Agriculture Issues and Policies

Bacillus thuringiensis

Cultivation, Applications in Agriculture and Environmental Safety

David P. Sanders
Editor

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DAVID P. SANDERS
EDITOR

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Chapter 2

HARNESSING THE POTENTIAL BENEFITS OF *BACILLUS THURINGIENSIS* FOR MANAGEMENT OF INSECT PESTS OF CASTOR (*RICINUS COMMUNIS L.*)

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ABSTRACT

The wonder bacterium *Bacillus thuringiensis* (Bt), since its discovery in 1901, has been extensively studied for harnessing its potential for management of insect pests. Bt is considered the most successful microbial insecticide and occupies more than 90% share in the microbial insecticide market. In India, oilseeds are cultivated primarily in dryland areas and their cultivation is constrained by several insect pests including foliage feeders, sap sucking pests, capsule borers, etc. Research efforts have been directed towards exploitation of Bt by using the whole organism to develop sprayable formulations as well as utilizing its cry genes in genetic engineering of crops for management of the major lepidopteran pests of

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oilseed crops like castor, sunflower, soybean, etc. Virulent local isolates of Bt have been isolated, identified, characterized, and utilized in development of formulations for field use. Cost-effective protocols for mass production of the virulent isolates have been developed employing solid state fermentation technique for enabling and promoting indigenous Bt production with low capital investment. Efficacy of the Bt formulations against major lepidopteran pests of oilseeds has been validated through multi-location testing under the All India Co-ordinated Research Projects (AICRPs) of the Indian Council of Agricultural Research (ICAR). Data for registration has been generated in accordance with the guidelines of the Central Insecticides Board (CIB), GOI and the technologies have been licensed and commercialized to the Indian bio-pesticide industry. This article describes the bottom-up-approach being followed to use Bt as a biopesticide and also to enhance host plant resistance against major foliage feeders through deployment of suitable cry genes.

Keywords*: Bacillus thuringiensis*, *cry* genes, genetic engineering, castor, pest management

INTRODUCTION

Agriculture along with its allied sectors constitutes the primary occupation and livelihood for 58% of Indian population and contributes to 15.4% India's Gross Domestic Product. Limited land resources, population increase and increased demand for food necessitate minimizing the crop losses due to insect pests. On-farm yield losses range from 10-30% while losses in oilseeds alone during harvesting, handling and storage are around 10% (Varaprasad and Duraimurugan, 2016). Management of several insect pests with microbial biopesticides based on fungi, bacteria, viruses and nematodes or their bioactive compounds has been gaining momentum in India due to their reduced environmental toxicity, target specificity and safety to non-target organisms (Kumar et al., 2019).

Bacillus thuringiensis (Bt) is the most popular and successful insect pathogen with over a century of history as a potent biocontrol agent for management of insect pests of agriculture, forest ecosystems and human vectors. Bt is a ubiquitous, gram positive sporulating soil bacterium that forms insecticidal crystal proteins during sporulation phase of its growth

cycle which cause specific toxicity in insects. Bt was first reported by Ishiwata during 1901 in Japan and named as *Bacillus sotto*. Ernst Berliner rediscovered it ten years later in 1911 in the province of Thuringen, Germany, from a diseased caterpillar of flour moth and classified it as type species *Bacillus thuringiensis*. The proteinaceous crystal production within the sporangium was detected by Hannay in 1953 who proved that the crystal protein was toxic to insects. This toxin was named as δ-endotoxin by Heimpel in 1967. The first commercial Bt product Sporeine became available in France during 1938. In the 1950s, widespread use of biocontrols began to take hold in USA as a host of research on Bt efficacy was published. More than 818 Bt toxins have been reported world-wide. There is still a continuous search for novel Cry proteins with toxic potential against various insect pests. Bt accounts for about 5–8% of *Bacillus* spp. population in the nature. Currently, the commercial utilization of Bt includes marketable pesticidal formulations as well as development of insect-resistant genetically modified crops employing the genes encoding the insecticidal toxins of this bacterium making several million dollars in global market. This has also resulted in significant lowering of usage of chemical pesticides coupled with the increase and survival of beneficial and non-target organisms.

Widespread adoption of cheaper but more toxic synthetic chemical insecticides in the second half of 20th century kept the research and development on Bt at a low ebb. An industrial process known as submerged fermentation, which allowed production of Bt on a large scale was developed by the Pacific Yeast Product Company that gave a fillip to development and application of new products of Bt, principally in niche markets where petroleum-based chemicals were not registered, ineffective, or uneconomical.

