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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR PROTEIN DETECTION IN COTTON: A QUALITATIVE APPROACH

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ABSTRACT

A qualitative assay was conducted to detect protein in cotton samples collected from the field under Cotton Research Unit, Dr. PDKV, Akola, at various growth stages throughout the season. A total of 53 samples were analyzed, which included leaf samples at different growth stages, square samples at 70 days and boll samples at 90 days stage. Of these, 38 samples tested positive for the target protein. Absorbance values in leaf samples initially increased at 60 days, ranging from 0.615 to 3.330, followed by a significant rise from 0.924 to 3.711 at 90 days stage. At 110 days, the absorbance values declined, ranging from 0.230 to 2.000. In square samples at 70 days, absorbance values ranged from 0.281 to 2.990, while in boll samples at 90 days, they ranged from 0.320 to 2.770. Additionally, the qualitative data was categorized to assess variations in the number of samples at each growth stage. The findings indicate variations in absorbance values across different growth stages of leaf, square and boll samples. This study provides insight into the temporal changes in absorbance of cotton plants, contributing to a better understanding of plant development.

***Key words*:** Absorbance, Cotton, Growth stages, Protein, Qualitative assay.

Introduction

Emerging from the Arabic word “quotn,” (Lee and Fang 2015), the word cotton shares a relationship with the *Gossypium spp.*, which came from the Arabic word “goz,” (Gledhill, 2008), signifying a delicate substance. *Gossypium spp.*, a member of the Malvaceae family, is cultivated for fiber in many diverse regions and is the most economically significant crop globally (Abdurakhmonov, 2013). Although cotton is usually grown as a shrubby annual in temperate environments, it is sometimes found as a perennial tree in tropical areas (Iqbal *et al.*, 2001). Out of the 50 identified cotton species, only 4 are grown commercially worldwide (Khadi *et al.*, 2010) with the other species existing in their natural habitat. Some of these species are categorized into American Cotton (*Gossypium hirsutum)*, Levant Cotton (*Gossypium herbaceum)*, Tree Cotton (*Gossypium arboreum)*, Sea Island Cotton (*Gossypium barbadense)* etc. (Gotmare *et al.*, 2000). Some cotton species tend to be cultivated in the developing world more than others because these types of cotton cannot adapt to change in environmental circumstances and low production (Amin *et al.*, 2017; Amin *et al.*, 2018). The production and productivity of cotton are suffering due to the changing atmospheric situation due to rising temperatures, conditions of biotic and abiotic stresses such as an increase in insect and pest infection, conditions of drought, salinity stress, etc. In the context of these conditions, the transgenic method is routinely being used to transfer biotic and abiotic stress-responsive genes in cotton and has shown great success leading to the development of various cotton varieties, such as *Bacillus thuringiensis* (Bt) cotton, which produced a sharp rise in cotton yield (Sawan, 2018; Hussain and Mahmood, 2020). With an

Table 1: Stock solutions used in qualitative assay.

Sr. No.	Buffers/ Solutions/ Sample preparation	Preparation
1	Extraction Buffer 10X	The concentrate powder was dissolved in 500ml distilled water and 25ml of Tween-20 was added. The solution was mixed well before use. The solution was further used when required by dilution to 1X and stored at 2-8°C.
2	Wash Buffer 10X	The concentrate powder was dissolved in 500ml distilled water and 2.5ml of Tween-20 was added. The solution was mixed well before use. The solution was further used when required by dilution to 1X and stored at 2-8°C.
3	Stop solution	27ml of 98% H_2SO_4 was added in 973ml of distilled water and stored at room temperature.
4	User positive control	The user positive sample was crushed into a uniform powder and 1ml of extraction buffer (1x) was added and waited for 10 minutes before use. The samples were freshly prepared.
5	User negative control	The user positive sample was crushed into a uniform powder and 1ml of extraction buffer (1x) was added and waited for 10 minutes before use. The samples were freshly prepared.
6	Unknown Samples	20mg sample was weighed into a microtube and crushed into a uniform powder by using seed crusher and 0.5ml of working extraction buffer(1X) was added to it. The mixture was kept for 30-60mins at room temperature before use.

early planting schedule, several bollworms created a threat to traditional varieties. This technology delivers inherent defence over such predators. Early detection systems that screen for genetically modified organisms (GMOs) and correspond to legal standards are necessary to prevent unintentional mixing of GM and non-GM crops (Arne Holst-Jensen, 2009). For the detection of transgenic crops, enzyme-mediated immunoassays have been widely used nowadays (Farid, 2002). ELISA permits the highly sensitive and selective quantitative/qualitative analysis of antigens, including proteins, peptides, nucleic acids, hormones, herbicides, and plant secondary metabolites (Seiichi Sakamoto *et al.*, 2018). In this study, qualitative assay method was used to detect the presence of Cry1Ac protein in different parts of cotton plant throughout the season.

