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Morphological and molecular identification of an invasive insect pest, fall army worm, *Spodoptera frugiperda* occurring on sugarcane in Andhra Pradesh, India

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Abstract
An invasive pest, fall army worm (FAW), *Spodoptera frugiperda* was noticed on 20 to 60 days old sugarcane crop (5-20%) at Regional Agricultural Research Station, Anakapalle and in some isolated pockets of Andhra Pradesh, India during February- March, 2019. Other than sugarcane, it was also reported on maize, sorghum, bajra and ragi in different districts of Andhra Pradesh during August-November, 2018. Early instars feed on sugarcane leaves scraping the chlorophyll leaving silvery transparent membrane. Later instars causing windows on leaves, leaving behind faecal pellets in whorls. In severely infested plants, large quantities of frass mass pellets were seen with mature larvae hidden in the whorls. FAW total life cycle ranged from 30-36 days on sugarcane during March-April months. In the present investigation both morphological and molecular characterization were studied for *S. frugiperda*. For molecular identification, partial amplification of mitochondrial DNA *COI* gene was done using specific primers *viz*., JM 76/JM 77 and confirmed its identity as *S. frugiperda* using BLAST programme of NCBI database. This confirms first report of *S. frugiperda* incidence on sugarcane from Andhra Pradesh, India. Further, a phylogenetic tree was constructed with very closely related *S. frugiperda* sequences from the NCBI database and it reveals that *S. frugiperda* has close resemblance with 'corn' strain. The population from Anakapalle has formed a separate clade in dendrogram and it has showed 99.75% resemblance with Mexico colony haplotypes of 'C' strain.

Keywords: Sugarcane, FAW, Andhra Pradesh, morphological, molecular identification

1. Introduction
Fall armyworm (FAW), *Spodoptera frugiperda* J.M Smith (Lepidoptera: Noctuidae) is one of the invasive and phytophagous pest. It feeds on leaves and stems of more than 80 plant species and causes considerable economic losses in several important crops such as maize, sorghum, rice, cotton, alfalfa, forage grasses and occasionally other crops in most of the countries with great potential for further spread and economic damage. FAW is native to the tropical and subtropical region of America and has invaded Africa, with the first detection being reported in Central and Western Africa in early 2016 [9] and in late 2016 and 2017 in parts of Southern, Eastern and Northern Africa. Reports of FAW occurrence on sugarcane up to 2018 were limited to Latin American countries only [1], subsequently other reports listed sugarcane as one of the hosts [2, 7]. Pashley [15] reported that there are at least two host strains of FAW commonly referred to as the rice and the corn strains.

FAW was reported on maize from the Indian sub-continent in Hassan, Chikkaballapur, Devanagere, Shivamogga and Chitradurga districts of Karnataka in 2018 by several workers [8, 10, 18, 19]. Reports from different states of India indicated that FAW primarily targeted maize in all the initial points of entry moving subsequently to other hosts like sweet corn and sorghum [13]. The pest also been reported mainly on maize in Maharashtra, Tamil Nadu, Andhra Pradesh and Telangana states of India during August- November, 2018. Within six months of its first occurrence in maize in different states of Southern India, the occurrence of FAW in sugarcane in India was reported for the first time from Maharashtra in September 2018 [3], [4] followed by Modakurichi and Pugalur districts of Tamil Nadu in November 2018 [11, 20].

Regardless of the status of the strains and their implications for host expansion and management of FAW, its occurrence and expansion primarily in south Indian states indicated

the ability of the pest to thrive in moderate tropical conditions where it is likely to remain round the year. Continuous generations of FAW, throughout the year which was reported in Africa, favored by the tropical and subtropical climate [16]. In the event of its spread to subtropical India, it is likely to survive there too with some variation due to the extremes of weather conditions. The present investigation was carried out with an aim to examine the population of *S. frugiperda* both morphologically and molecularly occurring on sugarcane with populations on other closely related hosts.

2. Materials and Methods

Incidence of fall army worm was recorded in sugarcane crop in isolated pockets of Visakhapatnam (5-10%), East Godavari (10-15%), West Godavari (5-20%), Krishna (10-20%) and Chittoor (10-15%) districts of Andhra Pradesh during February to April 2019.