Harnessing the potential of Bt for effective pest management necessitates identification of virulent isolates, development of protocols for their cost-effective production along with suitable formulations with an extended shelf-life at ambient temperatures. Extensive studies have been undertaken world over for detecting the presence of Bt in diverse ecological habitats *viz*., soil, stored product dust, dead insects, food grains, phyllosphere, and aquatic environments. A vast distribution and diversity of

Bt strains bearing putative genes with specific toxicity spectrum is reported from various habitats of India including rice fields ecosystems, Andaman and Nicobar Islands, desert soils of Rajasthan, phylloplanes of crops grown in Delhi, Western Ghats of Karnataka and Tamilnadu, Hill zone soils of Karnataka and as a whole from Southern, Northern states and North eastern states of India (Kaur and Singh, 2000; Asokan and Puttaswamy, 2007; Ramalakshmi and Udayasuriyan, 2010, Vimala Devi and Vineela, 2016; Subbannaet al., 2017). However, the efforts in India have been minimal reporting primarily new isolates of Bt as well as effective isolates against some insect pests like *Helicoverpa armigera* and *Spodoptera litura* through laboratory bioassays (Lalitha et al., 2012; Lone et al., 2017).

Castor (*Ricinus communis* L.) (Malpighiales: Euphorbiaceae) is an industrially important non-edible oilseed crop and its oil is used in the manufacture of several industrial products like lubricants, paints, plasticizers, pharmaceuticals, cosmetics, soaps, biopolymers, biodiesel, etc. (Ogunniyi, 2006; Shrirame et al., 2011). India, Mozambique, China and Brazil are the major castor growing countries in the world (http://www.fao.org/faostat/). India accounts for 79% and 87% of the world's castor area and production, respectively meeting about 90% of the world's castor oil requirement. Though castor productivity in India is more than world average, there are several production constraints. One of the major constraints is the excessive damage caused by insect pests. Among them lepidopteran foliage feeders *viz*., semilooper (*Achaea janata* L.), tobacco caterpillar (*Spodoptera litura* F.) and capsule borer (*Conogethes punctiferalis* Guen.) are of greater economic importance. It is estimated that castor yields are reduced by 17.2 to 63.3% due to the insect pests (Lakshminarayana and Duraimurugan, 2014). The ICAR-Indian Institute of Oilseeds Research (ICAR-IIOR), Hyderabad, India adopted a participatory bottom-up approach. Accordingly, research was initiated wherein the major problem of crop damage due to foliage feeders under rainfed cultivation of castor by resource poor farmers were addressed in Bt technology development right from identification of the effective strain to mass production through solid-state fermentation (SSF) and product development for commercial use through formulations of virulent Bt strains as well as

genetically engineering with toxin coding genes. We present hereunder an overview of the efforts embarked upon and the outcome thereof towards exploiting Bt for the management of lepidopteran insect pests in castor.

DEVELOPMENT OF BT AS A BIOPESTICIDE

Development of Sprayable Formulations of Bt

Research efforts for identification of virulent Bt isolates were initiated at ICAR-IIOR during 1998 with collection of soil samples from top soils at 5 cm depth from cultivated and fallow fields from Mahabubnagar, Nalgonda and Karimnagar districts of Telangana; Guntur and Bapatla districts of Andhra Pradesh; Nagpur in Maharashtra; Dharwad in Karnataka and various districts of Rajasthan. Selective isolations by the sodium acetate method of Travers et al. (1987) yielded around 200 Bt isolates. These isolates were multiplied in nutrient broth and the resulting Bt powders were screened through laboratory bioassays against major lepidopteran pests namely *H. armigera*, *A. janata*, *S. litura*, *S. exigua* and *S. frugiperda* and the potent isolates were identified (Vimala Devi et al., 2001; 2005; Vimala Devi and Vineela, 2016). These isolates were subjected to molecular characterization through PCR using gene specific primers as well as rep-PCR (Reddy et al., 2012a, b; Vimala Devi and Vineela, 2016). The next step was development of low-cost mass production protocols. Bt is an aerobic bacterium with specific growth requirements of pH 7.2-7.5 and temperature 30 ^{0}C for optimal growth, sporulation and toxin production. Hence, Bt production has been in the domain of multinational companies and traditionally multiplied through submerged fermentation requiring high capital investment thereby resulting in high cost of production. However, technology adoption in a developing country like India necessitates development of protocols that make the mass production economically feasible, so that these new technologies can compete in the open market.

