INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE IN
ARACHIS HYPOGAEA L. BY ASPERGILLUS FLAVUS
DERIVED ELICITORS

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Abstract: Aflatoxin production caused by Aspergillus flavus in peanut (Arachis hypogaea L.) seeds is a serious problem. Fungal components of Aspergillus flavus in form of fungal culture filtrate and mycelial cell wall fractions were used as elicitors to provoke the defense response in four different varieties (J-11, GG-20, TG-26 and TPG-41) of Arachis hypogaea L. Biochemical changes i.e., salicylic acid as signaling molecule and marker enzymes such as peroxidase, phenyl alanine ammonia lyase, β-1, 3 glucanase and lignin content. There was a substantial fold count increase in peroxidase, phenylalanine ammonia lyase activity, salicylic acid and β-1, 3 glucanase and lignin content in fungal culture filtrate and mycelial cell wall fractions treated plants of all varieties of groundnut than in control plants. Higher enzyme content was observed in fungal culture filtrate treated plants than mycelial cell wall fractions treated plants. Levels of aflatoxins were negligible in all FCF treated four varieties of groundnut plants stored seeds as compared to mycelial cell wall fractions and control plants seeds. The present results indicate that the use of fungal components (fungal culture filtrate and mycelial cell wall fractions) had successfully induced systemic resistance in four different varieties of groundnut plants against Aspergillus flavus.

Key words: Aflatoxin, Aspergillus flavus, Arachis hypogaea L.
exchange for the developing countries [4]. Health consequences related to consumption of aflatoxin-contaminated food include impaired growth in children, liver cancer, immuno suppression and synergism with hepatitis B and C viruses [5]. Progress has been made in an attempt to prevent aflatoxin contamination in crops through crop management and handling, microbial ecology and biocompetitive microbes and crop resistance through genetic engineering and conventional breeding [5].

Farmer prefers to opt fungicides to control the disease, but many of these chemicals the fungicides are ineffective in controlling A. flavus. As an alternative to fungicide application it may be possible to induce a plant defense response by exogenous application of certain chemicals in order to provide protection against A. flavus in groundnut. Various methods of control have been investigated including genetic control [6,7] biological control [8] chemical control [9]. All of these above strategies did not yield any promising results and also affected the environment. Hence there is a need to find alternative methods to control aflatoxin contamination and reduce the cost of disease.

As an alternative to these methods, it may be possible to induce a plant defence response by exogenous applications of certain chemical or biological agents in order to provide protection. Biological control enhanced resistance in several crop plants. Plants have a basic defence mechanism in which, sensing the presence of the pathogen and subsequent transduction of signal in the cell. The second part of the basic defence is the localization of the pathogen through hypersensitive reaction (HR). It is widely known that plants can defend themselves against pathogen infection through a variety of mechanisms, either local, constitutive or inducible [10,11]. The ability of plants to defend themselves actively with inducible and constitutive mechanisms has been extensively demonstrated in many plant systems [12]. For reducing severity of groundnut stem rot, biological control using antagonistic Pseudomonas fluorescens appears to be a potential management tool [13]. Several workers reported change in host defence activities after application of biocontrol bacterial strains against soil-born [14-17] and foliar fungal diseases [18]. Fungal components such as fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) used as elicitors might induce defence related enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (POX), β-1, 3 glucanase and salicylic acid which can mimic a defence mechanism in plants similar to the vaccination mechanism in humans and animals. Moreover, elicitors can be used as the source of biofertilizer and biological control which has its own benefits like cost reduction, ecofriendly in nature, not affecting any other organisms, when compared to chemical controlling agents. In the present study investigation is mainly focused on inducing the natural defence mechanism in groundnut plants against Aspergillus flavus contamination to improve the health and incomes of groundnut farmers by using its fungal components in the form of FCF and MCW.

MATERIALS AND METHODS

Selection of plants and fungal culture: Four varieties (J-11, GG-20, TG-26, and TPG-41) of groundnut growing in local area, Anand district of Gujarat were selected. The fungal culture of A. flavus (MTCC No. 4613) was procured from Junagadh Agriculture University, Gujarat. Fungal strains were maintained on potato dextrose agar medium at 25 ± 2°C.