Sugarcane crop was monitored regularly in research farm of Regional Agricultural Research Station, Anakapalle and recorded the incidence of *S. frugiperda* in 20−60 days aged crop during March- April months. The larvae collected on sugarcane were reared and maintained under laboratory condition for further studies. Some larvae were stored in 70% alcohol at 4ºC. Voucher specimens are being maintained at the Department of Entomology, Regional Agricultural Research Station, Anakapalle, Andhra Pradesh.

2.1. Morphological Identification

The FAW was identified morphologically based on larval, pupal and adult morphology and male genitalia. Images of 1st and 2nd instar larvae were taken with a stereo microscope with inbuilt camera using Capture Pro software (version 4.6).

2.2. Molecular identification

Since, the host plant is not a determinant for the identification of the colonizing strain, the molecular identification of the FAW strain is carried out at Regional Agricultural Research Station, Anakapalle.

2.3. DNA Extraction

Neonate larvae of FAW collected on sugarcane crop were maintained in stable conditions (26 ± 2 °C; 70 ± 5% RH and 12:12 h L: D) and reared on sugarcane leaves.

Genomic DNA was extracted from the neonate larvae of fall army worm by CTAB (Cetyl trimethyl Ammonium Bromide) method [5]. Neonate larvae (0.2 g) were taken from field collected larvae of fall army worm and were grounded using liquid nitrogen in sterilized pestle and mortar, then transferred to 1.5 ml eppendorf micro tubes. 600 µl of preheated (60 ^{0} C) 2 X CTAB extraction buffer (2%(w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20mM EDTA, pH 8.0) was added to the eppendorf micro tubes. The solution was incubated for one hour at 60^0 C in water bath with intermittent gentle stirring. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to this solution and mixed thoroughly. Subsequently, the mixture was centrifuged at 10,000 rpm for 20 min at 24 ^{0}C. Aqueous phase was separated and transferred to a fresh tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to this aqueous phase and mixed thoroughly, then centrifuged at 10,000 rpm for 20 min at 24 ^{0}C. These steps were repeated 2-3-times till a clear aqueous phase was obtained. To this clear aqueous phase, 0.6 volume of ice cold isopropanol and 0.1 volume of sodium acetate buffer (3M) were added and incubated at -20 ^{0}C for 30 minutes. DNA was precipitated by centrifuging at 10,000 rpm for 10 min at 4 ^{0}C. The precipitate was treated with 75% ethanol and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C, then the precipitate was discarded. The DNA was dried under a regular air flow for 20 min, re-suspended in 70µl TE buffer and stored at -20 ^{0}C. The presence of DNA in the samples was further confirmed by separating them on 1.2% agarose gel at 80 volts for 45 minutes using gel electrophoresis unit. The concentration of DNA was measured through spectrophotometrically using Denovix DS-11spectrophotometer.

2.4. PCR amplification of mitochondrial Cytochrome oxidase-I region

The identification of larvae of fall army worm, *Spodoptera frugiperda* based on morphological characteristics was complemented with the sequencing of the mitochondrial cytochrome oxidase region I. Primers used for amplification of CO1 gene were JM 76 (5-GAGCTGAA TTAGG (G/A)ACTCCAGG-3) and JM 77 (5-ATCACCTCC(A/T)CCTG CAGG ATC-3) [12]. Polymerase Chain Reaction was carried out in flat capped 200 µL volume PCR tubes obtained from M/s Tarsons, Kolkata, India. Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was performed in a 20 µl reaction mix containing 5X reaction buffer 25 mM $MgCl_2$, 0.01 mM dNTPs, 0.001 M primers, 1 U of TaqDNA polymerase (Genei) and 100 ng DNA total. PCR amplifications were performed on a DNA thermocycler (Eppendorf) with the following program: an initial denaturation at 97 ^{0}C for 6 min followed by 35 amplification for cycles of 94 ^{0}C for 1 min, 58 ^{0}C for 1 min, 72 ^{0}C for 2 min followed by final extension at 72 ^{0}C for 7 min.

2.5. Sequencing of the amplified Cytochrome oxidase-I region

The amplified samples were chromatographed by gel electrophoresis on 1.2% agarose. The gel was photographed using gel documentation system. Amplicons of 500 to 600 bp were selected for sequencing the COI region. For size selection a co-resolved 100 bp ladder was used. The amplified products were sequenced by Bioserve Biotechnologies (India) Pvt., Ltd., Hyderabad.

