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SIMPLE HIGH THROUGHPUT BIOASSAY TECHNIQUES TO EVALUATE TRANSGENIC PLANTS EXPRESSING ANTIFUNGAL GENES

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ABSTRACT: In recent years the genetic engineering has become very common due to its ability to create a room for the incorporation of desired traits in an organism. From smallest microorganisms to humans and plants, researchers have laid their hands and are being modified genetically. This technology has been exploited to a very large extent in the field of agriculture either to create a high yielding variety or improve the crops resistance to various diseases which are the main cause of yield loss, or to enhance any desired trait. In this context many transgenic plants have been developed which are resistant against different pathogens like bacteria, virus, fungi insect pests etc. Fungal infections are one of the most devastating and affect the crop yield to a large extent both during pre-harvest as well as post-harvest stages. Many attempts have been made by the researchers to combat these fungal pathogens by expressing one or more of antifungal genes like *chitinases*, *defensins*, PR proteins etc. Evaluating the bio-efficacy of these antifungal genes is very crucial in characterizing the transgenics expressing antifungal genes. For this designing a right bioassay is very important. This paper deals with few such simple bioassay techniques which work in both *in-vitro* and *in-vivo* conditions. The principle behind these bioassays are very practical as well as reliable and can be designed according to the host and pathogen involved in the study. The bioassays discussed herein are high-through put, simple and economical.

KEYWORDS: Aflatoxins, *Aspergillus flavus*, *Cercospora*, PDI, transgenic plants.

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1. INTRODUCTION

Even after adoption of various agricultural practices and agrochemicals, every year plant diseases result to approximately 12 % yield loss at the field level, to which are added 9 - 20 % during post-harvest stages [1]. Among the culprits causing this huge loss, the most devastating pathogens are fungi [2]. Though husbandry techniques and agrochemical usages reduce fungal inoculums and spread, it does not improve host resistance ability. In this context many plant pathogen related problem are yet to be identified and the role of multitude genes that are involved in immune responses after fungus infestation and the various pathways involved therein have to be elucidated [3][4][5][6][7][8]. Several soil born fungi attack plants causing disease [9][10]. *Aspergillus spp.* are considered as the most popular plant pathogens. Other important destructive fungal diseases in Groundnut are, early leaf spot caused by *Cercospora arachidicola* and late leaf spot caused by *Phaeoisariopsis personatum* [11] Many food crops such as ground nut, maize, barley, cotton seed, rice, wheat, tree nuts legume and spice crops are susceptible to fungal attack either in the field or during storage some of these fungal species can produce secondary metabolites, a diverse group of chemical substances known as mycotoxins which are a group of closely related heterocyclic compounds produced predominantly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. *Aspergillus flavus* is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as drought [12]. *Aspergillus flavus* produces aflatoxin B1(AFB1) and B2(AFB2) which are major types of aflatoxins, *Aspergillus parasiticus* produces B1, B2 along with G1 and G2. The produced aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are difuranocoumarin derivatives which contaminate a vast array of food and agricultural commodities [13]. Though Aflatoxin B1 usually predominant and is the most toxic, all four aflatoxins are potent hepatic carcinogen causing cancer of liver in wide variety of animal species including humans [14]. In general aflatoxins produce a number of adverse effects in a range of biological systems including plants animals and humans. Considerable progress has been made in identification and cloning of genes involved in plant defense responses. With the aid of plant molecular biology and biotechnology, a large number of antifungal proteins and peptides have been isolated and assessed through *in vitro* studies. Strategies like enhancement of plant structural defense, neutralization of fungal toxins and exploitation of antifungal genes from non-plant sources have been used to produce transgenic plants. Genetic engineering has made it possible by incorporating resistant genes from any species to improve disease resistance genetically. A genetically modified (GM) plant into which one or more genes have been artificially incorporated which in general does not exist under natural conditions of cross-breeding or natural recombination are commonly used by the scientist to develop transgenic plants with genes involved in these pathways in order to evaluate their effects in enhancing