Material and Methods

The present study was carried out on the field at Cotton Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. A total of 53 seeds were sown on the field and 53 plants were germinated. The leaf, square, and boll parts of each plant were screened by qualitative assay at different developmental stages of the cotton plant *i.e.* at 60, 90, and 110 days after sowing (DAS) for leaf samples, 70 DAS for square samples and 90 DAS for boll samples. The qualitative ELISA kits (make-Eurofins) for the detection of Cry1Ac/Ab protein were provided in ten plates or fifty plates format followed by Cry1Ac/Ab enzyme conjugate, extraction buffer concentrate, wash solution concentrate, TMB substrate, Cry1Ac/Ab positive and negative control respectively. The method for the preparation of stock solutions is given in Table 1. The standardized method for qualitative assay (Avrameas, 1969) and (Avrameas S, 1978) with slight modification was followed by using different working stocks (Table-1). The reagents were brought to room temperature before usage. 50µl of the enzyme conjugate was added in each well; 50µl of the working extraction buffer was added to the blank well. 50µl of both positive and negative controls were added to two separate wells each. To prevent cross-contamination, the contents of the plate were mixed properly. After covering the dish, the plate was incubated at room temperature for forty minutes. The contents of the wells were discarded properly into a sink to remove them and 300µl of wash solution was loaded in each well to carry out the washing method. To drain the wells, the plate was inverted on a paper towel. These steps were followed three times to remove as much liquid as possible from the plate after the final wash. 100µl of substrate was added in each well and incubated it for 20 minutes at room temperature and 100µl of stop solution was added to halt the reaction. The absorbance readings were calculated within fifteen minutes of adding the stop solution at 450 nm.

Results and Discussions

To validate the detection of Cry1Ac protein in various parts of cotton plant at different phases of growth, 38 of the 53 plants had the required protein verified. The absorbance was first measured at 450 nm in leaves at the stages of 60DAS, 90DAS and 110 DAS (Days after sowing). The range of absorbance values was determined to fall in between 0.615 to 3.330 for leaf samples at the 60 DAS stage, followed by 0.924 to 3.711 for leaf samples at the 90 DAS stage and 0.230 to 2.000 for leaf samples at the 110 DAS stage. Similarly, following 70 days after sowing, the absorbance values for the square sample were measured from 0.281 to 2.990 and for the boll samples; it went from 0.320 to 2.770. The details are

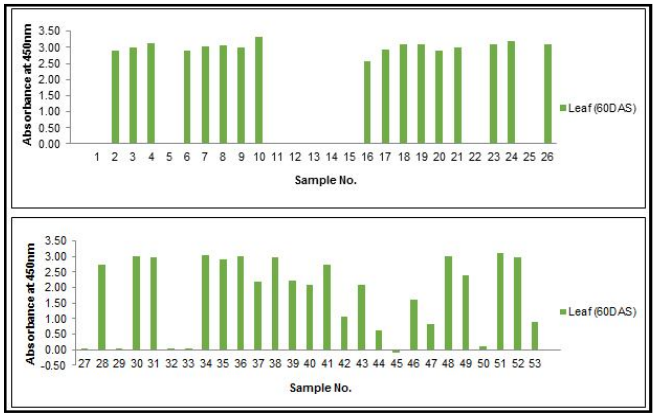

Fig. 1: Qualitative assay in 60DAS leaf samples.

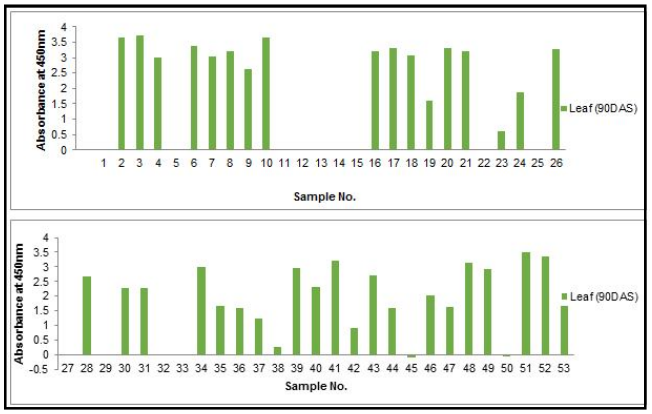

Fig. 2: Qualitative assay in 90DAS leaf samples.

