients deficient, without incorporating external inputs. Due to deficiency of micronutrients such as iron (Fe) and zinc (Zn), yield decline drastically. It limits more than macronutrients, but requirements of these plant nutrients are very less, but plants have self regulated mechanism, which secrete the phytosiderophore (PS) and mobilize the lower concentration of these metals to soil solution for easy uptake by plants. Phytosiderophore production is a general response of plants to Fe and Zn deficiency in particular. The uptake rate of PS-chelated Fe and Zn is 100 and 5 to 10 times higher than that of free Fe and Zn, respectively. Higher amount of carbon containing organic compounds enhanced the microbial activities in rhizosphere and alter the plant nutrient chemistry in soil. This article discussed the importance of PS in microbial activity in soil and nutrient uptake mechanism in plants.

Key words: Iron, phytosiderophores, rhizospheric microbial activity, zinc.

INTRODUCTION

One of the widest ranging abiotic stresses in world agriculture arises from low iron (Fe) and zinc (Zn) availability in calcareous soils, particularly in cereals (Berg et al., 1993; Palmiter and Findley, 1995). A higher Zn acquisition efficiency, further, may be due to either or all of the following: an efficient ionic Zn uptake system, better root architecture that is long and fine roots with architecture favoring exploitation of Zn from larger soil volume (Richardson et al., 1989), higher synthesis and release of Zn-mobilizing phytosiderophore (PS) by the roots and uptake of Zn-PS complex (Dotaniya et al., 2013a). Zinc and Fe are the two most important micronutrients in crop production. More than 50% of the Indian soils are suffering from zinc and iron deficiency. It is also a big problem in well aerated calcareous soil. The release of PS is one of the most important mechanisms which enhances the mobilization of Fe and Zn in soil and their uptake by crops (Ackland and McArdle, 1990;

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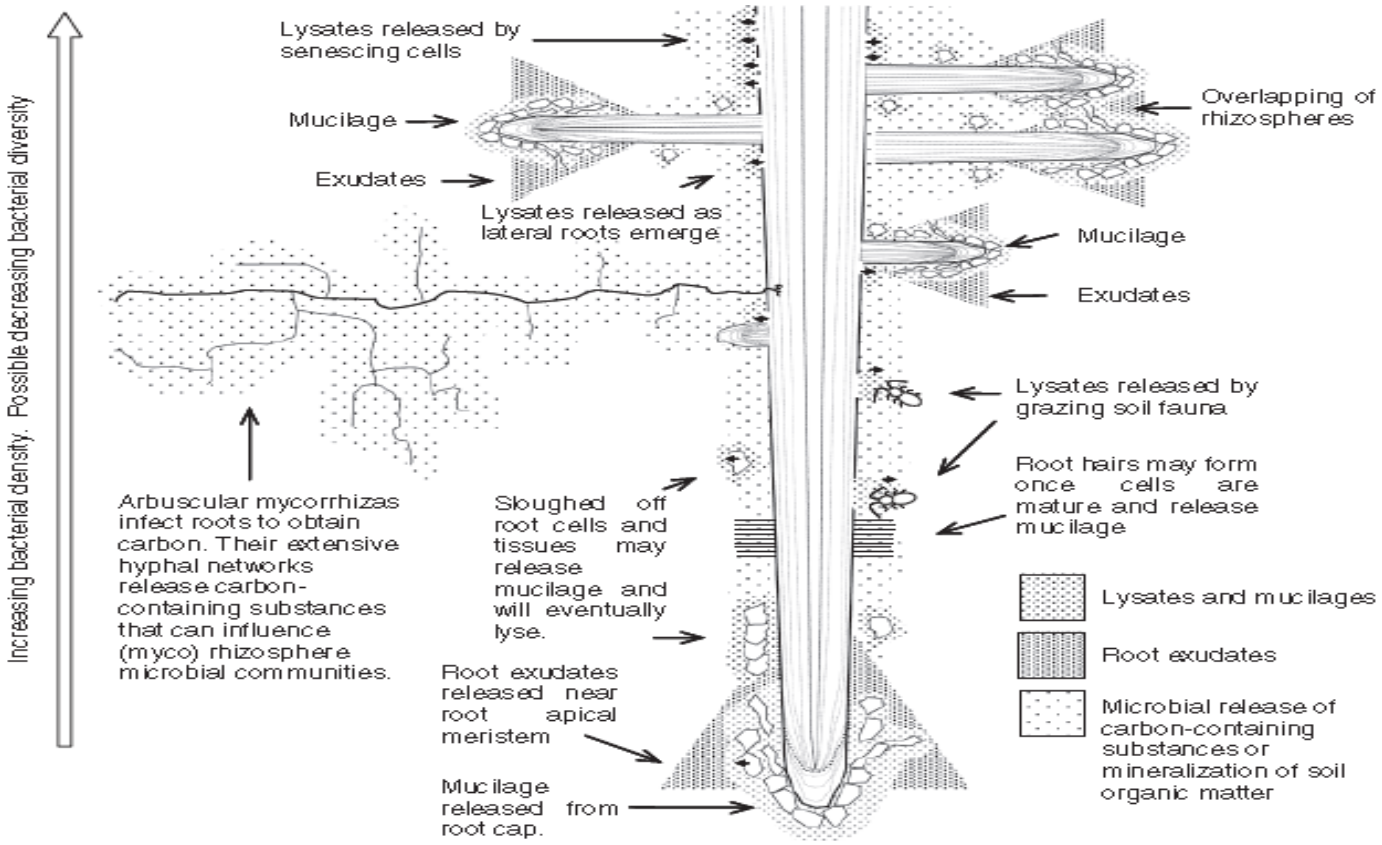

