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Health Implications of *Bt* Cotton Seeds in Animals and Man

Neeraj Singh Mewari[1], Tulsa Devi[2], RS Chauhan[3]

[1]PhD. Scholar, Department of Veterinary Pathology, College of Veterinary and Animal Sciences, G B Pant University of Agri. & Tech.Pantnagar-263145 Uttarakhand.

[2]Project Assistant, Department of Veterinary Pathology, College of Veterinary and Animal Sciences, G B Pant University of Agri. & Tech.Pantnagar-263145 Uttarakhand.

[3]Professor and Head, Department of Veterinary Pathology, College of Veterinary and Animal Sciences, G B Pant University of Agri. & Tech.Pantnagar-263145 Uttarakhand.

ABSTRACT

Cotton (*Gossypium* spp.) is an arborous plant from the Malvaceae family. Cottonseed meal is a by-product of cotton that is used for animal feeding because it is rich in oil and proteins. However, gossypol toxicity limits cottonseed use in animal feed. Gossypol is a phenolic compound produced by pigment glands in cotton stems, leaves, seeds and flower buds. Cotton is a long duration crop and is attacked by a number of insect pests. Earlier, insecticide quantity applied on cotton was highest but with the advent of *Bt* cotton, there is a great reduction in insecticide and pesticide usage as majority of the bollworms are controlled by it. *Bt* cotton refers to transgenic cotton which contains endotoxin protein inducing gene from soil bacterium *Bacillus thuringiensis*. It was discovered by a Japanese scientist Ishiwata in 1901. *B.thuringiensis* strains produces 3 types of insecticidal toxins that are Crystal toxins (Cry), Cytolytic toxins (Cyt) and vegetatively expressed insecticidal proteins (vip). *Bt*-delta-endotoxins which functions as oral toxins are ingested by insect and protoxins are proteolytically activated to trypsin-resistant active core δ- endotoxin in alkaline mid-gut. The active toxin binds to cadherin receptors present on the brush border membrane of the insect midgut. Cadherins process the toxins to form homo-oligomers, bind to specific receptors like alkaline phosphatases and aminopeptidases before causing pores in the epithelial membrane, resulting in osmotic lysis of the cells which results in cessation of feeding and finally mortality of the insect. Cry toxins have not been reported to be toxic to higher animals such as goats, sheep and cattle in any part of the world as scientific evidences indicate that the Cry toxins do not get activated under the acidic conditions of non-target animals such as goat, sheep and cattle. In one study of acute oral toxicity study in rats, *Bt* cotton seed material did not induce any treatment related observable toxic effects when compared with Non-*Bt* cottonseeds. Histological studies in another study revealed that there were no difference in cellular architecture in liver, heart, kidney and intestine of *Bt* and non-*Bt* diet fed rats. The Cry toxins Cry1Ac, Cry1Ab, Cry2Ab, Cry1F and Cry1C are considered to be safe to human beings. The stomach of humans, being the first organ of digestion which the *Bt* protein encounters, is acidic and contains proteases like pepsin which degrade the *Bt* protein. Thus, the alkaline conditions needed for pro-toxin solubilization and protease action required for toxin activation are absent in the stomach. More importantly, the human intestine lacks the specific receptors to which the activated *Bt* protein binds and initiates the physiological effect. However, there are several reports that *Bt* genes cause some serious problems to human health. *Cry1Ac* toxin at higher concentration have lethal cytotoxic and

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genotoxic effects on the human lymphocytes. The present review is an attempt to provide details from the available literature regarding uses of *Bt* cotton and its implications on animal and human health.

KEYWORDS: Health implications, Bt cotton, Gossypol, Animals, Man, Biosafety.

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INTRODUCTION

Cotton (*Gossypium* spp.) is an arborous plant from the Malvaceae family. It is one of the earliest plants that were cultivated by man and it has been used for over 4,000 years. It is primarily cultivated for fiber used in the textile industry and the oil from the cotton seed (Soto-Blanco, 2008). The most economically important cotton species is *G.hirsutum*, which is grown to produce 90% of the world's cotton (Bor'em *et al*., 2003). *Bt* cotton is genetically modified cotton crop that expresses an insecticidal protein whose gene has been derived from a soil bacterium called *Bacillus thuringiensis*, commonly referred as *Bt*. Many subspecies of *B.thuringiensis* are found in soils and are in general known to be toxic to various genera of insects but safe to other living organisms. *Bt* was first discovered by a Japanese scientist Ishiwata in the year 1901. *Bt* has been used as an insecticide for control of stored grain pests since 1938 in France and from 1961 as a registered pesticide in the USA and later in many other countries including India as sprays in cotton IPM programs to control insects. *Bt* toxins thus have several decades of proven selective toxicity to insect pests and with established safety record to non-target animals. Currently there are 67 recognized subspecies of *B. thuringiensis* most of which produce spores and insecticidal proteins. The *B. thuringiensis* strains produce three types of insecticidal toxins, crystal (Cry) toxins, cytolytic (Cyt) toxins and vegetatively expressed insecticidal proteins (vip). These toxins are highly specific to certain insect species. The *Bt* gene *cry1Ac* was used to develop the first Bt-cotton variety. The gene was transferred into the genome of cotton explants (tissue pieces) using a bacterium called *Agrobacterium tumefasciens*. The transformed cells were developed into a full GM plant now called *Bt*-cotton. In general, Cry1Ac toxins are highly specific to insects at species level, and are not known to cause any harm to non-target species such as fish, birds, farm animals and human beings. Currently, Cry1Ac, Cry2Ab and Cry1C have been approved for commercial cultivation in India. This review article presents an overview of the uses and implications of *Bt* cotton on animal and human health.