Preparation of fungal culture filtrate and mycelial cell wall fraction: Fungal culture filtrate (FCF) was prepared by inoculating 8mm agar plug of fungus A. flavus in potato dextrose broth (Hi-media, India) and incubated at 25±2°C for 28 days. Total protein in this filtrate was measured at every 7 days intervals up to twenty eight days by method described by Lowry et al. [19]. Twenty-one-day old fungal mat (7.9gm) was harvested by gentle filtration with muslin cloth, washed with sterile distilled water and crushed in liquid nitrogen and then suspended in 50 ml of 0.1M phosphate buffer (pH 7.4) to prepare mycelium cell wall fraction. Cell wall protein was also measured every week uptil twenty eight - days and the elicitor inoculum was selected based on the protein concentration.

Selection of concentration of FCF and MCW: Twenty-one-day old FCF and MCW of A. flavus were selected for elicitor treatment on the basis of higher protein concentration. Murashige Skoog’s (MS) basal medium [20] supplemented with different concentrations of FCF and MCW i.e. (5, 10, 20, 30…100%) were used for in vitro germination and growth of groundnut plants. Enzyme activities such as phenylalanine ammonia lyase (PAL) and
peroxidase were checked in fifteen days old in vitro grown plants at 24 hour intervals for one week. The concentration of FCF and MCW was standardized on the basis of elevated level of enzyme activity.

Groundnut varieties were raised under controlled irrigation conditions at Lingda, Anand district, Gujarat, India. The experiments were laid in randomized block design in a plot size of (10X10 m$^2$) for each treatment. Forty five days old groundnut plants of J-11, TG-26, GG-20, and TPG-41 were used for the treatment of elicitors. Initially, hypersensitive (HR) response (necrotic lesions) were observed by applying the 200 µl of 10% FCF and MCW of A. flavus on the adaxial surface of the leaves of different varieties of groundnut growing in the experimental field. Ten percent FCF and MCW were applied separately to the foliage of plants by using a hand-operated atomizer. Control plants were treated with Potato dextrose broth (PDB) and distilled water. Leaves from five young plants from each treatment were rando-mly excised at different time intervals after elicitation (24, 48, 72, 96, 120, and 144 hrs) with FCF and MCW to determine the activities of defence related enzymes.

Enzyme extract preparation: Specific enzyme activities such as phenylalanine ammonia lyase, peroxidase, β-1, 3 glucanase and salicylic acid were quantified. The experiment was repeated thrice under similar conditions and the analyses were performed in triplicate. One gram of leaf tissue was homogenized in 5ml of prechilled extraction buffer (0.1 M phosphate buffer, pH 7.0 added, 2mM polyvinyl pyrollidone, 0.1M β-mercaptoethanol) and centrifuged for 10 min at 9200g using Sigma refrigerated centrifuge, Laborzentrifugen GmbH, Osterode, Germany. The supernatant was collected and used as an enzyme extract.

Peroxidase activity: The reaction mixture for peroxidase activity consists of 1ml of 0.1 M potassium phosphate buffer (pH 7.0), 200µl of enzyme extract and 1ml of 0.01 M orthodianisidine. To this mixture, 500µl of 20mM H$_2$O$_2$ was added and the change in absorbance was measured at 436 nm up to 3 min at 15 seconds interval. One unit (U) of enzyme is defined as the change in the absorbance 0.1/unit (O.D/min) under specific assay conditions [21].

Phenylalanine ammonialyase activity: The phenylalanine ammonia lyase assay reaction consisted of 1ml of enzyme extract, incubated at 30°C with 900 µl of 150mM tris HCL (pH 8.5) containing 50mM L-phenylalanine. One unit of enzyme activity was calculated as the amount of enzyme that formed 1 mg cinnamic acid/hr. Deamination of phenylalanine to transcinamate was followed by measuring the absorbance of the mixture at 280 nm over a period of 5 min [22].