Biopesticides production in India is being promoted commercially by medium range entrepreneurs. However, Bt production was not undertaken

by them due to high capital investment for production as well as the difficulty in generating data for registration. In order to bring Bt in the reach of the average Indian farmer, a novel, simple, low-cost mass production methodology for Bt on the principle of solid-state fermentation (SSF), eliminating the need for a fermentor, was developed at ICAR-IIOR during the year 2002. One local isolate DOR Bt-1 belonging to *B. thuringiensis* var. *kurstaki*, isolated from a dead castor semilooper larva collected from farmers' fields at Kothakota, Mahabubnagar district of Telangana, was identified for the mass production. This process of mass production of Bt was carried out utilizing easily available and inexpensive media ingredients (Vimala Devi et al., 2005). Bt obtained through this process was formulated as a wettable powder and used for further studies. The process does not pose contamination problems and can be carried out effectively in a routine manner even by less skilled/unskilled people. All the growth requirements were addressed in the development of the production process through SSF. This was the first report of enabling Bt production through solid-state fermentation, without employing a fermenter, using agricultural wastes/byproducts like wheat bran and molasses. This methodology had the potential to enable large-scale localized production of Bt through establishment of cottage industry/micro-enterprises (Vimala Devi and Rao, 2005a; b). Based on this protocol, other virulent isolates were also multiplied against important pests.

Formulations developed at ICAR-IIOR include wettable powder (WP) formulations of two Bt strains DOR Bt-1 and 5, a suspension concentration (SC) formulation of DOR Bt-127, a water dispersible granule (WDG) formulation of Bt-127, two combination SC formulations of Bt with the entomopathogenic fungi *Beauveria bassiana* and *Nomuraea rileyi* (Vimala Devi et al., 2020; 2021).

Evaluation of Efficacy

All the formulations were evaluated through laboratory bioassays and field trials at ICAR-IIOR as well as farmers' fields (Vimala Devi and

Sudhakar, 2006; Vimala Devi et al., 2020). Data on LC_{50} and potency was generated against *H. armigera*, *S. litura* and *A. janata*. These formulations were then evaluated through All India Co-ordinated Research Projects (AICRPs) against *A. janata* and *S. litura* on castor, *H. armigera* and *S. litura* on sunflower, *S. litura* on soybean as well as *H. armigera* on pigeonpea. DOR Bt-1 formulation was found to be effective for control of castor semilooper @ 1.0 g/l, @ 2.0 g/l against pod borer in pigeon pea and head borer in sunflower while DOR Bt-127 SC formulation was found effective against *S. litura* and semiloopers in soybean as well as against head borer in sunflower @ 3.0 ml/l in multilocation trials under AICRPs and was found superior to the commercial Bt formulation Delfin WG that is overly expensive at Rs. 4000/- per kg.

Outreach Initiatives

ICAR-IIOR took the initiative of reaching out to farmers for creative awareness about the importance of the technology and facilitates its adoption by them. Outreach activities at Kothakota and Nallavelli villages included on-farm training programmes about the use and efficacy of Bt against lepidopteran larvae, its mode of action and safety to the natural enemies *viz*., parasitiods and predators. The trainings included identification of the various stages of the pests right from egg to the late larval instars as well as the various parasitoids. Emphasis was laid on undertaking the Bt sprayings when the larvae were in the early stages.

Another outreach initiative was the conduct of demonstrations with the formulations in farmers' fields. Each farmer was provided the IIOR Bt formulation for one acre and another acre was maintained as per his/her practice for pest management. Thus, farmers were made to observe the differences in between Bt sprayed fields and insecticide sprayed fields *viz*., natural enemies, frequency of sprays, yield etc. (Vimala Devi and Sudhakar, 2006).

The SSF technology for Bt production had the potential to enable large-scale localized production of Bt through establishment of cottage

industry/micro-enterprises. Hence, two microenterprises were established in association with NGOs - one with Society for Development of Drought Prone Area (SDDPA) and second with Grameena Mahila Mandali (GMM) at Mahabubnagar and Nalgonda districts, respectively. High school dropout boys and girls were trained in Bt production and the formulation was supplied to farmers for use in field for pest management. This initiative helped in faster spread of technology among the farmers and its successful adoption.

Intellectual Property Rights (IPR)

Intellectual property (IP) refers to creations of the mind, such as inventions; literary and artistic works; designs; and symbols, names and images used in commerce. The IP Rights usually give the creator an exclusive right over the use of his/her creation for a certain period. It was for the first time that a protocol was developed for Bt production through SSF. Since the Bt SSF technology had a commercial value, IP protection became utmost important. A provisional patent application for the process was filed by ICAR in 2002 and the complete patent application in 2003.

