

# The Role of *veA* in *Aspergillus flavus* Infection of Peanut, Corn and Cotton

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**Abstract:** The aflatoxin-producing fungus *Aspergillus flavus* is a causal agent of preharvest contamination of food commodities such as oil seed crops worldwide. Peanut, corn and cottonseed are among the oil seeds that are susceptible to aflatoxin contamination during invasion of these crops by *A. flavus*. Contamination of agricultural commodities with aflatoxins can result in serious economic hardships to producers and adverse health impacts in both humans and domestic animals. It is therefore of great importance to determine ways to control *A. flavus* dissemination, survival and toxin formation. In this study we demonstrate the role of the *veA* regulatory gene in the contamination of peanut, corn and cotton by *A. flavus*. Virulence of *A. flavus* on peanut and corn seeds was reduced in the absence of the *veA* gene product. Generation of air-borne asexual spores was reduced and production of aflatoxin and sclerotia in peanut seeds, viable or non-viable, or in viable corn seed was completely blocked when infected with the *A. flavus veA* mutant ( $\Delta veA$ ). *In planta* inoculation of cotton bolls also showed that conidiation was decreased in bolls inoculated with the  $\Delta veA$  strain and spread of the  $\Delta veA$  strain to seed in locules adjacent to the inoculated locule was less than observed with the wild-type *veA* strain. As observed in peanut and corn, no aflatoxin was produced in seed harvested from cotton bolls inoculated with the  $\Delta veA$  strain while aflatoxin was present in seed from wild-type *veA* inoculated bolls.

**Keywords:** Aflatoxin, *Aspergillus flavus*, *veA*, Seed infection.

## INTRODUCTION

*Aspergillus flavus* is a soil-inhabiting, filamentous fungus that saprophytically utilizes a wide range of organic substrates. Though *A. flavus* is considered a saprophyte, it is also an opportunistic pathogen [1] that can invade agronomically important oil seed crops such as corn, peanut and cottonseed that are under biotic or abiotic stress. Among the toxic secondary metabolites produced by this organism are the aflatoxins (AFs) with aflatoxin B1 being the most potent natural carcinogen known [2-5]. AFs are polyketide-derived compounds and the genes encoding enzymes involved in their biosynthesis are found clustered [6-8]. The adverse effects of AF contamination in crops such as corn can be much more serious in developing countries because in these regions the largest proportion of the crop is used for human consumption and there is a limited capacity to monitor the level of AF contamination in the grain [9]. Ingestion of food contaminated with high levels of AF has been implicated in acute toxicoses that often results in death while chronic, low-level exposure can lead to liver cancer, immune suppression, and stunting of growth in children among other pathological conditions [10, 11]. The liver is the primary target organ in animals and humans where AFB1 is metabolized to the toxic and carcinogenic AFB1-epoxide form by cytochrome P450 enzymes [12-14]. From an economic perspective, the total annual loss to the U.S. corn industry alone has been estimated at approximately \$200 million. This includes losses from market rejections,

livestock losses, and the costs of AF sampling and testing [15]. Despite the many advances made in the biochemistry, genetics, and ecology of *A. flavus* and AF production, current control strategies fail to effectively eliminate AF contamination. Additional knowledge of the genetic mechanisms that control *A. flavus* morphogenesis, survival, and its ability to invade host tissues [16, 17] could lead to the development of novel biotechnological control strategies, contributing to the reduction of pre-harvest AF contamination.

*A. flavus* air-borne conidia (asexual spores) are an efficient means of dissemination for the fungus, leading to vast field infestations. In addition, under adverse conditions *A. flavus* is able to survive by forming numerous spherical, resistant structures called sclerotia [18-20]. High concentrations of AFs may occur in both conidia and sclerotia of *A. flavus* and certain toxicities associated with *A. flavus* infection have been attributed to the combined activities of AFs and other metabolites present in sclerotia [21]. Sclerotia are proposed to be vestigial forms of sexual cleistothecia, produced by other *Aspergillus spp* (*i.e.* *A. nidulans*), that lost the capacity to produce spores [22]. For this reason, it is likely that genes homologous to those known to govern the formation of cleistothecia could be involved in the formation of sclerotia in *A. flavus* and other sclerotial-producing *Aspergilli*. Supporting this hypothesis, we have shown that the *veA* regulatory gene, required for cleistothecial production in *A. nidulans*, is also essential for sclerotial production in *A. flavus* and *A. parasiticus* [23-25]. Interestingly, *veA* has only been found in the fungal kingdom (Calvo, unpublished data).