2.6. Identification of *S. frugiperda* through partial amplification of cytochrome oxidase-I region

Molecular identification of *Spodoptera frugiperda* was done using partial amplified nucleotide sequences of *COI* region through NCBI.BLAST programme and checked for homology. The COI generated sequence was deposited in NCBI Gene Bank database.

3. Results and Discussion

The incidence of fall army worm at Regional Agricultural Research Station ranged from 5 to 25 per cent in different experimental plots with maximum incidence recorded in young crop (20 days old) which is planted during the month of March, 2019. The populations were collected and studied for both morphological and molecular characterization.

3.1. Field symptoms

In sugarcane, the incidence of FAW was noticed on young crop (20-60 days) which was planted during February-March months. Early instars scraped the chlorophyll leaving silvery

transparent membrane which resulted in white elongated patches (Fig.1). Later instars causing windows on leaves, leaving behind faecal pellets in whorls (Fig.2) and foliar damage by cutting down the leaves. In severely infested plants, large quantity of frass mass pellets could be seen in the whorl with mature larva visible or hidden in the whorl (Fig.3). Older leaves showed patches of dry frass on outer leaves.

The results are in conformity with Chormule *et al.,* [3, 4] who first reported the occurrence of FAW in sugarcane in September 2018 in Maharashtra, India and during November 2018 in Tamil Nadu by Srikanth *et al.,* [20] who reported the occurrence of *S. frugiperda* from two districts in the sugarcane belts of M/s Sakthi Sugars Ltd., Modakurichi, Erode district, and M/s E.I.D. Parry (India) Ltd., Pugalur, Karur district, Tamil Nadu.

Fig 1: Scraping of chlorophyll by young larva

Fig 2: Windows on leaf lamina caused by mature larva

Fig 3: Mature larva and faecal pellets in whorl

3.2. Biology of fall army worm, *Spodoptera frugiperda* on sugarcane

The rearing of FAW was initiated with larvae collected from sugarcane fields at the research farm of Regional Agricultural Research station, Anakapalle, Andhra Pradesh. The observations were made during March- April, 2019 under laboratory conditions at the Department of Entomology. The larvae were reared on sugarcane leaves, on which incidence was noticed under laboratory conditions in stable conditions (26 ± 2 °C; 75 ± 5% RH and 12:12 h L: D). Fall armyworm completed its life cycle when it fed on sugarcane leaf bits under laboratory conditions. The incubation period ranged from 2- 3 days with a mean of 2.5 days. The larva passed through six instars over a period of 13 -14 days. During the prepupal period the full-grown larva stopped feeding, turned dark greenish-gray and the bright brown colour. Duration of the pupal period was about 8 to 9 days and pupal period is in conformity with Débora *et al.,* [6] who reported the pupal period of *S. frugiperda* on maize as 8.54 days.

The total life cycle of male and female ranged from 30-34 and 32-36 days, respectively. The female adult survived for 10 days with a range of 9-11 days compared to male (8 days) with a range of 7 - 9 days during March- April months at RARS, Anakapalle, Andhra Pradesh, India. Sharanbasappa *et al.,* [18] reported that the total life cycle of male and female of FAW was observed to be 32-43 and 34-46 days, respectively. Under laboratory conditions, the larvae fed on hosts like sorghum, cabbage, tomato, groundnut and sugarcane but not on rice.

3.3. Morphological Identification

3.4.1. Egg: Egg laying occurs on the inner side of the whorl and also on the upper surface of the leaf in a mass deposited in layers (Fig.4). The eggs are dome shaped brownish yellow coloured and loosely covered with pale yellowish coloured frass.

Fig 4: Egg mass & hatching of eggs

3.4.2. Larval stages: First and second instar larva are green-ish in colour with black colur hair on dorsal side with black head (Fig.5), while the final instars are with dark grey head and dull grey body with white subdorsal and lateral white lines. The mature larva is with a typical inverted 'Y' on head capsule and with distinct black spots on the body. Arrangement pattern of black spots is square on 8th and trapezoidal on 9th segment (Fig 6).