Figure 1. Origin of various pools of rhizodeposition (Dennis et al., 2010).

Bar-Ness et al., 1992). Peanut/maize intercropping was a sustainable and effective agroecosystem that evidently enhances the Fe nutrition of peanuts in calcareous soils by the influence of PS (Xiong et al., 2013).

PHYTOSIDEROPHORES

Phytosiderophores are organic substances (such as nicotinamine, mugineic acids (MAs) and avenic acid etc) produced by plants (Figure 1) (Mori and Nishizawa, 1987) under Fe-deficient conditions, which can form organic complexes or chelates with Fe^{3+}, and increase the movement of iron in soil (Ueno et al., 2007). It is non proteineous, low molecular weight acids released by the graminaceous species under the iron (Wallace, 1991) and Zn deficiency stress. The PS mobilize micronutrients Fe, Zn, Mn and Cu from the soils to plant in deficient condition (Takagi et al., 1984).

Characteristics of phytosiderophores

1) These are molecules with high affinity for Fe^{3+}, and remove the Fe^{3+} from minerals and contribute towards their dissolution.
2) These Fe-chelates are highly soluble and stable over a wide pH range.
3) They are of crucial importance for the zinc and iron transport in soils and its supply to plants.
4) Zn-PS have similar structural confirmations as Fe-PS and a similar regulatory mechanism for the biosynthesis and/or release of PS under both Zn and Fe deficiencies.
5) A plant releases PS at higher amounts about a few hours to the onset of the light period. Under continuous darkness or continuous light, the rate of release of PS is lower.
6) There has been observed a sharp rise in PS production three hours after onset of the light period, which gradually declines thereafter.

IRON DEFICIENCY: A GLOBAL CONCERN

Fe deficiency chlorosis in crop plants is a widespread nutrient problem particularly in calcareous soils in arid and semiarid regions, which often results in significant yield losses (Mortvedt, 1991). Such yield reductions have been reported in many crops, such as upland rice, maize and sorghum (Jolley et al., 1996; Dotaniya et al., 2013b).