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A number of studies have demonstrated that fungal morphogenesis is associated with natural product biosynthesis, including mycotoxin production (reviewed in [26, 27]). We hypothesize the existence of common genetic regulatory mechanisms that govern both toxin production and morphological differentiation. In previous studies we have demonstrated that the same *veA* regulator that controls morphogenesis is also necessary for the production of several secondary metabolites, including AF in *A. flavus* and *A. parasiticus* [23-25], and sterigmatocystin (ST) in *A. nidulans*, where ST is the penultimate precursor in the AF biosynthetic pathway [28]. Interestingly, *veA* regulates the biosynthesis of other natural products indicating that *veA* is a global regulator of fungal morphological differentiation and secondary metabolism [23, 28].

Until now the role of *A. flavus veA* has been characterized on laboratory medium only. In this study we have taken the next step by evaluating the importance of *A. flavus veA* on the ability of the fungus to colonize and produce AF during growth on a variety of common, natural substrates such as peanut, corn and cottonseed.

## MATERIAL AND METHODS

### Strains and Culture Conditions

An *Aspergillus flavus* SRRC 70 isolate in which *veA* has been replaced by the *A. parasiticus niaD* marker gene ( $\Delta veA::niaD$ , referred to in this manuscript as  $\Delta veA$ ) [23] and an isogenic *A. flavus* 70 (pSL82) harboring a wild-type *veA* gene (referred to as *veA+*) were utilized in this study. The methods used to obtain the *A. flavus veA* strain used in this study are detailed in Duran et al., [23]. Three transformation controls (*veA+*) and five  $\Delta veA$  strains were obtained. Initial characterization of these strains showed that all *veA+* control strains had a consistent wild-type phenotype. The  $\Delta veA$  mutant phenotype was also consistent [23] and similar to those described in other *Aspergillus* spp. [24, 28]. Based on the consistency of these results, one representative *veA+* and  $\Delta veA$  strain were chosen for further studies, including functional genomics [29], and the present infection studies. Conidia of each culture were collected in 0.01% Tween 80 from point inoculated PDA plates that had been grown at 30°C in the light, a condition that promotes conidiation. Conidial suspensions were quantified with a hemacytometer under a light microscope and used as inoculum for the seed and plant infections.

### Seed Inoculations

#### Peanut

Two peanut seed lines were used in this study, Flow Runner and Southernrunner (kindly provided by Dr. C. Holbrook, USDA, Georgia). The seeds were prepared following previously described procedures [24, 30]. In brief, first, seeds were shelled, the cotyledons separated, and the embryo removed. Then seeds were weighed so that all the peanuts used were approximately 0.6 g. For non-viable seed experiments, the cotyledons were autoclaved in a liquid cycle for 30 min. For viable seed experiments, cotyledons were surfaced sterilized by immersion in 10 % Clorox bleach for 1 min followed by two washes in sterile distilled water for 1 min. Seeds were then inoculated with either the *veA+* or  $\Delta veA$  strains and incubated as follows: For each strain, four

peanut cotyledons were placed in sterile glass Petri dishes, resting on 3-MM chromatography paper (Whatman, Maidstone, England) saturated with sterile deionized water. The Petri dishes also contained a moisture reservoir with 5 ml of water. One hundred microliters containing  $2.0 \times 10^6$  spores were spread on top of each cotyledon adaxial face. Cotyledons were incubated for 7 days at 30°C in the light ( $25 \mu\text{E m}^{-2} \text{s}^{-1}$ ). The experiment was repeated twice with similar results. To further demonstrate that the *veA* deletion is the sole cause of the phenotype observed in this study, additional peanut infection experiments were performed with two additional *veA+* control and  $\Delta veA$  mutants and consistent (no significant difference,  $P < 0.01$ ) results were obtained (data not shown). In all seed-inoculation experiments, Koch's postulates were determined after spore-counting to verify the identity of the *Aspergillus* strains infecting the seed.