Development of stable formulations of Bt in combination with entomopathogenic fungi by IIOR was the first report. A patent has been granted for the process (Indian patent no. 315134). Bt and entomopathogenic fungi have diverse modes of action thereby creating more stress on the target insect since Bt acts through per ingestion while fungi infect the insect through the cuticle. This in turn leads to faster kill of the larvae and effective against older larvae as well.

Registration with Central Insecticides Board (CIB)

The import, manufacture, sale, transport, distribution and use of microbial pesticides in India is regulated under the Insecticides Act, 1968 and rules framed there under. Registration of bio-pesticides in India was

approved around the year 1998. Biopesticides of botanical and microbial origin were included in the schedule of the Insecticide Act 1968.

Since micro-enterprises were established for enabling localized production and sale of the DOR Bt-1 W.P. formulation to the farmers in Mahabubnagar and Nalgonda districts of Telangana, registration of the formulation became essential. DOR Bt-1 formulation was therefore registered provisionally with the CIB in 2005 under the trade name KNOCK.W.P. [Registration No. CIR–511/2005(256)] under section 9(3b) with the Central Insecticides Board, Govt. of India, and was the first formulation of a microbial pesticide from the Indian Council of Agricultural Research, registered for commercial use and also the first from public sector.

Technology Licensing

Medium range entrepreneurs promote commercial production of biopesticides in India. However, the high capital investment for Bt production as well as the difficulty in generating data for registration did not enable commercial production and promotion of Bt. Data generation by the IIOR for the purpose of registration encouraged several medium range firms to approach IIOR and request for licensing of the technology during the year 2003. IIOR started licensing the Bt production and formulation technology to the bio-pesticide industry in India from July 1, 2006. The technology package included DOR Bt-1 strain along with data for provisional registration and training in the production technology through SSF. The technology has been licensed to more than 40 biopesticide firms in India. This initiative enabled the firms to seek registration of the formulation with the Central Insecticides Board and undertake its commercial production. Majority of the firms who received Bt-1 license, obtained the provisional registration and sold their Bt formulations under different trade names *viz*., Cezar, Caterpillin, JAS BT, VBT, R.B. Bt, Prasar, Dipole, Beater etc. Companies have now started receiving permanent registration. Thus, DOR Bt technology has successfully reached the end user *i.e*., the average Indian farmer and is being used in insect pest management on several crops.

Access and Benefit Sharing

ICAR-IIOR was the first institute to set an example in access and benefit sharing in accordance with the Biodiversity Act, 2014. IIOR shared 3% of the licensing fee with Biodiversity Management Committee (BMC) at Kothakota, Mahabubnagar district through the Telangana State Biodiversity Board (TSBB). The BMC was constituted by TSBB at Kothakota since the DOR Bt-1 isolate was obtained from that village. BMCs have the responsibility of using these funds for betterment of the respective village including creation of awareness on conservation of biodiversity. For this initiative, IIOR received the prestigious UNDP award as runner up under the category "Successful Mechanisms/Models for Access and Benefit Sharing" at the "India Biodiversity Awards 2016."

GENETIC ENGINEERING THROUGH DEPLOYMENT OF BT*CRY* GENES

One of the strategies for genetic enhancement of resistance to insect-pests is development of transgenic plants through incorporation of suitable insect resistance candidate genes (Sharma et al., 2000). Transgenics are imperative for agriculturally important crops and the 113-fold hectare increase between 1996 and 2018 makes biotech crops the fastest adopted crop technology in agriculture. Of the global biotech area of 192 million ha, genetically engineered (GE) varieties expressing one or several insecticidalgenes from Bt were grown on a total of 101 million hectares worldwide,reaching adoption levels above 80% in some regions (ISAAA, 2017; 2018; Romeiset al., 2019). With the advent of genomics, several genes in the fatty acid metabolic pathways and triacyl glycerol assembly; genes controlling the noxious proteins ricin and RCA and disease resistance genes have been characterized. Hence, there is vast scope for genetic engineering of castor so as to mitigate the damages caused by the lepidopteran insect-pests and improve castor for seed quality traits.

Identification of Candidate Bt Proteins Effective Against the Foliage Feeders

Host plant resistance is the most desirable option for insect control. Development of transgenics for resistance against lepidopteran insect pests would facilitate the development of genotypes resistant to these biotic stresses through breeding programmes. Due to narrow genetic resources conferring resistance to insect pests in castor, there is a need for utilizing the biotechnological tools for alien gene transfer. Before embarking on the programme of genetic engineering, it is vital to identify suitable insect resistance genes, which could be deployed into castor against the major pests. Several candidate genes, such as crystal protein (Cry) genes of Bt produced during the sporulation stage, vegetative insecticidal Bt proteins (VIPs) induced during the vegetative stage, proteinase inhibitors, lectins, α-amylase inhibitors, insect chitinases, novel genes of plant origin, etc., are deployed into crop plants for imparting protection against insect-pest. However, the most commonly used and commercially exploited insect resistance genes are the Bt*cry* genes from Bt.