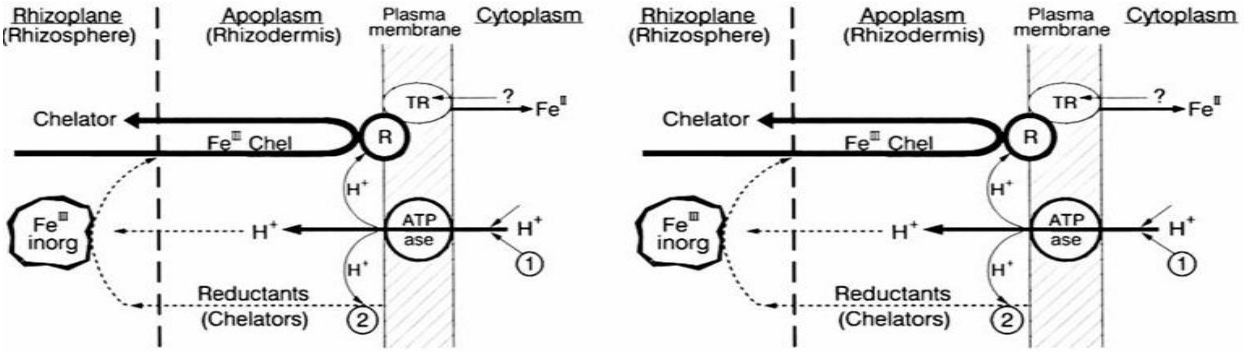

Figure 2. Strategy of Fe Acquisition by plants (Tagliavini and Rombola, 2001).

Grazing induced Fe-deficiency chlorosis in wheat was also reported (Berg et al., 1993). Soil amendments and foliar sprays of Fe are common methods to correct Fe deficiency (Bashir et al., 2010). However, these methods are expensive, time-consuming and may not be effective for more than one cropping season. Alternatively, breeding of plant genotypes with higher efficiency in the acquisition of Fe from the soil is a realistic approach (Kobayashi and Nishizawa, 2012). Selection for resistance, however, is difficult because of heterogeneous soil and highly variable environmental conditions that affect expression of Fe-deficiency chlorosis in the field (Nozoye et al., 2011). Yellow stripe 1 (ys1) and ys3 are recessive mutants of maize (*Zea mays* L.) that show typical symptoms of Fe deficiency, that is interveinal chlorosis of the leaves (Tomoko et al., 2013).

A lack of understanding of the factors influencing chlorosis expression has also impeded the development of reliable screening methods in the laboratory, controlled greenhouse, or environmental-chamber environment (Jolley et al., 1996). So the development of reliable Fe-deficiency chlorosis screening criterion is a necessary prerequisite for significant improvement of Fe-deficiency chlorosis resistance. Recently, many studies suggested that non-proteinogenic amino acids (PS) release has been linked to the ability of species and genotypes to resist Fe- deficiency chlorosis (Hansen et al., 1996; Romheld and Marschner, 1986). Therefore, PS release has been suggested as a selection criterion for Fe efficient graminaceous monocots.

ZINC DEFICIENCY: A GLOBAL CONCERN

Low availability of Zn in calcareous soils is one of the widest ranging abiotic stresses in world agriculture particularly in Turkey, Australia, China and India. Global studies initiated by the Food and Agriculture Organization (FAO) reported Zn deficiency in 50% of the soil samples collected from 25 countries (Hansen et al., 1996). It is one of the most widespread nutritional constraints in crop plants, especially in cereals. Among cereals, wheat and rice in particular, suffer from its deficiency. The yield reduction up to 80% along with reduced grain Zn level has been observed under Zn deficiency (Fageria et al., 2002). This deficiency is a serious implication for human health in countries where consumption of cereal-based diets predominates. Further, plants grown on zinc-deficient soils tend to accumulate heavy metals, which again is a potential human health hazard.

STRATEGY OF FE AND ZN ACQUISITION BY PLANTS

Iron and Zn deficiency induced chlorosis represents the main nutritional disorder in plants grown on calcareous and/or alkaline soils because of an extremely low solubility of soil Fe. Mechanisms of Fe acquisition in higher plants have been grouped into Strategy I and II (Figure 2). Strategy I plants (Tagliavini and Rombola, 2001), which include dicotyledons and non-graminaceous monocotyledons, respond to Fe deficiency by extruding both protons and reducing substances (phenols) from the roots, and by enhancing the ferric reduction activity at the root plasma membrane. This strategy is similar to the Zn acquisition by plants. The solubilized Fe must be reduced from Fe^{+3} to Fe^{+2} on the plasma membrane before Fe^{+2} is transported into the root cell through a specific Fe^{+2} transporter. Strategy II plants (graminaceous species) synthesize and secrete Fe-chelating substances, mugineic acids (MAs) from their roots to dissolve sparingly soluble Fe compounds in the rhizosphere (Figure 3) (Marschner et al., 1986) and affected by soil bacteria (Chattopadhyay, 2006; Dipanwita and Chattopadhyay, 2013). Iron is transported across the plasma membrane as a complex of PS- Fe^{+3} through a specific transport system without prior reduction.