GOSSYPOL

Cotton fiber and oil production generate byproducts rich in fat from oil and protein which are used for animal feeding. However, this plant contains a toxic compound, gossypol (Soto-Blanco, 2008). Gossypol is produced by pigment glands in cotton stems, leaves, seeds, and flower buds. The pigment glands are small black spots distributed throughout the cotton plant but their greatest concentration is in the seeds (Soto-Blanco, 2008; Rogers *et al*., 2002; Kenar, 2006; Alexander *et al*., 2008). Two gossypol forms have been observed, free and bound (Alexander *et al*., 2008). The bound form is produced via covalent bonds between gossypol and the free epsilon-amino groups from lysine and arginine (Soto-Blanco, 2008; Bressani *et al*., 1964; Fernandez *et al*., 1995) through the browning or Maillard reaction (Soto-Blanco,2008). However, this reaction reduces the availability of amino acids for absorption by the animal with lysine being the most affected (Fernandez *et al*., 1995). Total gossypol production is influenced by several factors, including weather conditions and cotton species. Considering weather conditions, gossypol production is positively correlated with the rainfall rate and negatively correlated with temperature (Pons Jr. *et al*., 1953). Cottonseed may contain concentrations greater than 14,000mg/kg of total gossypol and 7,000mg/kg of free gossypol (Alexander *et al*., 2008). However, after oil extraction from the seeds, up to 0.6% is available following solvent extraction, but approximately 0.06% is available, if the extraction process involves mechanical pressure and heat treatment (Nicholson, 2012). In addition to its harmful effects, gossypol and its derivatives have potential therapeutic use. These compounds showed *in vitro* action against some viruses such as human immunodeficiency virus (Polsky *et al*., 1989; Yang *et al*., 2012) and H5N1 influenza virus (Yang *et al*., 2012; Yang *et al*., 2013) and several bacteria and yeasts (Margalith, 1967; Yildirim-Aksoy *et al*., 2004; Turco *et al*., 2007; Anna *et al*., 2012). Gossypol is a promising treatment for leukemia (Balakrishnan *et al*., 2008), lymphoma (Johnson, 2008), colon carcinoma (Wang *et al*., 2000), breast cancer (Poznak *et al*., 2001; Ye *et al*., 2007), myoma (Han *et al*., 1987), prostate cancer (Jiang *et al*., 2012) and other malignancies (Tuszynski and Cossu, 1984; Wu *et al*., 1989; Badawy *et al*., 2007; Ko *et al*., 2007; Chien *et al*., 2012; Hsiao *et al*., 2012; Wong *et al*., 2012). Furthermore, it was used in China, in 1970, to treat uterine fibroids, endometriosis, and uterine bleeding in women (Han *et al*., 1987).

GOSSYPOL POISONING

Gossypol poisoning has been reported in many species, including broiler chicks (Henry *et al*., 2001), pigs (Haschek *et al*., 1989), dogs (West, 1940; Uzal *et al*., 2005), sheep (Morgan

et al., 1988) and goats (East *et al*., 1994). Monogastric animals, such as pigs, birds, fish, and rodents, are more susceptible to gossypol toxicity than ruminants (Kenar, 2006; Alexander *et al*., 2008; Randel *et al*., 1992; Zhang *et al*., 2007). Moreover, young ruminants are more sensitive to gossypol compared with adult ruminants (Soto-Blanco, 2008) because gossypol is not bound during ruminal fermentation, as it occurs in animals with fully functional rumens. However, if the gossypol intake overwhelms the ruminal detoxification capacity, free gossypol may be absorbed at hazardous concentrations even in adult ruminant animals (Willard *et al*., 1995). General signs of acute toxicity are similar among animal species and include respiratory distress, impaired body weight gain, anorexia, weakness, apathy, and death after several days (Soto-Blanco, 2008; Alexander *et al*., 2008; Kerr, 1989; Morgan *et al*., 1988; Rogers *et al*., 1975; Holmberg *et al*., 1988; Risco *et al*., 1992; Zelski *et al*., 1995; Fombad and Bryant, 2004). Heart failure was reported in calves (Holmberg *et al*., 1988; Hudson *et al*., 1988), lambs (Morgan *et al*., 1988) and dogs (Patton *et al*., 1985). The postmortem findings in ruminants include pulmonary edema, yellowish liquid in the chest and peritoneal cavities, gastroenteritis, centrilobular liver necrosis, and hypertrophic cardiac fiber degeneration. In calves, the major pathologic findings are ascites, visceral edema, acute centrilobular hepatocyte necrosis, kidney damage, and cardiovascular lesions (Chauhan, 2018). Increased pneumonia has also been observed, likely due to an increased sensitivity to secondary infections (Morgan *et al*., 1988; Holmberg *et al*., 1988; Risco *et al*., 1992; Zelski *et al*., 1995). Pigs may present reduced weight gain, anorexia, respiratory distress, cardiac insufficiency, coughing, and exercise intolerance. Necropsy findings include fluid accumulation in the body cavities; edema and congestion in the liver, lung, and spleen; and cardiac hypertrophy with degenerated muscle fiber (Haschek *et al*., 1989). Anemia is often observed in animals fed cottonseed. In fact, gossypol is a highly reactive compound that readily binds to minerals and amino acids. Binding with iron forms a gossypol-iron complex, which inhibits the absorption of this metal. The consequent iron deficiency affects erythropoiesis. Furthermore, gossypol promotes increased erythrocyte fragility (Randel *et al*., 1996; Lindsey *et al*., 1980; Zhang *et al*., 2007; Mena *et al*., 2004). Gossypol also stimulates the eryptosis (apoptosis-like erythrocyte death) by increasing cytosolic Ca^{2+} activity resulting in cell membrane scrambling and contraction, which contributes to anemia (Zbidah *et al*., 2012). Gossypol also affects thyroidal metabolism (El-Mokadem *et al*., 2012; Tang and Wong, 1984; Rikihisa and Lin, 1989; Lin *et al*., 1990; Udoh *et al*., 1992). Some studies with male (Rikihisa and Lin, 1989) and female (Lin *et al*., 1990) rats showed decreased blood concentrations of T4 and T3 after dosing with gossypol. On the other hand, gossypol dosing resulted in increased T3 serum concentrations without affecting T4 in rats (Tang and Wong, 1984) and sheep (EL-Mokadem *et al*., 2012). The histopathological evaluation of thyroid glands from male rats dosed with gossypol revealed follicular degeneration and atrophy (Rikihisa and Lin, 1989). The thyrotropic cells in the pituitary gland, which are specialized for TSH synthesis and secretion, showed hypertrophy, hyperplasia, and degranulation after gossypol dosing in rats (Udoh *et al*., 1992). In addition to such effects, gossypol is hepatotoxic (Kakani *et al*., 2010; Blevins *et al*., 2010; Deoras *et al*., 1997; Fonseca *et al*., 2013; El-Sharaky *et al*., 2010; Manabe *et al*., 1991). Ascites and hepatocyte degeneration (strong cytoplasmic eosinophilia and nuclear pyknosis) were observed in rats that received a single intraperitoneal gossypol dose of 25mg/kg BW (Deoras *et al*., 1997) or 30mg/kg BW (Fonseca *et al*., 2013). Rats that received lower gossypol doses (15mg/kg/day for four weeks or 30mg/kg/day for two weeks) showed morphological changes in the liver, as observed through electron microscopy, which were characterized by mitochondrial vacuolation, an enlarged endoplasmatic reticulum, an expanded perinuclear space, and collagen fiber proliferation in the perisinusoidal space (Wang and Lei, 1987). Gossypol affects male and female gametogenesis and promotes embryo lesions (Gadelha *et al*., 2011). The gossypol toxicity formale reproduction was reported in several studies showing that it inhibits spermatogenesis, which decreases the sperm count and spermatozoid motility and viability (Randel *et al*., 1992; El-Sharaky *et al*., 2010; Hahn *et al*., 1981; Heywood *et al*., 1986; Ga'fvels *et al*., 1984; Chenoweth *et al*., 1994; Gu and Anderson, 1985; Lagerl'of and Jone, 1985; Fomes *et al*., 1993; Chongthammakun *et al*., 1986; Hong *et al*., 1989; Brocas *et al*., 1997;Chenoweth *et al*., 2000; Yuan and Shi, 2000). Gossypol also affects female reproduction and ruminant females tolerate higher dietary gossypol concentrations than non-ruminant females (Randel *et al*., 1992; Lin *et al*., 1985; Brocas *et al*., 1997; Gray *et al*., 1993), probably due to the ruminal detoxification. Female exposure to gossypol has been associated with interference with the estrous cycle, pregnancy, and early embryonic development (Randel *et al*., 1992; Randel *et al*., 1996; Gadelha *et al*., 2011). Gossypol interfered with rodent estrous cycles (Lin *et al*., 1985; Adeyemo *et al*., 2007) and pig granulosa cell function (Basini *et al*., 2009). Gossypol may cause a reduced number of leukocytes and primarily lymphocytes, which affects the immunocompetence of the organism (Braga *et al*., 2012). *In vivo* and *in vitro* mouse experiments also demonstrated that gossypol has immunosuppressive activity (Xu *et al*., 2009), which operates by affecting lymphocytes through inhibiting proliferation and inducing apoptosis (Xu *et al*., 2009; Quintana *et al*., 2000). Mice that received gossypol had significantly decreased numbers of lymphocytes in the thymus and mesenteric lymph nodes (Sijun *et al*., 2012), in the total spleen