Insecticidal δ-endotoxins of Bt have acquired great significance because of their specificity to target pests, non-toxicity to humans and beneficial insects, toxicity at low concentration and environmental friendly nature. It is known that Btvar*kurstaki* (Btk) strains produce several lepidopteran toxic proteins such as Cry 1Aa, Cry 1Ab, Cry 1Ac, Cry IIAandCry 1B. Information on the reaction of the major lepidopteran pests attacking castor to Cry proteins in the toxin specificity database (http://wmv. glfc.forestry.ca/bacillus/web98.adb) is limited. Studies were carried out at ICAR-IIOR to assess the efficacy of various purified crystal Bt proteins which are lepidopteran pest specific against major defoliator pests of castor. Bioassays were done against neonate larvae of semilooper (*A. janata*), tobacco caterpillar (*S. litura*), hairy caterpillars (*Spilosoma obliqua* and *Euproctis fraterna*) using Cry toxins (Cry1Aa, 3A, 2B, 1C, 2A, 1E, 1Ac, 1F, 9A, 1Ab) at concentrations ranging from 4 to 1500 ng/cm^2 using leaf paint assay. With regard to semilooper, the proteins 1Aa, 1Ab, 1E and 2A were most effective, resulting in 100% within 48 hours while the other proteins

gave no or delayed mortality at the highest concentration tested. Among the effective proteins, Cry1Aa was found to be superior to other proteins in giving early mortality even at lower concentrations (125 ng/cm^2) (Sujatha and Lakshminarayana, 2005). In case of tobacco caterpillar, none of the proteins gave 100% mortality even 96 hours after treatment at the highest concentration (1500 ng/cm^2) tested except for Cry1Aa which gave 50% mortality at 1500 ng/cm^2. Increasing the concentration of the proteins up to 3000 ng/cm^2 also failed to cause larval mortality. However, feeding cessation in terms of low larval weight was recorded in treatments with Cry1Aa and 1Ab (Lakshminarayana and Sujatha, 2005). The proteins 1Aa, 1E, 1Ab were effective against Bihar hairy caterpillar, *S. obliqua* while 1Ac, 1Aa were effective against hairy caterpillar, *E. fraterna*. Based on these studies, the genes *cry1Aa, cry1Ab, cry1Ac* and their fusion genes were identified for deployment into castor for conferring resistance to the foliage feeders.

The genes deployed in castor were *cry1AcF* (Manoj Kumar et al., 2011); *cry1Aa, cry 1Ec* (Sujatha et al., 2009) and *cry1Ab* (Malathi et al., 2006). The *cry1EC* and *cry1AcF* genes are deployed for conferring resistance to *S. litura*, while c*ry1Aa* and *cry1Ab* were used for *A. janata*. Keeping in view the superiority of Cry1Aa protein against all the target pests, genetic transformation of castor has been initiated using the respective gene with plant codon usage. *S. litura*, a polyphagous lepidopteran insect pest on castor is reported to be tolerant to most of the known δ-endotoxin proteins. Hence, a hybrid δ-endotoxin protein of Cry1Ea and Cry1Ca was developed by replacing amino acid residues 530-587 in a poorly active natural Cry1Ea protein with a highly homologous 70 amino acid region of Cry1Ca in domain III and was designated as Cry1Ec (Singh et al., 2004). The solubilised Cry1Ec made from *E. coli* was 4-fold more toxic to the larvae than Cry1Ca, the best known δ-endotoxin against *Spodoptera* sp. The hybrid endotoxin conferred complete protection against *S. litura* when deployed in tobacco and cotton. Based on these studies, the genes *cry1Aa, cry 1Ab* and *cry 1Ec* were selected to be deployed in castor for conferring protection against the major lepidopteran foliage feeders.