The synthesis of mugineic acid is induced by Fe-deficiency. The chemical constituents, number and amount of mugineic acid synthesized and secreted into the rhizosphere may differ among species and even cultivars (Xiong et al., 2013). In general, the amount of

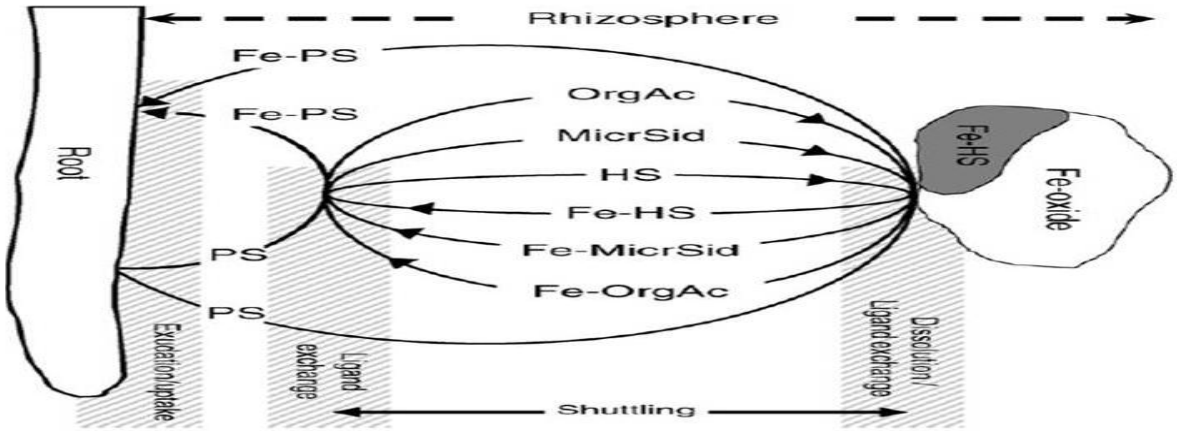

Figure 3. Schematic representations of important processes in strategy II iron acquisition (Dotaniya et al., 2013a).

MAs secreted correlates positively with the ability of the plants to tolerate Fe deficiency. But siderophore produced by microbes also enhanced the Fe uptake. If siderophores and PS are present at similar concentrations, Fe is preferentially bound to the siderophores, which may even remove Fe from the Fe-PS complex. In contrast to many bacterial siderophores, rhizoferrin from the fungus *Rhizopus arrhizus* has only a slightly higher affinity towards Fe compared to PS (Crowley and Gries, 1994; Zelenev et al., 2005). Rhizoferrin is a good Fe source for barley, probably because of exchange of Fe from rhizoferrin to the PS (Yehuda et al., 1996). It can be amply surmised from the available literature that Zn and Fe efficiency of cereals under deficiency is regulated by several factors, most importantly, the presence of an efficient Zn^{2+}, Fe^{+2} and PS complex uptake system.

Manipulation of phytosideriophore biosynthesis and release is a promising strategy to improve Fe and Zn efficiency in cereal crops (Wallace, 1991). In Alice maize cultivar, Zn uptake decreased with increasing stability constant of the chelate in the order: $ZnSO_4$ (greater than or equal to) Zn-desferrioxamine > Zn-PS > Zn-EDTA. Adding a 500-fold excess of free PS over Zn to the uptake solution depressed Zn uptake in maize mutant ys1 almost completely (von Wiren et al., 1996). It may be quite plausible that iron and zinc deficiency tolerance of graminaceous species can also be achieved through manipulation of key enzymes of PS biosynthesis that is Nicotianamine synthase (NAS) and Nicotianamine amino-transferase (NAAT). This will help in reducing and may be even totally eliminating the application of zinc and iron fertilizers to the soil.

EFFECT ON MICROBIAL ACTIVITIES IN RHIZOSPHERE

The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms (Giri et al., 2005). Soil which is not part of the rhizosphere is known as bulk soil. The rhizosphere contains many bacteria that feed on sloughed-off plant cells, termed rhizodeposition and the proteins and sugars released by roots (Curl and Truelove, 1986). It is a densely microbial populated area of soil in which the roots must compete with the invading root systems of neighboring plant species for space, water, and mineral nutrients, and with soil-borne microorganisms, including bacteria, fungi, and insects feeding on an abundant source of organic material (Ryan and Delhaize, 2001).