Fig 5: 2nd - 3rd instar larva with four black spots arranged in a square on 8th and trapezoidal on 9th segment

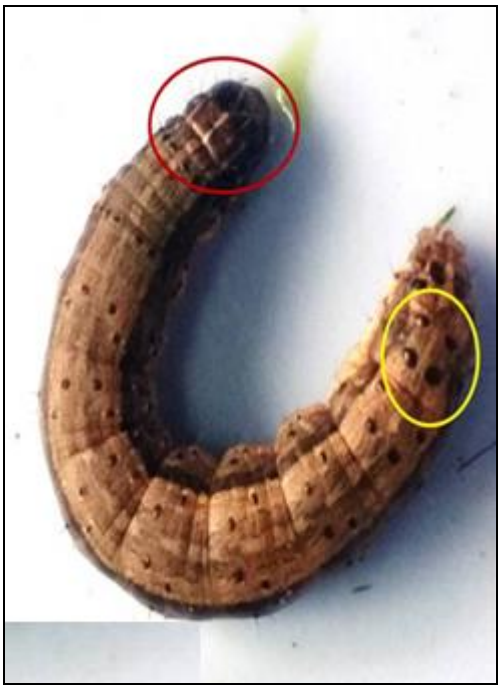

Fig 6: Mature larva of FAW with a typical inverted 'Y' on head capsule and with distinct black spots on the body

3.4.3. Pupa: Pupa is reddish brown in color and pupation occurs in the soil or sometimes in the leaf folds (Fig 7).

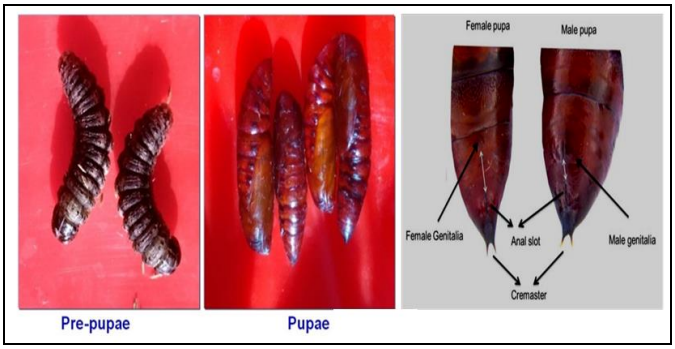

Fig 7: Pre-pupae and Pupae of FAW

3.4.4. Adult: Sexual dimorphism is clearly evident in the adult moths. Forewing of male is shaded with gray and brown, with triangular white patch at the apical region and circular spot at the center of the wing. The forewings of females are uniform grayish brown to a fine mottling of gray and brown. Female Adult hind wing is silver-white with a narrow dark border in both male and female (Fig.8). The morphological characters of adult described here are similar as reported earlier [18-20]. The present study obviously provides basic information about the biology and external morphology of FAW on sugarcane.

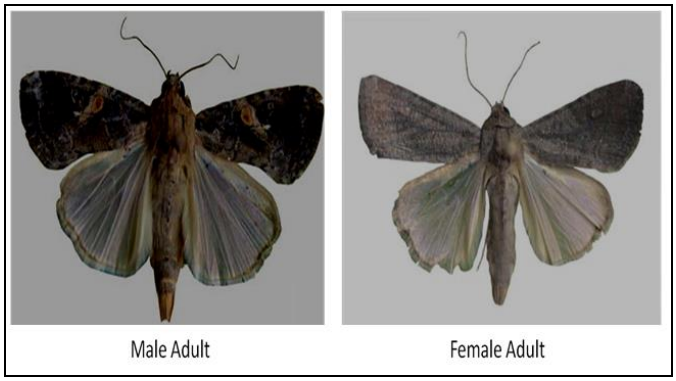

Fig 8: Male and female adults of FAW

3.4. Molecular identification of *Spodoptera frugiperda* based on mitochondrial cytochrome oxidase region analysis

DNA was extracted from neonate larvae of *S. frugiperda* and a 547-bp region of the mitochondrial DNA cytochrome oxidase subunit I (CO I) gene was amplified using PCR and specific primers *viz*., JM 76 and JM 77 and then sequenced (Bioserve Biotechnologies (India) Pvt., Ltd., Hyderabad**).** The test sequence was compared with available database sequences in NCBI and BOLD database. The sequence based homology confirmed the population as *Spodoptera frugiperda*. The COI region sequence was deposited in NCBI database with an accession number, MK908223.