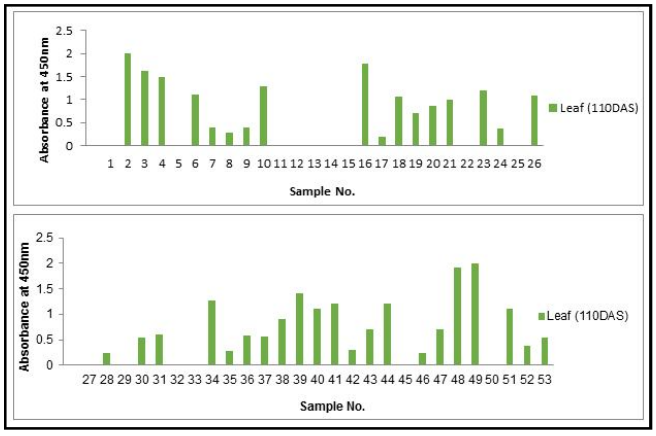

Fig. 3: Qualitative assay in 110DAS leaf samples.

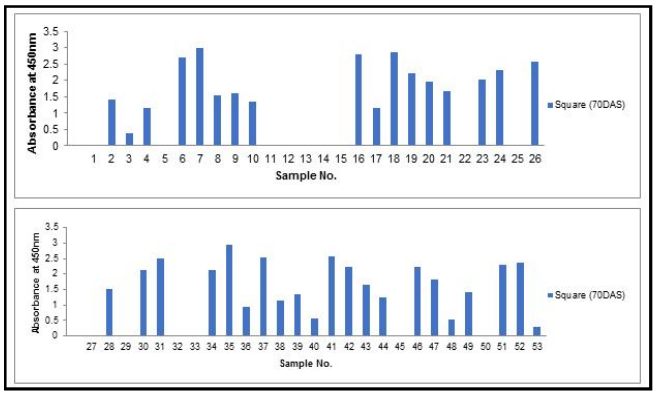

Fig. 4: Qualitative assay in 70DAS square samples.

mentioned in Fig. 1, 2, 3, 4 and 5 respectively.

Based on the range of absorbance measured for each plant, the numbers of samples were classified on the basis of absorbance values. The outcome illustrated that in the case of leaves at 60 days stage, the range of absorbance values in samples were first limited and subsequently grew higher. In the same way, it was found that sample evaluation was higher in the case of leaf samples at the 90-day mark, and that sample count sharply decreased as absorbance values ranged up by 3.00, respectively. When the absorbance value exceeded 3.00, few samples were found in the square samples at the 70DAS stage. The starting range was smaller at absorbance greater

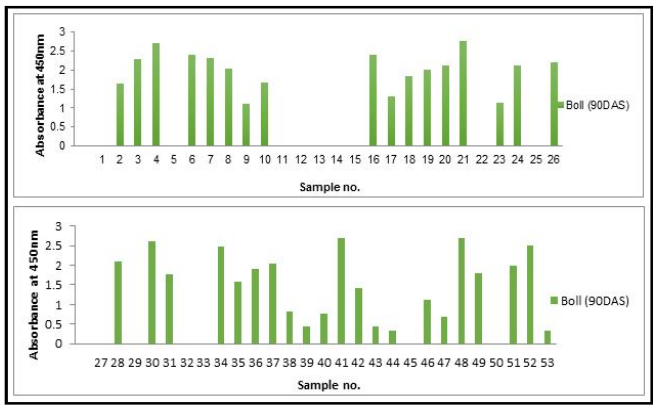

Fig. 5: Qualitative assay in 90DAS boll samples.

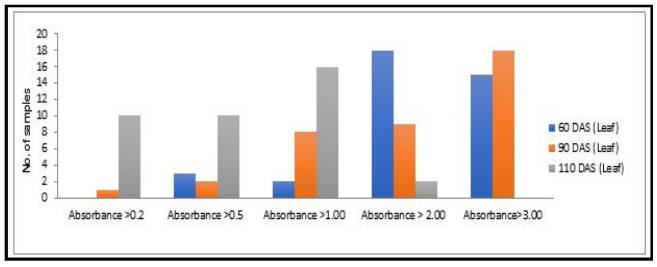

Fig. 6: Classification of leaf samples by qualitative assay at different growth stages.