In 1904, the German agronomist and plant physiologist Lorenz Hiltner first coined the term "rhizosphere" to describe the plant-root interface (Figure 4), a word originating in part from the Greek word "rhiza", meaning root (Hiltner, 1904; Hartmann et al., 2008). Microbial population is more affected by the amount and type of C in soil (Akiyama et al., 2005). Under long term study, it was found that microbial population is greater in organic soil as compared to inorganic farming plots (Tu et al., 2005). In general 10-20% more biomass was measured in organic soils (Gelsomino et al., 2004). High secretion of PS in soil, improved the soil fertility and nutrient mobility in soil (Colmer and Bloom, 1998). Microbial biomass is an indicator of soil microbial activities. Generally, in crop production, more biomass means more fertile soil, which is a good indicator of plant nutrient (Becard et al., 1992, 1995; Trieu et al., 1997). Root secretions may play symbiotic or defensive roles as a plant ultimately engages in positive or negative communication (Stintzi and Browse, 2000; Stotz et al., 2000), depending on the other elements of its rhizo-sphere such as available nutrients, water, space CO_2 concentration and C. In contrast to the extensive progress in studying plant-plant, plant-microbe (Keyes et al., 2000) and plant-insect interactions that occur in above ground plant organs such as leaves and stems, very little research has focused on root-root, root-microbe, and root-insect interactions in the rhizosphere

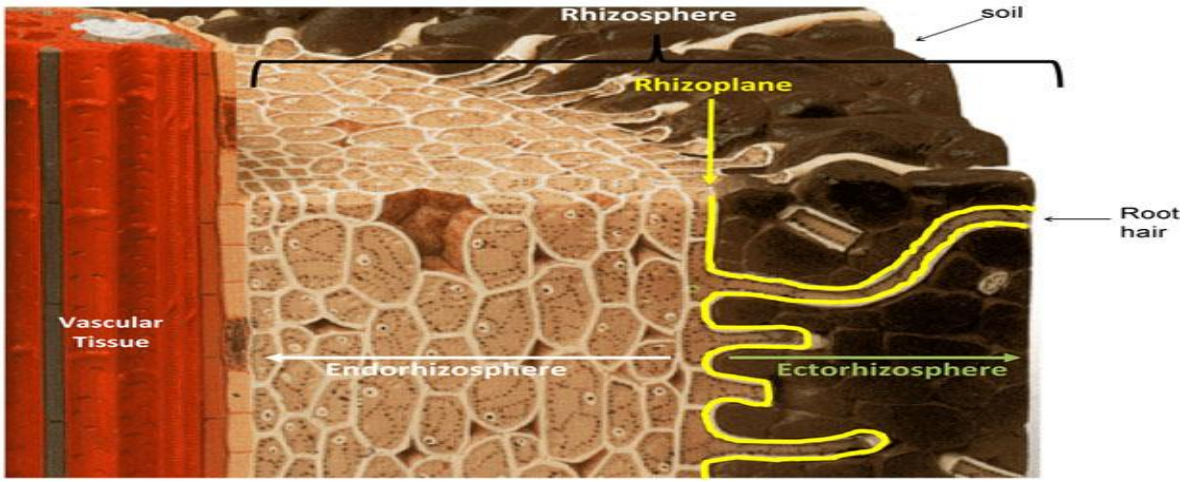

Figure 4. Structure of the rhizosphere in soil (McNear, 2013).

(Shannon et al., 2002). Bacterial siderophores are usually poor Fe sources for both monocot and dicot plants (Bar-Ness et al., 1992; Crowley et al., 1992; Walter et al., 1994). However, in some cases, microbial siderophores have alleviated Fe deficiency-induced chlorosis in dicots (Jurkevitch et al., 1988; Sharma et al., 2003; Wang et al., 1993; Yehuda et al., 2000). On the other hand, plant-derived Fe-PS complexes appear to be a good Fe source for bacteria (Jurkevitch et al., 1993; Marschner and Crowley, 1998).