Barcode of life (BOLD) database specimen identification results showed 99.75 per cent similarity to Mexico colony haplotypes of 'C' strain (Gen Bank: KF872171.1, KF872172, KF872173.1, KF872174.1, KM362176, KF872181, KF872182) and 99 per cent resemblance with the population of Vizianagaram ('R' strain) which was reported on maize from Andhra Pradesh, India [13].

3.5. Phylogenetic analysis of partial cytochrome oxidase I region

Using maximum parsimony method in MEGA software (ver. 10.0.5), a phylogenetic tree was constructed by considering most relevant sequences from NCBI database along with sequence data of our investigation. Separate grouping was obtained through COI region sequences where in *Spodoptera frugiperda* sequences were clustered in to two clades as

depicted in Fig. 9. The FAW population collected on maize during Aug-Nov, 2018 in Andhra Pradesh, India were clustered in one group and the population of *S. frugiperda* collected on sugarcane during March, 2019 at Regional Agricultural Research Station, Anakapalle was made in to a separate clade and it is more similar (99.75%) to Mexican colony haplotypes of *S. frugiperda* ('C' strain) collected on corn crop [14] and 99% similarity with the population of Pedabathevalasa, Vizianagaram ('R' strain) collected on maize from Andhra Pradesh [13].

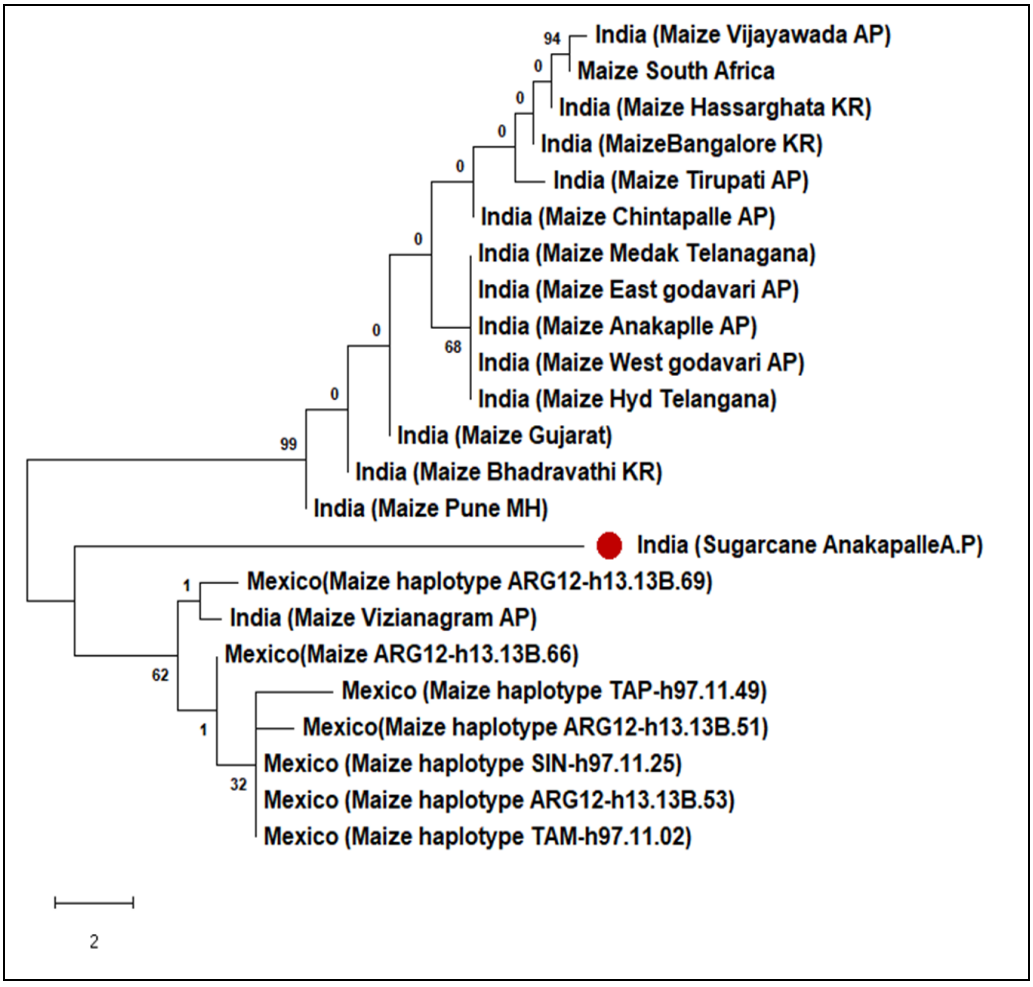

Fig 9: Dendrogram with Co Region Sequences of Spodoptera Frugiperda Using Maximum Parsimony Method