disease resistance [15]. Such a technology has been vastly used nowadays by the biotechnologist to combat the plant pathogens and increase the crop economy. Bioassay or biological assay is an experimental set up in which the aim lies in comparing the potential of the treatment on an acceptable scale either on insects, plants or animal. These bioassays should also stimulate field conditions to ensure predictability of control efficacy in the field from data obtained through lab measured resistance. Bioassay involves: 1.Stimulation 2.Organism (plant, animal, insect) and 3.Reaction i.e. the response produced by the organism due to the application of stimulus. Bioassays for plant disease bio control agents are often tests or evaluation systems designed to efficiently screen isolates for control of plant pathogens, usually fungi or bacteria, in a regulated environment. These bioassays may exclude the host (in *vitro)* or include it *(in vivo)* [16]. Here we depict few such Bioassays (both in-vitro and in-vivo) which have been used to evaluate and screen the transgenic plants expressing antifungal genes for their resistance against fungal pathogen. The bioassays discussed are high throughput, simple and economical.

2. MATERIALS AND METHODS

Isolation of *Cercosporaarachidicola* and *Phaeoisoriopsis personatum*: Overnight incubated matured Tikka disease (Early leaf spot and late leaf spot) infected groundnut leaves spotted portion of the infected leaves were cut, sterilized by using 0.1% mercuric chloride and washed. The sterilized bits of both early and late leaf spot infected leaves were inoculated on *Cercospora* specific Richard's medium; the plates were incubated at room temperature for 10-12 days. After 10-12 days plates were covered with white mycelia growth and stored under refrigeration for further studies. For purification, the hyphal tips were taken and placed on a Richard's medium in slants culture and the culture was stored in refrigeration for further use.

In-vitro leaf bioassay against *Cercospora* spp.

Fresh matured healthy groundnut leaves from both transgenic and control plants were collected separately and washed thoroughly under tap water leaves are then dried and were surface sterilized with 0.1% mercuric chloride for 2-5 minutes, followed by thorough rinses with sterile water for 3-4 times. These leaflets from the respective plants were placed on the 4-5 days old *Cercospora* pure culture in petriplates grown on Richard's medium. The plates were then incubated at 37^{o}C for further observation.

In-vivo bioassay of transgenic crops with *Cercospora* spp.

The culture of *Cercospora* spp. were multiplied on Richard's broth enriched with finely ground and filtered groundnut leaf extract at the rate of 10 ml/liter and autoclaved at 121^{0}C for 15 minutes and at 1.1 kg/cm^{2}. 1-2 ml of surfactant (Tween-20) was added to the finely ground mycelial bits and were sprayed (2 X 10^{3}) on under the leaf surface of matured transgenic and wildtype plants.The sprayed

plants were covered with polythene bags for 48 hours to create suitable environmental condition for infection to occur.10 days later spore suspension of *Cercospora* spp. made in sterile water from field collected leaf samples was sprayed at the rate of spore suspension concentration was 2 X 10 ^{3}/ml. Observation were recorded on number of spots on each leaflet on each plant and scored at different scales /grades at 10 days intervals until the harvest of the crop. Finally by taking the mean of each observation the Percent Disease Index was calculated by the following formula

$$PDI(\%) = 1 + \frac{sum\ of\ individual\ disease\ ratings}{number\ of\ leaf} \text{x} \frac{100}{maximum\ examined\ grade\ value}$$

For the estimation of disease incidence, disease was recorded using modified 9-point scale [17] as given below:

1 = No disease; 2 = 1 to 5% disease severity; 3 = 6 to10% disease severity; 4 = 11 to 20% disease severity; 5 = 21to 30% disease severity; 6 = 31 to 40% disease severity; 7 =41 to 60% disease severity; 8 = 61 to 80% disease severity; 9= 81 to 100% disease severity

Bioassay of groundnut transgenics against *Aspergillus flavus*

Isolation of *Aspergillus flavus* from groundnut seed: *Aspergillus flavus* was isolated from surface sterilized infected groundnut seeds of *Aspergillus flavus*. After confirmation of *Aspergillus flavus* with microscopic study from the suspected seeds, culture plates were made on the potato dextrose agar (PDA) medium. Pure culture on the potato dextrose agar (PDA) medium was kept at room temperature for 10 -12 days. After 10-12 days plates were covered with green colour mycelia with enormous sporulation and these plates were stored under refrigeration for further studies.