Salicylic acid level: Fresh leaf tissue was chopped and placed in 10ml hexane and stored overnight at 4°C. The hexane extract was collected and filtered by using 0.22 µm filters. HPLC analysis was carried out by using C-18 ZORBAX ODS column (4.6 mm ID X 250 mm, 5 µm particle size, Agilent Technologies, Waldbrown, Germany) with the solvent system consisting of equal volume of methanol: phosphate buffer (pH 5.5, 0.02M), with flow rate 1 ml/min at 35°C to quantify the salicylic acid levels. Samples were monitored at 315 nm [23]

β-1, 3 glucanase activity: The activity of β-1, 3 glucanase was determined by measuring the release of reducing sugars by using laminarin as substrate and glucose as standards. The reaction mixture consisted of 100 µl of enzyme extract, 300 µl of 1M sodium acetate buffer (pH 5.3) and 0.5ml of 4% (w/v) laminarin for β-1, 3 glucanase activity [24]. The reaction was carried out at 40°C for 2 hours. The reaction was stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min on a boiling water bath, vortexed and its OD was measured at 500nm using UV spectrophotometer. The specific activity of β- 1, 3 glucanase was expressed as glucose released units/gm fresh leaf weight.

Aflatoxin level measurement: The aflatoxin level was estimated by using [25] twenty five gram of groundnut sample was taken into Erlenmeyer flask and 100ml of acetonitrile and water were added in 84:16 ratios. Samples were shaked for 1 hour and filtered the sample and HPLC analysis was carried out by using Puri Tox$^{SR}$ column (TC-A100 or TC-M160 quantitative filter paper 15 X 125mm test tube) with the solvent system consisting of mobile phase Acetonitrile and water (1:8) with flow rate 0.5 ml/ min at 35°C to quantify the salicylic acid levels. Samples were monitored at (Emission= 430 and Excited=369 nm).

RESULTS

Protein concentration in fungal culture filtrate and mycelial cell wall fractions of A.flavus was measured
Fig. 1: Induction of peroxidase enzyme activity in groundnut plants of J-11, GG-20, TG-26, TPG-41 varieties upon foliar spray applications of 10% each of fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) derived from Aspergillus flavus. Data points are the mean of three applications in three sets of the experiment. Error bars indicate standard deviation.

Fig. 2: Induction of phenylalanine ammonia lyase (PAL) enzyme activity in groundnut plants of J-11, GG-20, TG-26, TPG-41 varieties upon foliar spray applications of 10% each of fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) derived from Aspergillus flavus. Data points are the mean of three applications in three sets of the experiment. Error bars indicate standard deviation.
Fig. 3: Induction of salicylic acid level in groundnut plants of J-11, GG-20, TG-26, TPG-41 varieties upon foliar spray applications of 10% each of fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) derived from Aspergillus flavus. Data points are the mean of three applications in three sets of the experiment. Error bars indicate standard deviation.

Fig. 4: Induction of β-1, 3 Glucanase activity in groundnut plants of J-11, GG-20, TG-26, TPG-41 varieties upon foliar spray applications of 10% each of fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) derived from Aspergillus flavus. Data points are the mean of three applications in three sets of the experiment. Error bars indicate standard deviation.
at different time intervals up to twenty eight days. There is a gradual increase in the protein concentration in fungal culture filtrate and mycelial cell wall fraction. Protein concentration in fungal culture filtrate was higher (3.82mg/ml) in twenty one day old culture after the inoculation, whereas the protein concentration of mycelial cell wall fraction was 1.2mg/ml and then decreased gradually. The FCF and MCW of twenty one day old cultures were harvested and used as elicitors for treatment.

Various concentrations (5, 10, 20, 30 and 100%) of FCF and MCW of A. flavus were selected for elicitation in in vitro grown plants on MS basal medium. FCF and MCW at ten percent concentration showed higher fold induction of PAL (8 fold at 96 hrs) and peroxidase (5 fold at 96 hrs) activity in groundnut plants when compared to other selected concentrations of FCF and MCW. Both the fungal components at ten percent concentration were further used for elicitor treatment.