Tissue Culture and Regeneration in Castor

Availability of an efficient and highly reproducible system of tissue culture regeneration is a prerequisite for genetic transformation experiments. Castor proved to be highly recalcitrant to manipulations *in vitro* which is a major bottleneck in the development of transgenic castor (Sujatha et al., 2008). Earlier studies during 1960s were confined to endosperm culture and the interest was mainly due to the large endospermic seeds that enabled easy culturability. However, these resulted in continuously growing cultures which lacked the ability for organogenic differentiation. Tissue culture studies were once again undertaken by researchers during 1980s for obtaining whole plantlet regeneration from seedling tissues of castor. Experiments were restricted to the use of young seedlings and the ability to regenerate complete plants was rather limited. Plant regeneration was mainly from the pre-existing meristematic centers (Athma and Reddy, 1983; Reddy and Bahadur, 1989; Molina and Schobert, 1995; Alamet al., 2010), and a maximum of 40 and 47 shoots from embryo axes and shoot tip explants, respectively, was reported (Sujatha and Reddy, 1998). Callus-mediated shoot regeneration from hypocotyl explants, young stem segments, leaves and cotyledonary leaves are reported but the morphogenic differentiation was sporadic, unreproducible with very low frequency of shoot regeneration and few shoots (1-5) per responding explants (Reddy et al., 1987; Genyu, 1988; Bahadur et al., 1992; Sarvesh et al., 1992). Subsequently, Ahn et al., (2007), Sujatha and Reddy (2007) and Ganesh Kumari et al., (2008) have reported relatively higher shoot induction frequencies from seedling explants with around 22 to 24 shoots per explant and the use of growth adjuvants and amino acids for improving the caulogenic ability. Preincubation of cotyledon explants from mature seeds cultured on medium with 5 µM thidiazuron (TDZ) in dark for seven days resulted in a maximum of 25 shoots per explant (Ahn and Chen, 2008). Nevertheless, the reproducibility of these methods across laboratories and different genotypes is rather limited.

Owing to the caulogenic response of cultured explants of castor, efforts were made at refinement of protocols for meristem based proliferation. Of

the meristematic explants tested, embryo axes possessed high proliferative ability on TDZ supplemented media when compared to shoot apices and nodal explants (Sujatha and Reddy, 1998). This protocol using mature seed derived embryo axes was used for genetic transformation of castor (Sujatha and Sailaja, 2005; Malathi et al., 2006). To unravel the reasons for recalcitrance of castor tissues, transcriptomic profiles of cultured hypocotyl tissues of castor were compared with that of jatropha and sunflower which have great prosperity for regeneration (Sai Sudha et al., 2019). Differential gene expression analysis indicated downregulation of genes involved in auxin biosynthesis and homeostasis, vacuolar transporter genes, Wuschel gene, shoot root like transcription factors and histidine containing phosphotransfer proteins while genes like DELLA and brassinosteroid LRR receptor kinases were upregulated resulting in regeneration recalcitrance. There is a need for assessment of a large number of genotype and growth regulator combinations for determining the caulogenic ability and also to draw valid conclusions about the recalcitrance of castor tissues *in vitro*. Till such time a highly efficient and reproducible system of plant regeneration is developed, genetic transformation experiments have to rely on meristem-based shoot proliferation system.

Development of Transformation Protocols

Castor leaves are susceptible to crown gall disease caused by *Agrobacterium tumefaciens* (Lippincott and Haberlein, 1965). However, recalcitrance *in vitro* has been a major problem for undertaking plant transformation experiments in castor. McKeon and Chen (2003) obtained genetically engineered plants by employing the method of *Agrobacterium*-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6.620.986). The first successful attempt to develop a stable transformation system for castor using embryo axes from mature seeds has been described by Sujatha and Sailaja (2005). The protocol exploits the proliferative ability of embryo axes on TDZ (0.5 mg/l) supplemented media followed by selection on appropriate antibiotic

multiplication and elongation on media supplemented with BA (0.5 and 0.2 mg/l). Elongated shoots were rooted on a media fortified with 200 mg/l NAA. A similar shoot proliferation method with minor modifications has been adopted for direct gene transfer using particle gun bombardment method (Sailajaet al., 2008). Malathi et al., (2006) developed *Agrobacterium* mediated method while Kumar et al., (2011) optimized *in planta* method of transformation for deployment of *cry* genes. The several transformation studies for genetic transformation of castor continue to rely on meristem-based proliferation (Chen et al., 2013; Patel et al., 2015; Sousa et al., 2017).