cell population (Sein, 1986) and in the capacity of blood and lymphatic cells to produce antibodies after sheep erythrocyte immunization (Sein, 1986; Sijun *et al*., 2012). Furthermore, the spleen and lymph nodes from mice receiving gossypol had decreased CD^{4+} thymocyte populations and increased CD^{8+} lymphocyte populations (Sijun *et al*., 2012).

WHY DO WE NEED *Bt*-COTTON?

Cotton is a long duration crop and is attacked by large number of insect pests throughout its growth and development. The three bollworms, American bollworm *Helicoverpa armigera*, Pink bollworm *Pectinophora gossypiella* and the Spotted bollworms, *Earias vittella* and *Earias insulana* are major pests and cause serious threat to cotton production resulting in significant yield losses. Before the introduction of *Bt* cotton, insecticide quantity applied on cotton was the highest, relative to other cultivated crops. Cotton bolls are highly vulnerable to hidden insects such as the American bollworm, pink bollworm and spotted bollworm. Bollworms, especially the pink and spotted bollworms are hidden feeders and generally do not come into direct contact with insecticide sprays. Three crops, paddy, cotton and pigeon-pea are major consumers of insecticides in India. The use of Cry toxins to develop GM crops in paddy, pigeonpea, chillies and soybean has the potential to reduce the use of hazardous insecticides on food crops (Kranthi, 2012).

HOW DOES *Bt*-COTTON KILL INSECTS?

The Cry1Ac, Cry2Ab, Cry1C, Cry1F etc., belong to the class '*Bt*-delta-endotoxins' which function as oral toxins. The delta-endotoxins are ingested and the protoxins present in the crystals are proteolytically activated to trypsin-resistant active core δ-endotoxin in the alkaline mid-gut. The active toxin traverses the peritrophic membrane to bind cadherin receptors present on the brush border membrane of the insect midgut. The cadherins process the toxins to form homo-oligomers and bind to specific receptors like alkaline phosphatases and aminopeptidases before causing pores in the epithelial membrane, resulting in osmotic lysis of the cells. This results in cessation of feeding and finally causing mortality (Kranthi, 2012).

IS *Bt*-COTTON REALLY SELECTIVELY TOXIC TO INSECTS?

The Cry toxins are specifically toxic to specific classes of insects. For example, the Cry1Ac is toxic to three species of cotton bollworms, but is less toxic to the tobacco caterpillar, *Spodoptera litura* and is non-toxic to other classes of insects which are sap-sucking pests such as mealybugs, jassids, aphids, whiteflies etc. Other Cry toxins as Cry1F and Cry1C are more toxic to tobacco caterpillar *Spodoptera litura* and relatively less toxic to the cotton bollworms. The Cry1Ac is mainly toxic to the bollworms (cotton bollworm, pink bollworm and spotted bollworm), semiloopers and hairy caterpillars. *Bt*-cotton expressing Cry1Ac is non-toxic to other non-target organisms such as beneficial insects, birds, fish, animals and human beings. Laboratory and field studies carried out in India showed that the Cry1Ac protein deployed in *Bt*-cotton did not have any direct effect on any of the nontarget beneficial insects (Kranthi, 2012). Hilbeck *et al*. (2012) confirmed their earlier findings that Cry1Ab toxin increases mortality of the two-spotted ladybird beetle, *Adalia bipunctata* larvae.

HOW *Bt* COTTON IS DEVELOPED?

- **(a)** Identification of effective gene or genes
- **(b)** Gene transfer technology
- **(c)** Regeneration ability from protoplasts, callus or tissues
- **(d)** Gene expression of the product at desired level
- **(e)** Proper integration of genes so that are carried for generations by usual means of reproduction.