Before application of fungal components as elicitors hypersensitive response (HR) was detected by using the fungal components directly and necrotic lesions were observed at the site of elicitor applications after 24 hrs on the adaxial surface of leaves of groundnut plants. The lesions were more prominent and distinct in FCF treated leaves than MCW treated leaves, where the intensity of lesions was inconspicuous.

Defence responses were checked by estimation of peroxidase activity (POX), phenylalanine ammonia lyase (PAL) activity, lignin content, salicylic acid (SA) level and β-1, 3 glucanase activity in the plants treated with fungal components. Various enzyme activities were observed significantly higher in the plants treated with ten percent FCF and MCW than the controlled ones. (Figs. 1-4).

A significant increase in peroxidase activity was detected in the A. flavus FCF and MCW treated four varieties of groundnut J-11, TG-26, GG-20 and TPG-41 plants by spectrophotometer. However, treatment with FCF recorded significantly higher enzyme activity compared to MCW treatment. The POX activity increased transiently by 51 fold in GG-20, 32 fold in TG-26, 19 fold in TPG-41 variety at 96 hrs, 11 fold in J-11 variety at 120 hrs in FCF treated plants (Fig. 1) and gradually decreased thereafter. The fold count of POX activity was observed to be high throughout the whole set of experiment in FCF treated plants when compared to the controlled and MCW treated plants. The POX activity was observed in MCW treated plants as 13 fold in TG-26 variety, 40 fold in GG-20, 16 fold in TPG-41 variety at 96 hrs and 4 fold in J-11 variety at 120 hrs and then gradually decreased but was erratic.

Higher lignin content was induced in J-11, GG-20 and TG-26 varieties after treatment of FCF and MCW than in TPG-41 variety. The lignin content was also estimated and remained at a very low level in untreated plants. Lignin content increased drastically by 23 fold in J-11 variety and 29 fold in TG-26, GG-20 variety and 19 fold in TPG-41 at 96 hours after treatment with FCF of A. flavus. MCW of A. flavus also elicited the lignin 8 fold in TG-26, 16 fold in J-11, 7 fold in TPG-41 and GG-20 at 96 hrs. FCF of A. flavus elicits higher lignin content compared to MCW of A. flavus.

Enhanced induction of PAL was also evident in all the treated varieties. The PAL activity was observed at 96 hrs 9 fold in GG-20, and 12 fold increases in TPG-41 at 96 hrs, 12 fold in TG-26, 29 fold in J-11 at 120 hours. However, treated groundnut plants with elicitors exhibited significantly higher PAL activity than those in controlled plants. The rise in PAL activity was transient and decreased after 96 hours in all varieties except J-11 variety, but it was high throughout the whole set of experiments when compared to controlled plants (Fig 2). The induction of PAL activity varied among different varieties after MCW treatment. MCW treated plants showed increase in PAL activity at 7 fold in GG-20, 9 fold in TG-26 and 7 fold in TPG-41 at 96 hrs, 18 fold in J-11 variety at 96 hrs. Higher PAL activity was observed in FCF treated plants rather than MCW treated plants.

Induction of salicylic acid levels varied in different varieties of groundnut treated with FCF. Significant

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Sample</th>
<th>Aflatoxin B1(µg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GG20-UT</td>
<td>140.39</td>
</tr>
<tr>
<td>2.</td>
<td>GG20-FCF</td>
<td>0.966</td>
</tr>
<tr>
<td>3.</td>
<td>GG20-MCW</td>
<td>7.8</td>
</tr>
<tr>
<td>4.</td>
<td>J-11 UT</td>
<td>148.92</td>
</tr>
<tr>
<td>5.</td>
<td>J-11-FCF</td>
<td>19.92</td>
</tr>
<tr>
<td>7.</td>
<td>TPG-41-FCF</td>
<td>136.26</td>
</tr>
<tr>
<td>8.</td>
<td>TPG-41-MCW</td>
<td>11.2</td>
</tr>
<tr>
<td>9.</td>
<td>TG-26-UT</td>
<td>208.29</td>
</tr>
<tr>
<td>10.</td>
<td>TG-26-FCF</td>
<td>16.10</td>
</tr>
<tr>
<td>11.</td>
<td>TG-26-MCW</td>
<td>18.8</td>
</tr>
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</table>