The organic compounds released through these processes can be further divided into high and low molecular weight (HMW and LMW, respectively). By weight, the HMW compounds which are those complex molecules that are not easily used by microorganisms (mucilage, cellulose) make up the majority of C released from the root (Chin-A-Woeng et al., 1997); however, the LMW compounds are more diverse and thus have a wider array of known or potential functions (Bauer and Mathesius, 2004). Rooting density has a large effect on uptake per unit PS secretion as a result of overlap of the zones of influence of neighboring roots (Von Wiren et al., 1996). The list of specific LMW compounds released from roots is very long, but can generally be categorized into organic acids, amino acids, proteins, sugar, phenolics and other secondary metabolites which are generally more easily used by microorganisms. It provides the C source of energy and food, because of plenty of organic compounds released from roots enhanced the microbial activity and population. Further increase in microbial population accelerates the competition for water, C and space also (Baudoin et al., 2003).

EFFECT OF FERTILITY AND ATMOSPHERIC CO_2 CONCENTRATION ON PHYTOSIDEROPHORE

Root exudates is secreted from root in two way: (1) actively released from the root and (2) by diffuseness which are passively released due to osmotic differences between soil solution and the cell (Dakora and Phillips 2002), or lysates from autolysis of epidermal and cortical cells. These organic compounds may be sugar, non-protein amino acids mugineic acid (of barley) and avenic acid (of oats) (Darrah, 1991). Das and Dkhar (2011) conducted a research with various organic and inorganic fertilizers and their effect on physico-chemical properties of rhizosphere (Table 1). They observed that the application of vermicompost resulted in most pronounced growth of microbial population compared to inorganic treatment. Also, application of organic treatments showed increased rhizosphere soil physicoche-mical properties which in return lead to the increased microbial population which is of great importance in nutrient availability of the studied soil (Kundu et al., 2013). The soil microbial population also secrets a significant amount of sidero-phores in soil, however it promotes the root exudates from plants (Bais et al., 2001). The root exudates play an important role in root microbe interactions. Flavonoids are present in the root exudates of legumes that activate *Rhizobium meliloti* genes responsible for the nodulation process (Peters et al., 1986). Fertilizer and lime applications typically result in increased bacterial numbers and decreased fungal biomass (Lovell et al., 1995).

Bacterial communities in the rhizosphere are not static, but will fluctuate over time in different root zones, and bacterial composition will differ between different soil types, plant species, plant growth seasons and local communities (Semenov and Brooks, 1999). Changes induced in the soil by the growing root provide additional niches for soil microbes. Soil types and growth stages are important factors in shaping rhizobacterial community structure (Latour et al., 1996; Seldin et al., 1998; Herschkovitz et al., 2005) and may be the strongest factor affecting bacterial communities in potato rhizo-

Table 1. Physico-chemical properties of rhizosphere soil influenced by organic and inorganic fertilizers (Das and Dkhar, 2011).

Treatment	pH	Moisture content	SOC (%)	Total N (%)	Av- P (µ/g)	K (mg/g)	Soil Respiration (mg/g)	MBC (µ/g)
Plant compost	5.6	24.90	1.80	0.32	1.18	0.04	65.1	1015.0
Vermicompost	5.4	24.24	1.50	0.31	2.66	0.05	66.11	2145.7
Integrated plant compost	5.6	24.68	1.75	0.35	2.01	0.04	64.56	1385.1
FYM	4.6	23.82	1.27	0.31	2.24	0.08	56.5	940.9
Control	4.9	23.39	1.60	0.28	2.01	0.05	56.56	656.5
NPK	4.9	23.39	1.60	0.35	2.68	0.04	62.89	798.9