Once identification of bollworm inhibiting genes has been achieved, molecular biologists have step by step solved the problems to achieve perfect transgenics. In case of cotton, Agrobacterium-mediated gene transfer technique has been essentially used (Firozabady *et al*., 1987). Although now for direct gene transfer to protoplast, biolistic gene transfer techniques are available. The regeneration of cotton plants from callus and somatic embryogenesis have so far been restricted to few 'Coker' genotypes. All cotton genotypes are not amenable to regeneration and that is one big hurdle in gene transfer. There are reports of induction of somatic embryogenesis has also been reported from china and Australia but in India, attempts to repeat it with Indian genotypes have been unsuccessful. To circumvent the problem of genotype-limited regeneration of callus or leaf tissues, transformation and regeneration from meristematic tissues was attempted which was found useful. Using Cry 1 Ab and Cry 1 Ac genes, transgenic cottons with perfect integration, expression and reproduction was achieved first in USA in 1987. There are four important methods of foreign gene (DNA) transfer in crop plants viz. plasmid method, particle bombardment, direct DNA uptake and micro-injection (Stewart, 1991). These methods are also known as systems of DNA delivery for genetic transformation. The soil borne bacterium *Agrobacterium tumifaciens* (termed as Nature's Genetic Engineering) is used for development of transgenic plants. This method has three main limitations viz. host specificity, somaclonal variation and slow generation. There are two main advantages of Agrobacterium mediated DNA transfer method. Firstly, this method has some control over the copy number and site of integration of transgene which is not possible in particle bombardment method. Secondly, this is a cheaper method of genetic transformation than particle

bombardment method. Perlak *et al*. (1990) transferred successfully the Cry 1 Ac gene to cotton via Agrobacterium with CaMV promoter and the Cry protein produces by transgenic cotton was found highly toxic to bollworms. This method was later used extensively by others. The particle bombardment method in which the foreign DNA is delivered into plant cells through high velocity metal particles, has some advantages over the Agrobacterium mediated method of DNA transfer, This method does not exhibit host specificity. Hence, it can be effectively used for the development of transgenic plants in various plant species. Moreover, this method is technically simple than Agrobacterium mediated DNA transfer method. In this method, there is no need of isolating protoplast. The other two method viz. direct DNA transfer and microinjection technique are rarely used for developing transgenics in cotton. Currently, two DNA delivery system, viz.(1) Agrobacterium mediated gene transfer, and (2) bombardment of cells with plasmid DNA coated particles, are widely used for development of transgenic (genetically engineered) plants in cotton (Umbeck *et al*., 1987; Firozbady *et al*., 1987; Finer and McMullen, 1990). The first two workers used Agrobacterium method while the last workers used biolistic method of gene transfer in cotton for developing transgenic plants. More than 37 transgenic plants have been developed in cotton so far by these two methods.

Anti-viral and anti-cancerous properties of Gossypol

Some amino acids substituting the aldehyde groups of gossypol not only reduced the cytotoxicity of gossypol but also enhanced the antiviral activities of gossypol against HIV-1 and H5N1 (Yang *et al*., 2012; Yang *et al*., 2013). This study further indicated that amino acid derivatives of (-)-gossypol could bind to the gp41 hydrophobic pocket and blocked the formation of the cell fusion activated gp41 core to inhibit HIV-1 mediated membrane fusion and subsequent viral entry (An *et al*., 2012). Results of this study showed that the (+)-derivative was more active against H5N1 than the corresponding (-)- derivative. The anti H5N1 activity of chiral gossypol derivatives and its analogs depended on the availability of the phenolic groups and on the physicochemical properties of the substituents in the chiral gossypol derivatives and its analogs. Amino acids replacing the aldehyde group of chiral gossypol were more helpful to enhance the activity against H5N1 than the other substituents of the aldehyde group. With regard to the mechanism of action, chiral gossypol derivatives and its analogs could be novel entry inhibitors against H5N1, likely targeting to the HA2 protein. Gossypol is a natural phytochemical found in cotton seeds (*Gossypium* spp.) and *Thespesia populnea* and displays potential anti-cancer activities. Its antitumor properties have been studied in a variety of tumors (lymphoid, hematologic and solid tumors). Gossypol suppresses cell proliferation, induces autophagy and apoptosis in colorectal cancer, HT-29, HCT116 and RKO cancer cell lines (Lan *et al*., 2015). According to one study by Levin *et al*. (1987), cotton textile workers in Shanghai have a decreased risk of lung cancer. The reduction in risk was found among smokers and nonsmokers in men, and among light smokers and nonsmokers in women, and tended to affect cell types other than adenocarcinoma. The risks were not particularly low among workers with intense or long-term exposures to cotton dust. It is noteworthy that cotton dust has other components such as plant phenolics (NRC, 1982) that may inhibit carcinogenic responses (Mukhtar *et al*., 1984; Newmark, 1984). Also, proteolytic enzymes in cotton dust may stimulate protease inhibitors in bronchial mucus (Milton and Chawla, 1986), with potential anticarcinogenic activity (Yavelow *et al*., 1983). Finally, certain fatty acids found in the lungs of cotton workers might inhibit tumor growth through enhancement of cellular differentiation (Lynn *et al*., 1986).

Impact of *Bt* cotton on soil health

All available evidences show that there have been no adverse or significant effects on soil health in terms of soil biology and ecology by growing *Bt* cotton. In general, the Cry proteins released in root exudates and from plant residues of *Bt* cotton had no consistent, significant and long-term effect on the soil micro-flora. From the available literature, there is little evidence about crystal protein accumulation in soils, even after years of continuous *Bt* cotton cultivation. Based on the research from cotton-growing nations, there was no solid substantiation that points out adverse effects on soil health or fertility in terms of soil biology and ecology following cultivation of transgenic cotton. Comparing soils from the *Bt* and non-*Bt* cotton fields, some differences were evident with regard to the microbial community structure and their population. However, most of these observations were of a transient nature. Furthermore, since a majority of these studies were not statistically significant, it cannot be inferred that the differences were due to the inserted *Bt* transgene (Velmourougane and Blaise, 2017). In one study by Kumari *et al.* (2015), the effect of transgenic *Bacillus thuringiensis* (*Bt*) cotton residues on soil microbiological activity was investigated. Greenhouse study was carried out during the 2011 wet season (March to August) at Institute of Agricultural Sciences, Banaras Hindu University. It was experimented on three different soil orders that included entisol, inceptisol and alfisol. *Bt* cotton (var NCS-138) and its non-transgenic isoline (var.NCS-138) were grown until maturity along with one control treatment. Microbial population count, Dehydrogenage activity and Microbial Biomass Carbon (MBC) were estimated following standard protocols. The decomposition of cotton crop residues resulted increased micro-flora populations and microbial biomass carbon (MBC). When residue was retained, non- *Bt* cotton

showed higher populations of micro-flora as well as MBC that of *Bt*-cotton. Results from the study revealed that a significant reduction (7.5%) of the dehydrogenase activity was there in case of *Bt*-cotton. The interaction effect between soil type and varieties was found to be non-significant for the soil micro-flora populations for different sampling stages throughout the incorporation period. These results suggested that *Bt*-transgenic cotton tissues have no apparent effect on soil microbial activity.