Table 1: Aflatoxin level in the different varieties of groundnut
increase in fold count of SA level at 5 fold in J-11 and TG-26, 3.4 fold in GG-20, 3.0 fold in TPG-41 variety at 96 hrs of elicitation was noticed (Fig 3). The activity decreased gradually thereafter. The level of SA was high throughout the whole set of experiment as compared to the controlled plants. In MCW treated plants, the induction of SA levels varied among different varieties. The observed SA levels were lower in MCW treated plants rather than in the FCF treated plants.

β-1, 3 glucanase activity in different varieties of groundnut was significantly high in A. flavus FCF treated plants than MCW treated and control plants. Increase in β-1, 3 glucanase activity was observed at 17 fold in GG-20, 6.8 fold in TG-26 and 4.4 fold in TPG-41 variety at 96 hrs and 37 fold in J-11 at 120 hrs. β-1, 3 glucanase activity was elicited at 96 hrs in GG-20, TG-26 and TPG-41 varieties whereas in J-11 variety at 120 hours (Fig 4). The elicitation of β-1, 3 glucanase activity varied among different varieties of groundnut after treatment with MCW at different time interval and was significantly higher than controlled ones. However, this activity was lower when compared to FCF treated plants.

Aflatoxin levels were checked in the FCF and MCW treated groundnut plant (J-11, TG-26, GG-20, TPG-41 varieties) seeds after one year of storage. (Table 1) The level of aflatoxin was negligible in the FCF and MCW treated plants then compared to the control plants. The minimum edible level of aflatoxin in groundnut is 20 µg/Kg.

**DISCUSSION**

The molecules hailing during plant pathogen interaction either from plant or pathogen are known as elicitors inducing plant defence gene that ultimately lead to broad spectrum resistance [26]. The defence responses in *Arachis hypogaea*. L was provoked by both FCF and MCW of *Aspergillus flavus* elicitor. The fungal components in form mycelial cell wall and fungal culture filtrate mimic to a certain extent the real pathogen and provoke a large number of different defence related molecules including peroxidase, phenylalanine ammonia lyase, salicylic acid, lignin content and β-1, 3-glucanase. This defence related enzymes are known to protect the plants from fungal attack either by direct inhibition of pathogen or indirect activation of host defence response including cell wall thickening [27,28]. In this study, induction of systemic acquired resistance with fungal components on activating the traits involved in defence mechanism was demonstrated. Similarly Patel et al. [29] demonstrated that the use of fungal (FCF and MCW) derived from *Fusarium sps* has successfully induced systemic acquired resistance in *in-vitro* propagated banana (*Musa paradisiaca*) plantlets. The concentration of FCF and MCW as elicitors was determined on the basis of increased activities of PAL and peroxidase in *in vitro* treated plants. The fungal components at 10% concentration provoked both the enzyme activities compared to other concentrations tested. The elicitor concentration was fixed at 10% and used for elicitation in field grown plants. This study clearly demonstrated that FCF from *A. flavus* induced resistance in different varieties of groundnut plants against pathogens, whereas MCW from *A. flavus* did not induce much resistance on the basis of higher fold count induction of enzyme activity.

Hypersensitive response (HR) is characterized by a deposition of different phenolic compounds, synthesis of diverse phytoalexins and accumulation of pathogenesis related (PR) proteins [30]. In the present study, the HR response, appearance of necrotic lesions, on leaves of FCF treated plants probably indicates that the plants react to the pathogen elicitors by synthesizing the defence related enzymes there by protecting themselves against the pathogen. However, the appearance of HR response is insignificant in MCW treated plants as compared to FCF treated plants. The disease incidence is not seen in the field in both the treatments.

In our study, significant increase in peroxidase activity in terms of fold count in different varieties of groundnut may be one of the expressions of defence reactions after treatment. These results are in agreement, similar to the reports on induction of systemic resistance in groundnut by foliar applications of systemic resistance in groundnut by foliar applications of biocontrol agents/elicitors. [16,18,31].