Development of Transgenic Events

The vector-mediated and direct gene transfer methods were employed for transformation of castor, DCS-9 (Jyoti) using appropriate vectors containing the Bt fusion gene *cry1EC* driven by enhanced 35S promoter (Sujatha and Sailaja 2005; Sailaja et al.,2008; Sujatha et al.,2009). About 93 putative transformants were regenerated following selection on hygromycin and kanamycin. The integration, inheritance and expression of the introduced genes was demonstrated up to T_4 generation by PCR, Southern analysis and ELISA analysis. Field bioassays against *S.litura* and *A. janata*, conducted for eight events in T_1 to T_4 generations under net confinement conditions resulted identification of promising events bestowed with resistance to the two major defoliators. The same procedure with minor modifications was used for production of semilooper resistant transgenic castor by incorporating a synthetic δ-endotoxin *cry1Ab* gene driven by CaMV 35S promoter which recorded feeding inhibition, reduced larval growth and 88.9 to 97.3% mortality of *A. janata* as compared to the untransformed control plants (13.9%) (Malathi et al., 2006). The construct harbouring the insect resistance gene carried the herbicide resistance gene (*bar*) for selection of putative transformants. The presence of introduced gene, its stable integration, expression and inheritance was confirmed through PCR, Southern analysis, ELISA and progeny tests. However, characterization of transgenics harbouring the *cry1Ab* was confined to the

analysis of T_1 generation plants, and their reaction to the target pest was evaluated under laboratory. Transgenic castor with *cry1AbcF* gene against *S. litura* resulted 22.7 to 96.6% mortality (Kumar et al., 2011).

Bioassays and Field Evaluation for Event Selection

Among the four studies where transgenic events were developed, event selection was carried out with stable transgenic lines harboring the *cry1Aa* and *cry1Ec* genes for imparting insect resistance. Events harboring *cry1Ab* and *cry1ACF* genes were subjected to laboratory bioassays against *A. janata* and S. *litura*, respectively, while the events harboring *cry1Aa* and *cry1Ec* genes were tested for their level of resistance to the two foliage feeders both under laboratory and field conditions. Characterization of the transgenic castor events with the *cry1Aa* gene against lepidopteran insect pests in laboratory bioassays revealed that the mortality of *S. litura*and *A. Janata* ranged from 20 to 80% in different transgenic events and the weight reduction of surviving larvae over the control larvae after 8 days of feeding was 28.4–87.2% in the case of *S. litura* and 27.9–78.1% for *A. Janata* (Tarakeswari et al., 2019). In different events carrying the *cry 1Ec* gene, average mortality in laboratory bioassays against *S. litura* was 20–40% and 30–70% against *A. janata*. Reduction in larval weight of the surviving larvae at 8 days after treatment was 20–70% in case of *S. litura* and 20–80% for *A. janata* (Sujatha et al., 2009). For assessing the reaction in the field, heavy incidence of both the major foliage feeders was ensured by encaging the experimental plots with nets and encouraging natural built up of *A. Janata* and through artificial releases of *S. litura*. Under such high level of pest load of both the pests, the untransformed control (DCS-9) was completely defoliated including spike damage, while the events harboring the *cry* genes (*1Aa* and *1Ec*) showed defoliation less than 25% leaf damage. Transgenic events displayed significant variations with regard to the level of defoliation based on the crop growth stage, generation in which they were subjected to the bioassays which was substantiated through protein expression analysis using ELISA. Events harboring *cry1Ec* gene *viz*., NBRI-PB-1, PCP 202-

AMT-1 and PCP 202-AMT-9 and with *cry 1Aa* genes *viz.,* AMT-894, AK1304-PB-1, AK1304-PB-4, and DTS-43) were found to be promising. As castor can tolerate defoliation up to 25%, the genes could be transferred to other agronomically superior genotypes or stacked together and tested for the bioefficacy of the introduced gene(s) in different genetic backgrounds.

Biosafety Concerns

Over the past 20 years, extensive laboratory and field-based studies of the non-target effects of crops producing Cry proteins revealed that insecticidal proteins used in commercialized Bt crops cause no direct, adverse effects on non-target species outside the order (i.e., Lepidoptera for Cry1and Cry2 proteins) or the family (i.e., Coleoptera, Chrysomelidae for Cry3 proteins) of the target pest(s). This also holds true for Bt plants with pyramided insecticidal proteins (Romeis et al., 2019). Besides, when Bt crops replace synthetic chemical insecticides for target pest control, this creates an environment supportive of the conservation of natural enemies. In respect to Bt-transgenic crops, the National Academies of Sciences, Engineering, and Medicine (NASEM, 2016) concluded:

> "On the basis of the available data, the committee found that planting of Bt crops has tended to result in higher insect biodiversity on farms than planting similar varieties without the Bt trait that were treated with synthetic insecticides." Earlier the European Academies have stated that "There is compelling evidence that GM crops can contribute to sustainable development goals with benefits to farmers, consumers, the environment and the economy" (EASAC, 2013).