***In-vitro* bioassay:** Transgenic groundnut and control groundnut seeds were surface sterilized using 0.1% mercuric chloride, rinsed with sterile water thoroughly and placed on sterile petri plate having sterile blotting paper. Spore suspension of *Aspergillus flavus* is prepared by harvesting the spores from the pure culture plates of *Aspergillus flavus* using a sterile loop and dispersing in required quantity of sterile water. The spore load is measured using haemocytometer and diluted accordingly to get a spore load ~ 1x10^{6}/ml. Tween-20 is added to the spore suspension to a final concentration of 0.5%. This spore suspension is then loaded on to the seeds in petriplates directly (3ml of spore suspension for 10 seeds in 90mm petriplate). The plates are incubated at 37^{0}C in high humid conditions and observed after 7-10 days for the infection and germination of the seeds.

***In-vivo* bioassay:**

Large scale multiplication of *Aspergillus flavus* spores: 1-2 kg of Bajra seeds are cleaned and imbibed in tap water overnight. These seeds are then distributed to culture bottles and autoclaved. 3ml of spore suspension is added to the sterile bottles containing Bajra seeds and incubated at 37^{0}C

for 15 days to get *A. flavus* spores on the bajra seeds.

Measuring Colony count in the soil: 1 g of dry soil from the soil used to grow the plants is serially diluted using sterile water. 1ml of each of 10^4 and 10^5 dilutions is used to inoculate separate PDA plates (pour plate). Plates are incubated at 37^0C for three days and number of *Aspergillus flavus* colonies formed is counted. The number of colony forming units (CFU)/g of soil are calculated by multiplying the respective dilution factor. A known amount of uniform soil is used to fill the pots. The initial soil cunt of the *Aspergillus flavus* Colony forming units (CFU/g) of soil is observed in randomly selected pots before sowing the seeds for germination. The seeds of the transgenic along with the wildtype are sown and maintained. After 40DAS the *Aspergillus flavus* CFU/g count of soil is measured again. At 50 DAS plants were subjected to the deliberate challenging of the *Aspergillus flavus* by loading the pot soil whose CFU/g of soil is less than 1000 with the *Aspergillus flavus* spore culture grown on Bajra seeds so as to increase the *Aspergillus flavus* CFU count to above 1000/g of soil. The count of *Aspergillus flavus* is maintained above 1000CFU till the harvest by repeating the *Aspergillus flavus* CFU count and supplementing the spores wherever necessary. Moisture stress was also imposed starting from 80 DAS till the time of harvest to facilitate the production of Aflatoxin by *Aspergillus flavus*. Moisture stress was given by irrigating the plants once in three days. The seeds were harvested from these challenged plants at 110 DAS. An aliquot of the seeds are put for germination in sterile petriplates (Seed Colonization experiment) and observed for infection and growth of the seeds. Another aliquot of seeds are incubated at 65^0C for 72h to arrest the further accumulation of aflatoxin. These seeds are then tested for the accumulation of the aflatoxin levels by HPLC method.

3. RESULTS AND DISCUSSION

In-vitro leaf bioassay against *Cercospora* spp

The Leaves from non-transgenic wild type plant and transgenic plants expressing Antifungal gene were challenged with pure culture of *Cercospora arachidicola*, after seven days of incubation under controlled room conditions it was observed that transgenic plants showed a delayed growth of mycelia on the leaves whereas there was an immediate and dense growth of mycelia was observed on the leaves of non-transgenic wildtype plants. (Fig.1)

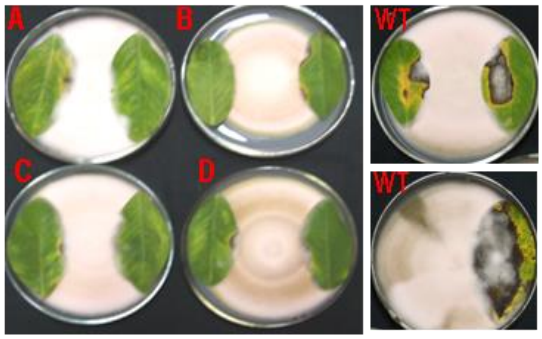

Fig.1: *Invitro* leaf bioassay against *Cercospora arachidicola*. A, B, C, D-leaves of different plants of Transgenic Groundnut. WT- Leaves of non-transgenic groundnut plants.