The increase in PAL activity in treated plants of different varieties of groundnut probably may be related to the biosynthesis of various defence chemicals in phenyl propanoid metabolism such as phytoalexins, phenols, lignin and salicylic acid that has direct or indirect effect on pathogens [32-34]. In the present study, *A. flavus* FCF treated groundnut varieties PAL activity was much higher than MCW.

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treated plants whereas the enzyme activity remains constant in control plants. The lignin content was also found higher in FCF treated plants of all varieties than compared to control and MCW treated plants. Lignification has been observed in many plant species following attempted infection by pathogenic fungi, bacteria, viruses and nematodes [35]. Several reports indicate the strong evidence that lignification is an important mechanism in disease resistance [36-38]. In comparison with controls in this study, the increased activities of β-1, 3-glucanase in all four varieties were evidence after challenged by FCF and MCW of A. flavus. The increases in fold activities of enzymes were higher in FCF treated plant than compare to MCW treated plants. Over expression of a soybean β-1, 3-glucanase in tobacco increases resistance to *phytophthora megasperma* and *Alternaria Alternata* [39,40].

*Aspergillus flavus* FCF treated plants showed higher level of salicylic acid in our study as compared to MCW treated and controlled plants of all varieties. Several researchers reported the activation of hypersensitive response after elicitation, triggers a systemic resistance response which includes the accumulation of signal molecule salicylic acid (SA) throughout the plant and extraction of the characteristic set of defence gene, including the PR gene i.e. β-1, 3-glucanase [41- 43].

*A. flavus* FCF treatment reduced the aflatoxin contaminations in different varieties of groundnut than compared to control plants. The results confirm the tolerance of the selected varieties to aflatoxin contamination and can play a significant role in the integrated management of the aflatoxin problem. Several approaches have been recognized to minimize aflatoxin contamination in agricultural commodities. These comprise breeding for resistance to fungal contamination in, good agricultural production, processing handling and storage practices. However, there has been little success in the development of resistant varieties of groundnut that are resistant to aflatoxins [43].

Several workers have undertaken studies for controlling aflatoxin contamination in groundnut, integrated pest management in groundnut in India [8,9] and demonstrated induction of peroxidase and polyphenol oxidase and β-1, 3 glucanase activities in *Arachis hypogaea* by inoculation with *Aspergillus flavus* and copurifies with a conglutin-like protein.

In the present study, the increase in fold count of enzyme activities i.e. POX, PAL, β-1, 3 glucanase, salicylic acid were higher in FCF treated plants than MCW treated plants, which gives concrete support to the fact that *A. flavus* secretary proteins in FCF and MCW when given as elicitors to different groundnut varieties (J-11, TG-26, GG-20 and TPG-41), can successfully induce systemic acquired resistance. Aflatoxin level in stored groundnut seeds (treated with FCF, MCW & D/W) also revealed that the treatment of *A. flavus* FCF and MCW drastically reduced the incidence of aflatoxin disease in the different varieties of groundnut. Effective disease control certainly leads to groundnut quality improvement and increase in yield.

From the present study, it can be concluded that higher accumulation of enzymes involved in phenylpropanoid metabolism and of PR-proteins in leaves in response to treatment with *A. flavus* fungal components (FCF and MCW) might have induced resistance against *A. flavus* in groundnut plants of J-11, TG-26, GG-20, TPG-41 varieties. The use of fungal components as biocontrol agents might reduce the pesticide residues in the environment and enhance the quality of the crop. Findings of this investigation might lead to development of biocontrol agents to control the Aflatoxin contamination of groundnut.

**Abbreviations:** FCF: Fungal culture Filtrate,  HR: Hypersensitive response, MCW: Mycelial cell wall fraction, PAL: Phenylalanine ammonia Lyase, PDB: Potato dextrose broth, POX: Peroxidase, PR Proteins: Pathogenesis related proteins, PVP: Polyvinyl pyrrolidone, SA: Salicylic acid

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