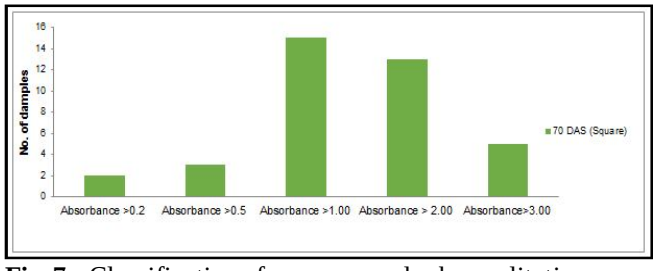

Fig. 7: Classification of square samples by qualitative assay.

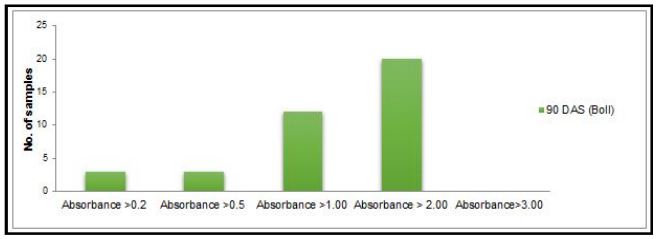

Fig. 8: Classification of boll samples by qualitative assay.

than 0.20, and the progressing range was higher at absorbance greater than 1.00. For boll samples, however, there were initially fewer samples but eventually more when the absorbance exceeded 1.00. The maximum number of samples was found when the absorbance was more than 2.00, but it was also noted that samples were absent when the absorbance was more than 3.00. The details are mentioned in Fig. 6, 7 and 8 respectively.

Alkaline phosphatase (ALP) and horseradish peroxidase (HRP) were employed in the so-called enzyme immunoassay, a similar technique to qualitative assay (ELISA) that was used and assessed by utilizing enzymes (Avrameas S, 1978) and (Nakane and Kawaoi, 1974). Sandwich ELISA has been proven by Wang S. *et al.,* (2007) as a more convenient approach for Cry1Ac protein detection. Although 52 genotypes collected from farmers were subjected to an immunostrip test by Cheema *et al.,* (2015) to detect Cry1Ac, 45 genotypes were confirmed to be Cry1Ac positive. Singh A. K. *et al.,* (2016) used a qualitative assay to find the Cry1Ac protein in two distinct transgenic cotton plants. In the initial Bt cotton plant, a total of 36 transgenic lines were examined; 25 lines had the required protein verified, while 11 lines had none. Similarly, out of the 70 Bt lines evaluated in the second Bt cotton plant, 27 were found to express the cry1Ac protein positively and 43 to do so in a negative manner. The detection of Cry1Ac protein on fifteen distinct seed samples, five-leaf samples, and one corn sample was validated by Rupula Karuna *et al.,* (2019). Of the fifteen seed samples, twelve had been determined to have Cry1Ac protein, whereas the remaining three were negative.

Conclusions

In conclusion, our study revealed variations in absorbance values across different growth stages of the cotton plant. At the 60DAS leaf stage, the highest absorbance was found in Sample No. 10 (3.33), followed by Sample No. 24 (3.19) and Sample No. 4 (3.13) respectively. Similarly, the lowest absorbance was observed in Sample No. 53 (0.89), followed by Sample No. 47 (0.82) and Sample No. 44 (0.61). At 90DAS leaf stage, the highest absorbance was found in Sample No. 3 (3.71), followed by Sample No. 2 (3.67) and Sample No.10 (3.66) respectively. At 110 DAS leaf stage, the highest absorbance was found in Sample No. 2 (2.00), Sample No. 49 (2.00), followed by Sample No. 48 (1.92) and Sample No. 16 (1.78). At 70 DAS square stage, the highest absorbance was found in Sample No. 7(2.99) followed by Sample No. 35 (2.95) and Sample No. 6 (2.69). Similarly, the lowest absorbance was observed in Sample No. 53 (0.28) followed by Sample No. 3 (0.37) and Sample No. 40 (0.55). However, at the 90DAS boll stage, the highest absorbance was found in Sample No. 21 (2.77) followed by Sample No. 4 (2.70) and Sample No. 30 (2.62) respectively. Similarly, the lowest absorbance was observed in Sample No. 44 (0.32), Sample No. 53 (0.34) and Sample No. 39 (0.45). It was also noticed that the number of samples gradually decreased in case of 70 DAS square stage and 90DAS boll stage as compared to leaf samples at different growth stages.

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