The results are in conformity with Chormule *et al.*, [4] who reported that the sugarcane fall armyworm from Kolhapur, Maharashtra is undeniably belongs to 'corn' strain. Mahadeva swamy *et al.*, [13] reported that mtCOI (5') based sequences of FAW collected on maize from Andhra Pradesh, Madhya Pradesh, Maharashtra, Tamil Nadu and Telangana in Aug-November, 2018 aligned with "R" strain with minimal genetic diversity exhibiting no host/ location specific variations.

The present findings are in conformity with Pashley [15] who reported that there are at least two host strains of FAW commonly referred to as the rice and the corn strains. These biotypes are sympatric and morphologically identical but differ in physiological characteristics and reproductive isolation. In two forms of FAW, the 'R' strain was predominant on rice and pasture grasses and the 'C' strain on maize, cotton and sorghum [16]. Molecular diversity studies of 22 FAW populations collected on maize, sweet corn and sorghum from five tropical states of India revealed the prevalence of "R" strain (Rice) and not "C" strain (Corn) [13]. But, the pest was not observed on young paddy in the sugarcane habitat in our preliminary surveys. The relative biomass of sugarcane presenting a huge niche in the habitat may be governing the colonization of sugarcane by the "C" strain, known to occur on maize, cotton and sorghum elsewhere ([16]. The high dispersal ability and reproductive capacity, and the likely absence of diapause in tropical climate may accelerate expansion of its geographical range within the country and neighboring countries [18]. Analysis of mtCOI (5') based sequences of FAW reported on maize from Andhra Pradesh, Madhya Pradesh, Maharashtra, Tamil Nadu and Telangana in Aug-November, 2018 revealed that these populations from India aligned with "R" strain with minimal genetic diversity exhibiting no host/ location specific variations [13]. It appears that while the "R" strain has colonized on maize, sweet corn and sorghum, "C" strain has started adapting to sugarcane. Determination of the populations attacking sugarcane in Maharashtra as "C" strain [4] indicated that both strains of *S. frugiperda* have entered India. It appears that while the "R" strain has colonized maize, sweet corn and sorghum [13] "C" strain has started adapting to sugarcane. Hence, the present study suggests a possible invasion by single genetic stock of FAW in India which requires haplotype analysis.

3.6. Alternate hosts in sugarcane ecosystem:

Grass weeds *viz., Cenchrus ciliaries* and *Cynodon dactylon* were identified as alternate hosts in sugarcane ecosystem. Neonate larvae of fall army worm were found to feed on these

grasses by scraping chlorophyll and cutting down by leaves resulting in white patches on leaves and faecal pellets in whorls Fig. 10 Damage symptoms of FAW on *Cenchrus ciliaries*).

Fig 10: Damage symptoms of FAW on *Cenchrus ciliaries*).

4. Conclusions

During our preliminary observations, incidence of FAW was observed in sugarcane after rabi maize which might acted as a predisposing factor for the FAW incidence on sugarcane as the larva shift from maize to sugarcane after 40 to 50 days. FAW incidence on sugarcane was noticed up to 60 days and thereafter it gets reduced. In the early stage of cane development, regular monitoring is necessary to assess FAW incidence and to adopt suitable management strategies. It is evident from the present studies that spreading of *S. frugiperda* may occur as it is migratory and it may shift to other hosts and survive in the absence of maize and maintain the population in India throughout the year.

Further, investigations on genetic variations in populations of FAW in different sugarcane growing areas of Andhra Pradesh and biology need to be studied. Since, no economic thresholds are available for FAW in sugarcane, assessment of field incidence levels on the basis of damage symptoms or larval presence on the leaves or whorls will help in decision making for insecticide application. Based on this information, we could outline and adopt crop specific prophylactic and curative management tactics to prevent its proliferation in sugarcane.

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