Implications of *Bt* Crops on Mycotoxin Contamination

Fumonisins are produced by *Fusarium* spp. such as *Fusarium verticillioides* and *Fusarium proliferatum*. They are known to cause leukoencephalomalacia in horses and other equine species and pulmonary edema in swine. The Food and Drug Administration has set advisory or guidance levels for fumonisins in maize and maize products at 2−4 ppm (CAST Report, 2003; NTP Report, 1999). Maize plants stressed by heat and drought are susceptible to simultaneous infection by *A.flavus* and *Fusarium* spp., resulting in kernels contaminated with both aflatoxins and fumonisins. *Fusarium* spp. also produce other mycotoxins including zearalenone, which causes vulvovaginitis (hyperestrogenism) in swine, and deoxynivalenol (DON), which causes feed refusal, nausea and vomiting (CAST Report, 2003). The various studies carried out on an experimental level, both under artificial and natural conditions have shown a reduction in fumonisin content in the kernels of the transgenic hybrids compared to the corresponding isogenic control group (Masoero *et al.*, 1999; Munkvold *et al*. 1999; Pietri and Piva, 2000; Bakan *et al.*, 2002; Hammond *et al.*, 2004). The decrease in fumonisin concentration in the maize ear of the GM plant compared to the corresponding isogenic one, as demonstrated in the data obtained from the experimental fields, is due to the more significant correlation that exists between the ECB (European corn borer) infestation and the infection of *Fusarium verticillioides (moniliforme)* that is the principal producer of fumonisin. Indeed, the damage caused to the maize ear by the second generation of the ECB larvae is the preferred site of penetration of *F. verticillioides*, the infection of which is favoured by hot and damp climatic conditions during the phase from flowering to harvesting (the same conditions are also ideal for the infestation by ECB larvae). For this reason, when the damage caused by the infestation of *Ostrinia nubilalis* is the principal cause of penetration, the defense from such insects leads to a lowering in the concentration of fumonisin in the maize ear. The use of late hybrids and delays in harvesting are factors that favour this type of positive interaction.

Is cottonseed oil healthy?

Cottonseed contains hull and kernel. The hull produces fibre and linters. The kernel contains oil, protein, carbohydrate and other constituents such as vitamins, minerals, lecithin, sterols etc. Cottonseed oil is extracted from cottonseed kernel. Cottonseed oil, also termed as "Heart Oil" is among the most unsaturated edible oils. It need not be as fully hydrogenated for many a cooking purposes as is required in case of some of the more polyunsaturated oils (CICR Technical Bulletin, 2003).

Cottonseed oil is a commonly used vegetable oil that is derived from the seeds of cotton plants. A whole cotton seed contains about 15 to 20 percent oil. Cottonseed oil must be refined to remove gossypol. This naturally occurring toxin gives the oil, yellow color and protects the plant from insects. Unrefined cottonseed oil is sometimes used as a pesticide. This toxin has also been linked to infertility and liver damage. Cottonseed oil is used in cooking and is also used as a home remedy for certain skin conditions and ailments. Like olive oil, cottonseed oil is high in polyunsaturated fat which can help lower LDL ("bad" cholesterol) and increase HDL ("good" cholesterol). But, it is also contains saturated fat, which has the opposite effect on cholesterol and increases the risk of heart disease. Cottonseed oil contains high concentrations of vitamin E, fatty acids and antioxidants that have many benefits for the skin, including moisturizing, anti-aging and anti-inflammatory properties (www.healthline.com/nutrition/are-vegetable-and-seed-oils bad#bottom-line). Volate *et al* (2010) showed that gossypol reduced tumor growth and slowed or killed three prostate cancer cell lines. Animal and human studies have found that it prevented tumour growth and spread in some breast cancers.

Cottonseed Oil Quality

Fats and oils are made up of triglycerides, three molecules of fatty acids joined to a glycerol molecule. The chain length of the fatty acids and their organization on the glycerol backbone vary greatly, although in most of the edible oils it is with 16 and 18 carbons. Fats and oils are a combination of fatty acids, both saturated (C14:0, 16:0, etc.) and unsaturated (C 18:1, 18:2, 18:3). Some fats, such as lard, palm and coconut oils, have higher concentrations of saturated fatty acids than other oils and are referred to as saturated fats, even though they contain some percentages of unsaturated fatty acids. Cottonseed oil is among the most unsaturated oils, others being safflower, corn, soybean, rapeseed and sunflower seed oils. Cottonseed oil has a ratio of 2: 1 of polyunsaturated to saturated fatty acids and generally consists of 65-70% unsaturated fatty acids including 18-24% monounsaturated (oleic) and 42-52% polyunsaturated (linoleic) and 26-35% saturated (palmitic and stearic) (CICR Technical Bulletin, 2003).

Cottonseed Oil - Nutritional Aspects

Cottonseed oil is cholesterol free, as are all oils extracted from plants. Linoleic acid is the major polyunsaturated fatty acid found in cottonseed oil. With three times as much unsaturated as saturated fatty acid, cottonseed oil is considered as a healthy

vegetable oil and is one of the few oils advised for reducing saturated fat intake. Cottonseed oil is described by scientists as being "naturally hydrogenated" because of the levels of oleic, palmitic, and stearic acids in it. This renders it a stable frying oil without the need for additional processing that could lead to the formation of trans fatty acids. When it is partially hydrogenated, its monounsaturated fatty acids actually increase. When hydrogenated to a typical Iodine Value of about 80, its fatty acid profile changes to 50% monounsaturated, 21 % polyunsaturated, and 29% saturates all well within health guidelines. Another of cottonseed oil's benefits is its high level of antioxidants -tocopherols that contribute to its long life on the shelf. Studies show that these natural antioxidants are retained at high levels in fried products, preserving their freshness and creating longer shelf life. Cottonseed oil is often used as the standard for measuring flavour and odour qualities of other oils. Cottonseed oil has a mild taste. It is generally clear with a light golden colour, but like most oils, the degree of colour depends on the amount of refining. Clear, colourless oils are not necessarily better oils, but may have been refined more severely. Cottonseed oil is light, non-oily consistency and high smoke point make it most desirable for stir-fry cooking, as well as for frying (CICR Technical Bulletin, 2003).