***In-vivo* bioassay against *Cercospora* spp.**

Spore suspension of *Cercospora arachidicola* was sprayed underneath the healthy leaves of non-transgenic wild type and transgenic plant expressing antifungal genes, ten days after spraying of spore suspension spots indicating the infection was observed and PDI was calculated. In non-transgenic wild-type plants de-foliage of the older leaves was observed and the younger leaves showed large number of spots caused by *Cercospora*, whereas transgenic plants showed varied results. In most of the transgenic plants (Resistant plants) very less number of spots was observed in older leaves and the most of the younger leaves showed no spots. In rest of the transgenic plants (Moderately Resistant plants) older leaves showed large number of spots (no de-foliage) and the younger leaves showed less number of spots. (Fig.2)

Fig.2: In vivo bioassay at whole plant level against *Cercospora arachidicola*. A-Resistant Transgenic Groundnut. **B**-Moderately Resistant Transgenic Groundnut. **WT**-Non transgenic wildtype groundnut plants.

***In-vitro* seedling bioassay against *Aspergillus flavus*.**

The spore suspension of $1X10^7$ spores/ml of a high virulent *Aspergillus flavus* strain was inoculated directly on the sterilized seeds of non-transgenic wildtype plants and transgenic plants expressing antifungal genes and incubated at 37^0C under high humid conditions. After seven days of incubation it was observed that wild type non-transgenic seeds showed weak/no germination and were infested completely by the spores of *Aspergillus flavus* while the e transgenic seeds showed healthy germination with less or no infection (Fig.3)

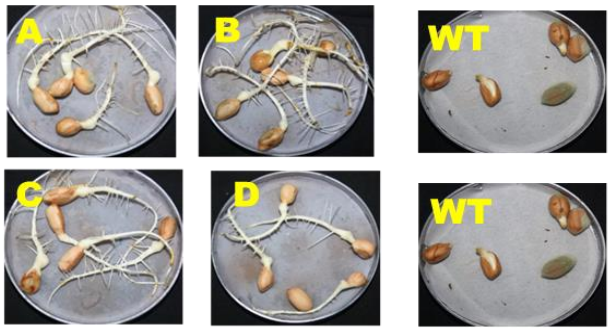

Fig.3: *Invitro* seedling bioassay against *Aspergillus flavus*. A,B,C,D-seedlings of different plants of Transgenic Groundnut. WT- seedlings of non-transgenic groundnut plants.

***In-vivo* bioassay against *Aspergillus flavus*.**

Non-transgenic wild type plant and transgenic plants expressing Antifungal genes were grown in uniformly filled pots. The CFU of *Aspergillus flavus* was maintained above 1000CFU/g of soil from 50 DAS by supplementing the spores grown on Bajra seeds to the soil 80DAS moisture stress was imposed to the plants till harvest. Seeds from respective plants were harvested 110DAS. A part of the harvested seeds was put for germination and observed for both infection and germination rates. After ten days observations were visually recorded for the Percent germination and percent damage by infection (Fig.4). Transgenic lines showed better germination percentages and lesser damage showing their resistance against the infection.

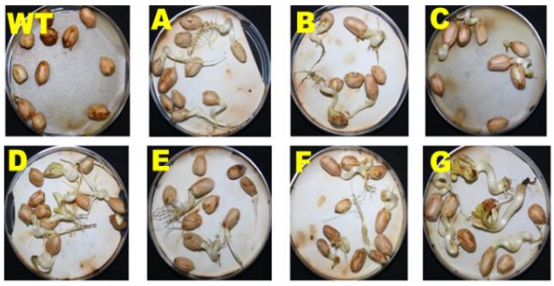

Fig.4: *Invivo* whole plant bioassay against *Aspergillus flavus*. A,B,C,D,E,F,G-seedlings harvested different plants of Transgenic. Groundnut challenged with *Aspergillus*. WT- seedlings of non-transgenic groundnut plants challenged with *Aspergillus*.