Following event selection, it is essential to determine the gene flow, protein toxicity and safety to beneficial organisms. In the case of castor, the main concerns are the toxicity of castor seeds due to ricin, an extremely toxic and water-soluble ribosome-inactivating protein which is also present in lower concentrations in other parts of the plant posing a problem in determining the toxicity and allergenicity of the transgenic castor events. The *Bt* genes for introduction in castor were selected based on the initial

bioassays of the purified proteins against the target lepidopteran pests. Risk assessment studies necessitate testing of the transgenic events/cry proteins on the non-target and beneficial organisms. Eri silkworm (*Samia cynthia ricini*) is an important economic insect used in the production of valuable silk commonly known as 'vanya silk.' The silkworm primarily reared on castor leaves and the process is called eri culture. When assayed, purified Bt crystal proteins (Cry1Aa, Cry1Ab, Cry1Ac) showed toxicity to eri silkworm. Among ten purified crystal proteins of Bt tested at concentrations ranging from 2.93 to 3000 ng/cm^2 for their toxicity to eri silkworm through protein paint bioassays using castor leaves, Cry1Aa (2.6 ng/cm^2) was highly toxic followed by Cry1Ac (29.3 ng/cm^2) and Cry1Ab (68.7 ng/cm^2). The Cry1Ca and Cry1Ea proteins exhibited moderate toxicity to eri silkworm larvae and resulted in 23% and 28% mortality, respectively at the highest concentration tested (3000 ng/cm^2) while other tested proteins were with low/non-toxic (Kumar et al., 2016). Evaluation of transgenic castor events (AK1304-PB-1, AK1304-PB-4 and AMT-894) expressing the insecticidal protein Cry1Aa against eri silkworm exhibited 20.2 to 78.5% reduction in larval weight (Tarakeswari et al., 2019). Hence, it represents the importance of long term perspectives and technical amenability of economic part of transgenic plant, for biosafety assay, in meeting the regulatory requirement for the successful and ecofriendly commercialization of transgenic crops. Further, studies need to be taken up for identification of proteins that are toxic to the target pests of castor but not attacking eri silkworm for development of genetically modified castor.

CONCLUSION AND FUTURE PERSPECTIVES

The indiscriminate uses of chemical pesticides have caused incalculable damage to every aspect of the environment. As a result, agriculturists world over are persuading farmers to turn to ecologically sound pest management technologies that do not harm the environment. Bt based biopesticides are promising alternatives to chemical pesticides and have opened up new vistas in insect pest management for safe and eco-friendly pest management.

Despite its high target specificity and environmentally favorable "green" characteristics, environmental sensitivity (photolysis and rain fastness) and short persistent effect reduces its potency and duration of efficacy against pests. In the recent years, nanotechnology has been used to improve the performance and safety of the microbial pesticides and to reduce the dosage needed in comparison to conventional formulations. Hence, improved formulations of the active ingredient are essential to make Bt formulations comparable to chemical insecticides in terms of the speed of kill. Formulations with small and narrow particle-size distribution will improve the coverage of sprays on the foliage. However, the requirements for generation of toxicology data need to be developed to enable registration and commercial exploitation for effective crop protection.

Host plant resistance is the most important aspect in the management of castor pests. Genetic engineering confers resistance in plants to biotic stresses and increases yield in several crops. The introduction of foreign genes by genetic engineering techniques requires an efficient *in vitro* regeneration system for the desired plant species. Such a system must be rapid, reliable and applicable to a broad range of genotypes. Difficulty in tissue culture-based regeneration and poor reproducibility of results is the major bottleneck for genetic transformation of castor. Despite the research expanded over the past four decades in tissue culture, a genotype-independent direct or callus-mediated adventitious shoot regeneration system remains the much sought-after goal in castor. There is an immediate need for development of a highly efficient, reliable and reproducible direct and callus-mediated tissue culture system as a prelude for genetic engineering of castor for desirable traits. Efficacy of crystal protein genes from Bt and other proteins against another dreaded castor pest *viz*., capsule borer (*Conogethes punctiferalis*) has to be evaluated for enhancing host plant resistance to all the major lepidopteran pests in castor. Potential impacts on biodiversity, non-target organisms, soil microbiota and toxicological studies for oil (crude and refined) on rat/mice need to be studied in detail from safety point of view, before their deliberate releases into the environment. This study represents a typical and unique example of using the Bt both as a biopesticide and also as a transgenic event and presents

a comprehensive account of the prospects and problems with each of the technologies and also at various development efforts to bring the products to a commercial stage.

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