IS *Bt*-COTTON TOXIC TO GOATS AND CATTLE?

Cry toxins have not been reported to be toxic to higher animals such as goats, sheep and cattle in any part of the world. Scientific evidence indicates that the possibility of Cry toxins killing goats and sheep is remote. The Cry toxins do not get activated under the acidic conditions of nontarget animals such as goat, sheep and cattle. Feeding studies did not show any toxicity symptoms that could lead towards extreme toxicity symptoms or mortality. A field study was carried out at CICR, Nagpur by a team of scientists led by a senior scientist of the Krishi Vigyan Kendra, for two years (2007-2009) by tethering six goats in one hectare of *Bt* cotton and one hectare of conventional cotton. The goats were fed on the crop continuously for four months and there were no differences in any biological aspects of the two sets of animals. The biochemical and health results clearly showed that *Bt* cotton was safe to goats (Kranthi, 2012).

EFFECT OF *Bt* COTTON ON LAB ANIMALS

Comprehensive biosafety studies were carried out by ICAR institutions with *Bt* cotton. First the safety of *Bt* Cry protein on lab animals such as rabbit, rat and guinea pigs. Various studies such as primary skin irritation test on rabbit, irritation to mucous membrane in rabbits, acute oral toxicity study in rats and skin sensitization study on guinea pigs were conducted. The results showed that *Bt* protein and *Bt*-Cotton seed powder were non-irritant to the skin of rabbits and vaginal mucus membrane. In case of acute oral toxicity study in rats, *Bt* cotton seed material did not induce any treatment related observable toxic effects when compared with Non-*Bt* cottonseeds. Studies on skin sensitization revealed that the repeated application of *Bt* cottonseed extract did not induce dermal sensitization (allergies) to the skin of any of the guinea pigs when compared with animals applied with extract of non-*Bt* cottonseeds (Kranthi, 2012).

EFFECT OF FEEDING *Bt* COTTON SEED MEAL TO BROILERS

Broiler chickens were tested by feeding of *Bt* cotton seed meal. This study was conducted at ICAR's Central Avian Research Institute, Izatnagar. Methodical studies were conducted with broiler chickens and tested for the effect of *Bt* protein. Birds were weighed at weekly intervals to observe weight loss or gain. After the 7^{th} week of study, 8 birds per treatment were sacrificed to study the effect of feeding CSM types on different carcass traits and development of digestive and immune organs. The results of the study revealed that the body weight gain and feed conversion efficiency, did not differ statistically over all phases of study. The protein and energy efficiencies of experimental diets fed to broiler chicken also remained statistically similar. The carcass traits (% of live weight) of broilers (blood loss, feather loss, dressed yield, eviscerated giblet, ready to cook yield and abdominal fat), cut up parts (breast, drum stick, thigh, back, neck, wings) and digestive and immune organs weights (heart, liver, gizzard, spleen, bursa) also remained statistically ($P<0.05$) similar to control. It was concluded that the solvent extracted *Bt* cottonseed meal can be included safely with maize or soybean meal based broiler diet up to 0-7 weeks of age (Kranthi, 2012).

EFFECT OF *Bt* COTTON ON FISH HEALTH

A systematic study was conducted with *Bt* cotton seed meal as a feed for Fish Common Carp and the side effects if any were tested in the fish food chain. This study was conducted at CIFE, Mumbai. A 60-day feeding trial was conducted on common carp fry. *Bt* cotton seed cake was included in the diet of common carp at 3 – level (10, 20, 30%) and compared with its non-*Bt* counterpart along with control group comprising of no cotton seed cake. Growth rate of *Bt* cotton seed cake fed group was comparable ($P< 0.05$) with that of control group and which and non-*Bt* counterpart as well. No mortality was found after feeding the *Bt* cotton cake, suggesting no adverse effect of *Bt* cotton seed cake (Kranthi, 2012).

FEEDING OF *Bt* COTTON SEED TO LAMBS

A trial was conducted at Central Sheep & Wool Research Institute (ICAR), Avikanagar for 120 days by continuous feeding on Weaner lambs at a higher plane of nutrition. Nutrient

(OM, CP and fiber fractions) and mineral (Ca, P, Mn, Co and Zn) contents were identical in *Bt*-cotton and non-*Bt* cotton seeds. The growth performance of lambs was similar on control, non- *Bt* cotton seed and *Bt* cotton seed included diets. The growing lambs consumed 168 g *Bt*-cotton seed per day and did not have apparent adverse effect on dry matter intake, nutrient utilization and nitrogen balance. Similarly, *Bt*-cotton seed intake of 0.681 % of body weight or 19.5 % of dry matter intake did not produce deleterious effect on performance and dry matter intake, thus palatability and growth performance was not a problem for *Bt* cotton seed feeding in lambs even under high plane of nutrition. Rumen fermentation characteristics *viz*, pH, TVFA and NH3-N concentrations was not influenced by feeding of GNC, non- *Bt* cotton seed or *Bt*-cotton seed in lamb diets. Heamatological observations did not change due to *Bt*-cotton seed feeding compared to non-*Bt* cottonseed or GNC feeding. Interestingly feeding of *Bt*-cotton seed increased RBC and decreased WBC in blood. Serum IgG level did not change due to *Bt* and non-*Bt* cotton seed feeding. Thus feeding of *Bt* cottonseed to lambs did not alter immunity and allergen status. Internal organs weights as g per kg empty live weight (ELW) indicated precise effect of *Bt* cottonseed feeding on internal organ changes. The weights of kidney, spleen, pancreas, heart, lung, penis, kidney fat, cole fat, GI tract, ingest and empty GI tract were not different among *Bt* cotton seed and non-*Bt* cotton. seed fed lambs. However, *Bt* cotton seed feeding increased liver weight, testicle weight and testicle fat g/kg empty live weight. The results were considered to indicate no detrimental effects (Kranthi, 2012).