The second part of the harvested seeds from each plant of each line were incubated at 70^0 C for 72 hours immediately after the harvest to lock in the Aflatoxin content present and kill any *A.flavus* if present. Further the seeds were checked for the levels of aflatoxin (AfB1) accumulation by HPLC. Almost all the transgenic seeds showed no aflatoxin accumulation. The average content of Aflatoxin B1(ppb) from seeds of 4 plants of each line ranged from0 to 18ppb as against 150ppb in wild type seeds.

DISCUSSION

The rationale behind the present study was to give an insight into the possible bioassay techniques that can be utilized to characterize and evaluate the transgenics harboring different antifungal genes. The study was carried in a set of transgenic ground nut plants over expressing combination of antifungal gene to elucidate the importance of selected bio-efficacy/bioassay experiments. Irrespective of the mode of action the antifungal transgene, the transgenic plants should show resistance to the target fungal pathogen. Based on the above reasoning, Bioassay experiments can be designed to evaluate the bio-efficacy of Transgenic plants both *in-vitro* and *in-vivo* and at all stages of plant growth from seedling level to harvesting stage by testing the transgenic plants against the target fungal pathogen. We started with an *in-vitro* bioassay where in detached transgenic leaves showed delayed infection by the mycelia even though the leaves were completely embedded on *Cercospora arachidacola* live culture. This clearly indicates that expression of the transgene evading the infection. Similarly in the *in-vivo* assay at the whole plant level the transgenic plants resisted the onset of tikka disease. Calculation PDI [18] wherein in the PDI of transgenic plant ranged from 5-20% which was considered as resistant PDI of some of the transgenic plants ranged between 25-50% which were considered as moderately resistant Plants showing PDI above 50% were considered susceptible. These variations in the bio-efficacy levels among the transgeneics might be attributed to varied levels of expression of the transgene. Similar line of study was also carried out by designing *In-vitro* bioassay experiment against *Aspergillus flavus* at seedling level. Very high levels of resistance against *Aspergillus* in transgenic seeds was observed showing that irrespective of the growth stage of the plants, bioassays prove to be a reliable technique. The infection of *A. flavus* and the subsequent aflatoxin production mainly occurs during the pre-harvest stages of the plant [19][20] and drought is said to be a predisposition factor for the aflatoxin production. Based on this an *in-vivo* bioassay was designed such that the infection was induced in the pre-harvest stage of the plant and conditions were made favoring for *Aspergillus flavus* to produce aflatoxins in the kernels. After harvest when put for germination, the seeds of wild-type plants showed acute levels of infection which indicated that the conditions created in the experiment were indeed favorable for the infection of *Aspergillus* and at the same time healthy germination in the transgenic seed indicate that the antifungal gene resisted the infection in whole plant. After harvest when the aflatoxin content were quantified in the seeds revealed very high levels of accumulation of aflatoxin up to 150ppb in wild type seeds while there was negligible amounts of aflatoxin detection in the transgenes indicating that the levels of aflatoxin contamination in seeds is directly proportional to the infection hence by avoiding the *A flavus* infection the dangerous levels of aflatoxin contamination can be prevented.

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4.CONCLUSION:

To conclude, bioassays play a key role in elucidating the effect of transgenes. These assays are simple, in-expensive and reliable technique which can be adopted for characterizing the transgenic plants. This bioassay can be experimented on any stage of the plant either In-vivo or In-vitro which adds to its advantages. Though this particular study was concentrated mainly on two devastating fungal pathogens *Cercospora arachidacola* and Aspergillus flavus, these assays can also be extrapolated by modifying the experiments based on the host plant, causative agent and the transgene.

CONFLICT OF INTEREST

The authors declare that no competing financial interests exist.

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