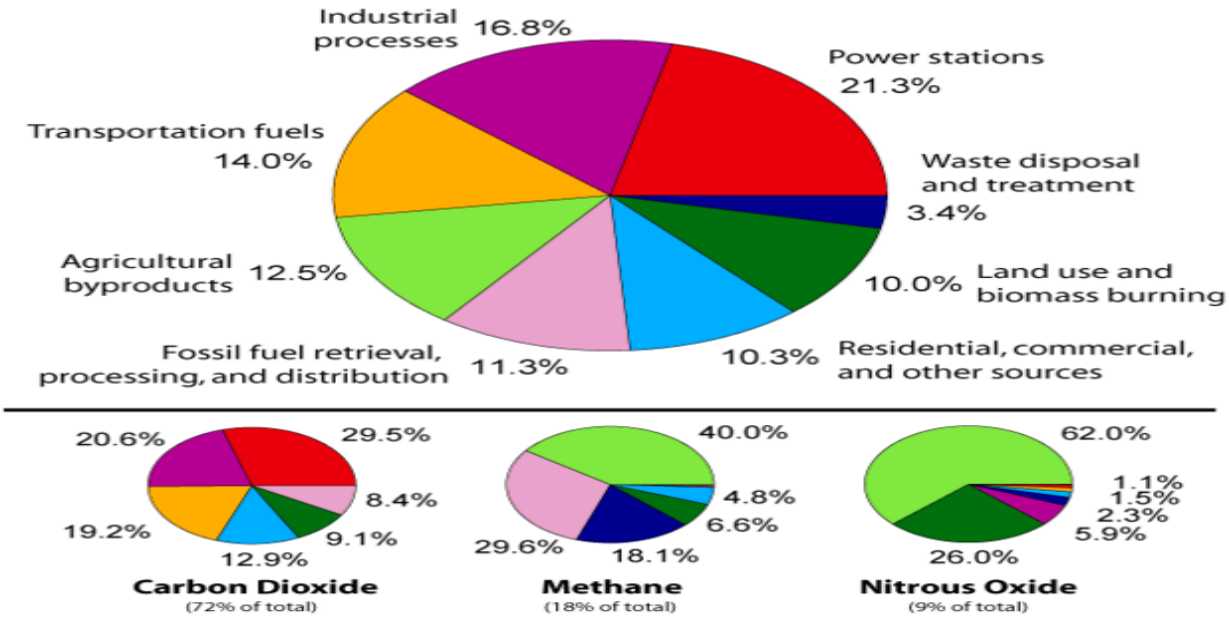

Figure 5. Annual greenhouse gas emissions by sector (www.e-education.psu.edu).

sphere (Van Overbeck and Van Elsas, 2008); plant species (Grayston et al., 1998; Smalla et al., 2001) and even 'cultivar (genotype) within the same species (Andreote et al., 2009). The rhizosphere is a highly dynamic environment for bacterial communities and even small topographical landform changes can alter environmental conditions that may accelerate or retard the activity of organisms (Ramette et al., 2005).

Soil microbial activities affected the physical, chemical and biological activities and ultimately crop production. Increasing environmental factors like CO_2 concentration and atmospheric temperature affected the root exudates and rhizospheric microbial population. Impacts of elevated CO_2 on soil ecosystems, focuse primarily on plants and a variety of microbial processes. The processes considered include changes in microbial biomass of C and N, soil enzyme activity, microbial community composition, organic matter decomposition, and functional groups of bacteria mediating trace gas emission in terrestrial and wetland ecosystems. Except from CO_2, other gases that is CH_4, N_2O and other gases play a significant role in global climate phenomena (Figure 5).

The cocktail of chemicals released is influenced by plant species, edaphic and climactic conditions which together shape and are shaped by the microbial community within the rhizosphere. There is still very little known about the role that a majority of the LMW compounds play in influencing rhizosphere processes (Cheng et al., 1996). A growing body of literature is beginning to lift the veil on the many functions of root exudates as a means of acquiring nutrients (acquisition of Fe and P), agents of invasiveness (that is allelopathy) or as chemical signals to attract symbiotic partners (chemotaxis) (rhizobia and legumes) or the promotion of beneficial microbial colonization on root surfaces (*Bacillus subtilis*, *Pseudomonas florescence*) (Bais et al., 2004, Park et al., 2003).

FUTURE NEED OF RESEARCH

1) More research should be on the biotechnological side, separation and insertion of high phytosiderophor responsible gene in crop plant, which is crucial for crop production in low fertility areas.
2) Also, research should be done on the use of alternative combat methods, against elevated CO_2 concentration without compromising positive effect on PS release.

CONCLUSIONS

A healthy crop production requires a good status of plant nutrient. It play crucial role in plant metabolism and ultimately in edible part. In nutrient deficient condition, plant growth is limited and poor yield is obtained. Phytosiderophors are secreted from plant root, and it is a life saving mechanism in plants. It enhances the plant nutrient uptake and improves the soil health. Iron availability is low in most aerobic soil, and microorganisms and plants release low molecular-weight compounds (chelators) which increase Fe availability. It specially enhances the uptake of Fe and Zn in lower concentration. Increasing root exudates in soil enhances the soil fertility level as well as microbial biomass. These soil microbes play vital role in nutrient transformation reactions in soil and nutrient uptake by crop plants.

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