EFFECT OF *Bt* COTTON ON LACTATING COWS

A comprehensive study was conducted with *Bt* cotton seed meal on milking cows. This study was conducted at NDRI, Karnal for four weeks. Sixteen crossbred (KS and KF) multiparous cows were adapted to test by feeding *Bt* cottonseed based diet. Mainly the *Bt* Cry protein side effect and absorption in the milk was tested. Milk yield and voluntary feed intake were recorded daily while milk samples were collected at the start of experimental feeding and thereafter at weekly intervals during the four weeks experimental period for the analysis of milk composition and to test for the presence of *Bt* protein. At the end, a blood sample from each cow was collected and plasma was separated to test for the presence of Cry 1Ac protein. Cry 1Ac protein in cottonseed, milk and blood samples was measured by ELISA method. The amount of Cry 1Ac protein in *Bt* cottonseed was 195.04 ng/g on fresh basis. Corresponding value in *Bt* concentrate mixture was 78 ng/g on fresh basis. Cows in both the groups improved their body weight during the study period and body weight gain in both groups was similar. Average milk yield during 28 days of experimental period in Non *Bt* (13.53 kg/day) and *Bt* (13.12 kg/day) groups did not vary significantly. During the experimental period the milk composition in terms of fat, protein, lactose, SNF and total solids content in *Bt* and Non-*Bt* were similar. Cry 1Ac protein was not detected in milk samples, drawn at 0, 7, 14, 21 and 28 day of feeding the experimental diet, as well as in plasma samples drawn on day 28 from the cows fed the *Bt* cottonseed based ration. Lactating dairy cows of both the groups did not show symptoms of any disease, maintained their health and performed in a similar fashion when fed with Non *Bt* and *Bt* cottonseed as a source of energy and protein supplement during the four week long experimental period. The present study results revealed that the Cry1Ac proteins were neither detected in the milk nor in blood of cows that were fed with *Bt* cottonseed during the four weeks trial. Further, there was no effect of *Bt* cottonseed containing Cry protein on milking cows. Hence, feeding of *Bt* cottonseed as a source of protein and energy in the ration of crossbred cows was considered to be safe and as nutritious as Non *Bt* cottonseed (Kranthi, 2012).

IS *Bt*-COTTON HARMFUL TO HUMAN BEINGS?

The Cry toxins Cry1Ac, Cry1Ab, Cry2Ab, Cry1F and Cry1C are considered to be safe to human beings. The stomach of humans, being the first organ of digestion the *Bt* protein encounters, is acidic and contains proteases like pepsin which degrade the *Bt* protein. Thus the alkaline conditions needed for pro-toxin solubilization and protease action required for toxin activation are absent in the stomach. More importantly, the human intestine lacks the specific receptors to which the activated *Bt* protein binds and initiates the physiological effect (Kranthi, 2012). There is a great need to experimentally prove the Bt cotton seed feeding to lactating animals and to clarify the apprehensions of the people about toxic material present in their milk using modern pathological techniques (Harit and Chauhan, 2020).

DOES *Bt*-COTTON IMPACT BIODIVERSITY THROUGH POLLEN-FLOW?

Cotton pollen is heavy and cross pollination happens mostly through pollinator insects such as honey bees and pollen beetles. Pollen-flow from *Bt*-cotton can contaminate non-*Bt* cotton varieties if compatible for crossing. However, the cultivation of GM *Bt* cotton hybrids, does not pose any risk to bio-diversity of naturally occurring Indian cotton or more specifically on the 'Western ghat biodiversity'. However, there is no possibility, whatsoever, of any of the native India Desi cotton species *Gossypium arboreum* and *Gossypium herbaceum* species getting genetically contaminated with GM *Bt*-cotton, so as to threaten the extant biodiversity. Desi cottons are diploid in their genetic constitution whereas the American cotton (*G. hirsutum*) is allo-tetraploid. Thus the Desi cottons species and the tetraploid cotton (all *Bt* cotton commercialized

in India are tetraploid cottons) are incompatible for cross-fertilization (Kranthi, 2012).

DOES *Bt*-COTTON DISRUPT ECOLOGY AND ENVIRONMENT?

Lu *et al*. (2012) showed that in the last 13 years GM crops delivered significant environmental benefits by reducing the insecticide usage by 50% and doubling the level of ladybirds, lacewings and spiders. Moreover, the study also stated that the environmental benefits extended to neighboring crops of maize, peanuts and soybeans.

Udikeri (2006) studied the dynamics of cotton aphids and predators in RCH 2*Bt* and non-*Bt* cotton hybrids. Laboratory feeding experiments using *Bt* and non *Bt* cotton were carried out to study the effect of *Bt* fed aphids on predator indicated no difference in incubation period, longevity of grubs and adults, fecundity and aphid consumption potential indicating safety of Cry1Ac to predator through intoxicated aphid host. Dong *et al*. (2003) reported only minor effects on some life table parameters in laboratory feeding studies with lacewings and predatory beetles and none with predatory bugs and spiders. There was some evidence of a reduction in numbers of predators and parasitoids which specialize on the *Bt* controlled bollworms, but also of increases in numbers and diversity of generalist predators such as spiders. A decrease in the parasitoid and predator populations can be associated with decrease in the densities of the pest populations on account of *Bt*-cotton.

DOES *Bt*-COTTON ENTER THE FOOD CHAIN?

The possible routes of *Bt*-cotton protein entering the food chain are, through human consumption of un-refined cottonseed oil, in which traces of *Bt* protein may be present with particulate seed residues or through consumption of meat or milk of the animals which fed on *Bt* cotton seed cake. However, ELISA tests showed that milk and meat were found to be free of Cry proteins. Thus the chances of *Bt* proteins entering the human food chain through milk and meat are low (Kranthi, 2012).

WHAT ARE THE BIOSAFETY TESTS CONDUCTED IN INDIA AND ABROAD?

A series of protocols have been formulated as pre-requisite for environmental release of genetically engineered (GE) plants in India. The bio-safety tests conducted in India and abroad are almost identical (Chauhan and Rana, 2010). These protocols include

1. Acute Oral Safety Limit Study in rats or mice
2. Subacute Feeding Study in rodents
3. Protein Thermal Stability
4. Pepsin Digestibility Assay
5. Livestock Feeding Study

These protocols address key elements of the safety assessment of foods and/or livestock feeds that may be derived from GE crops (Kranthi, 2012, Chauhan,1998; Singh *et al*., 2007; Telang and Chauhan, 2008).

WHO EXAMINES THE BIO-SAFETY TEST RESULTS FOR APPROVAL OF THE *BT* COTTON HYBRIDS?

India's regulatory system comprises of a three-tier mechanism. **Institutional Bio-Safety Committee (IBSC)** comprises of expert members and functions within the institution of technology developers. The IBSC periodically discusses and ratifies in-house proposals for GM experiments to be conducted within approved laboratories and contained greenhouses.

Review Committee for Genetic Manipulation (RCGM) comprises of 13 expert members with the Advisor DBT as member secretary, constituted by the Department of Biotechnology, Ministry of Science and Technology. The RCGM is empowered to approve (or not approve) applications for all small scale research activities in India designed to generate information on transgenic organisms. The RCGM also approves applications for experiments involving import of transgenic material (tissue, DNA, seeds, any other plant parts), limited field trials, bio-safety and toxicity studies. The role of RCGM extends to monitor the safety related aspects in respect of ongoing r- DNA projects & activities involving Genetically Engineered Organisms/Hazardous organisms and controlled field experiments of transgenic crops through the MEC (Monitoring and Evaluation Committee). The RCGM is actively involved in clearing and guiding public and private institutions in the development of transgenic crops and RDNA therapeutics

Genetic Engineering Appraisal Committee (GEAC) is a statutory body under the Ministry of environment and Forests (MoEF) The GEAC is the lead inter-ministerial body empowered to shape - by consensus - the Government's final disposition toward large-scale use and environmental release of GM organisms. The GEAC is chaired by the Additional Secretary of MoEF, co-chaired by an expert nominee from DBT, and it includes representatives from DBT, and other related Ministries. The GEAC examines applications for large scale field trials and commercial approval for transgenic crops and recommends decisions (Kranthi, 2012).

CAN THE FARMERS DETECT *Bt*-COTTON SEEDS OR PLANTS?

When '*Bt* cotton' was first commercialized on 5th April 2002 by Mahyco in India, the initial seed cost was high. 450 gms of the original *Bt* cotton seed costed Rs 1350 to 1650, About two years prior to the approval of *Bt*-cotton, illegal trade of *Bt*-

cotton seeds was happening in some parts of Gujarat. The illegal versions costed Rs 400 to 600. Lured by the low cost of fake *Bt* seeds, farmers would easily be trapped into buying them. But they would not know if the seeds really had the *Bt* gene in them. The *Bt* cotton seeds and plants look normal and it was not to separate them from normal seeds or plants. The Central Institute for cotton research Nagpur developed a simple test method like a litmus test paper that could tell the difference and identify the *Bt* gene in pure *Bt* cotton seeds within 10 minutes at Rs 10 per test. *Bt* cotton seed or leaf or tissue is taken. Then the tissue is crushed and the strip is dipped in 0.5 ml buffer taken in an Eppendorf tube. After 5 minutes, if 2 bands are coming in the strip, then the test is positive and when 1 band is appearing on the strip, then this test is negative. The test kits which can be used by farmers directly in the field and also at the shop to check the seed quality on the spot, were commercialized as '*Bt*-Express'. In the initial few years, from 2002 to 2005, hundreds of farmers mostly from Gujarat, Maharashtra and Andhra Pradesh used the kits at the sowing time to find out if the seeds they had purchased were genuine. The kits are in use now by farmers and seed testing laboratories across the country (Kranthi, 2012).

SOME OTHER STUDIES ON *Bt* COTTON

There are several reports that *Bt* genes cause some serious problem to human health. Bhat *et al*. (2011) studied the cytotoxic and genotoxic effects of *Cry1Ac* toxin from *Bt* cotton (RCH2) on human lymphocytes. The MIT test, cytokinesis blocked micronucleus and erythrolysis tests showed that high dose of *Cry1Ac* toxin decreased the cell survival ability up to 47.08% after 72 hour of incubation period. Only 2.52% of micronuclei were found in test samples. The *Cry1Ac* toxin also showed lethal effect on human leukocytes by their haemolytic action.

It was concluded that *Cry1Ac* toxin at higher concentration have lethal cytotoxic and genotoxic effects on the human lymphocytes.

Bt COTTON RELATED APPREHENSIONS

It is only in India that apprehensions were expressed by NGOs regarding sheep mortality at Warangal and Adilabad district of Andhra Pradesh due to grazing in *Bt* cotton fields. The issue was examined by the State Government and reports received from the Directorate of Animal Husbandry, Hyderabad and the Indian Veterinary Research Institute, Izatnagar, U.P. revealed that the sheep deaths might be due to high content of Nitrates/Nitrites, residues of hydrocyanide (HCN) and organophosphates which are common constituents of pesticides used during cotton cultivation and not due to *Bt* toxin (Kranthi, 2012).

CONCLUSION

Cotton is a commercial crop produced for its fiber which is universally used as a textile raw material. Cotton is a warm-weather shrub of Malvaceae family and the genus Gossypium grows naturally as a perennial. Gossypol is a yellow pigmented phenolic compound present in various parts of cotton plant. The ingestion of gossypol present in cottonseed and its products (cakes and meal) may promote clinical poisoning, liver damage, male and female reproductive toxicity and immunological impairment. The immunotoxicity of gossypol impacts animals by reducing their resistance to infections and by impairing the efficiency of vaccines. Extensive research is needed to develop more efficient and inexpensive technologies to reduce gossypol toxicity. Acreage and popularity of *Bt* cotton is increasing day by day as it plays a vital role to provide durable resistance against a wide range of insect species. Besides this, it significantly reduces small-molecule insecticide use for target pests controlled by *Bt* proteins. It reduces greenhouse gases emissions by minimizing field spraying with self-propelled sprayers or other motorized equipment. It also potentially decreases fumonisin levels in maize grain by reducing mycotoxin contamination of grain. Main purpose of introducing *Bt* cotton is to control bollworms. Other benefits include reduction in insecticide and pesticide usage, elimination of bollworm threats and enhancing seed cotton quality.

Bt cotton has played important role to sustain agriculture in all over the world for their maximum yield and other agronomic practices as well. The feeding of *Bt* cotton seed to animal has not been reported to have any adverse effect. Seed of *Bt* cotton and its cake do not have any adverse effect on digestion of animals. No allergic or toxic effect of use of *Bt* cotton seed and meal has been reported. Oil extracted from the seed of *Bt* cotton has not been found to have any adverse effect on human health. No adverse effect of *Bt* cotton on the environment has been reported by any of the countries where *Bt* cotton is commercially cultivated. However, with the passage of time, several biosafety and environmental issues arise with the use of different *Bt* genes. Several researchers have reported the toxic effects of *Bt* proteins of cotton and other crops on diverse range of non-target animal species including human being. Some findings revealed that *Bt* toxins affect human lymphocytes and other physiological characters when used in higher concentration. Now, it is the responsibility of the scientists and other concerned persons to bring awareness in people to develop new *Bt* cotton cultivars that assure no or very low toxicity on non-target organisms to minimize risks associated with *Bt* cotton technology.

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