#### Corn

Surface sterilized kernels of genotype B73 (a kind gift from Dr. P. Williams, USDA, Mississippi State) were immersed in a 0.01% Triton X-100 conidial suspension of either the *veA+* or  $\Delta veA$  strain ( $5.0 \times 10^6$  conidia per ml) and evaluated using a kernel screening assay (KSA) [31]. Briefly, following immersion in the fungal spore suspension, individual kernels were placed in plastic vial caps (20 mm dia. x 6 mm height) that were positioned side by side in an open 60 x 15 mm tissue culture plate bottom. One experimental unit consisted of four kernels in one plate bottom. A total of 10 culture plate bottoms containing four kernels each were placed in a clear tray (243 x 243 x 18 mm Nunc bioassay tray, Thomas Scientific, Swedesboro, NJ) lined with 3-MM chromatography paper (Whatman). The lid was placed on top of the tray and high humidity was maintained by adding about 30 ml of sterile deionized water to saturate the chromatography paper. Additional deionized water was added as needed during incubation to maintain humid conditions. The experiment was replicated 5 times. Kernels were incubated at 30°C in the light for 7 days after which they were removed from the trays for further analyses.

#### In-Planta Cotton Assay

Cotton plants (*Gossypium hirsutum* var. Coker 312) were greenhouse grown in 10-gal pots containing MetroMix 366 (Scotts). Plants were watered twice daily to field capacity and fertilized at planting with 15 g slow release Osmocote (19%N:6%P:12%K; Scotts). Greenhouse temperatures ranged from 21-35°C. Flowers were dated at opening and 24-25 dpa bolls were inoculated with either the *veA+* or  $\Delta veA$  spore suspensions. Twenty cotton bolls present on greenhouse grown plants were wounded in the center of one of the locules to a depth of approximately 2 mm with a 3 mm-diameter cork borer to simulate pink bollworm exit holes. Approximately  $10^4$  conidia in 10  $\mu\text{l}$  of 0.01% Triton X-100 were inoculated at the wound site and the fungus allowed to colonize the bolls for three weeks. Ten bolls inoculated with either of the fungi were collected and analyzed for fungal spread and aggressiveness. Fungal spread from the inoculated locule to the adjacent locules was measured by the amount of ergosterol present in the seed of the adjacent locules. Intercarpellary membranes adjacent to the inoculated locule were examined to measure fungal aggressiveness [32]. Membranes were rated as follows; 0, no symptoms; 1,

discolored on the surface next to the inoculated locule; 2, same as a rating of 1 plus slightly discolored on the opposite face; 3, highly discolored on both faces; 4, same as 3 plus pinhole tears; 5, same as 3 plus 1 mm or larger tears.

### Conidiation and Sclerotial Formation Studies

Conidia were released from the infected seeds by adding 3 ml of Tween 80 water and vortexing for one min. Conidia were counted with a hemacytometer (Hausser Scientific, Horsham, PA). Four peanut cotyledons and 8 corn seeds were used for counting per treatment. Sclerotia were visualized with a Leica MZ75 dissecting microscope. Micrographs were obtained using Leica MZ75 microscope attached to a digital camera (Spot Insight color Model #3.2.0, Diagnostic Instruments, Sterling Heights, Michigan).

### AF Analysis

Seeds (4 infected peanut cotyledons, 8 corn kernels, or 8 cottonseeds) were ground in liquid nitrogen. Ground material was added to 50 mL of sterile water. Then 25 mL of acetone was added to each sample and shaken on a rotary platform at room temperature for 1 h. Each sample was filtered through Whatman paper and added to a 250 mL separatory funnel. Seventy mL of methylene chloride (MeCl) was added to the funnel and inverted three times. The liquid phases were allowed to separate. A fluted funnel containing a filter filled with sodium sulfate was placed in a beaker to absorb the remaining water. The bottom layer in the separatory funnel was released through the sodium sulfate and collected in the beaker. Liquid extracts were allowed to dry overnight. Dried extracts were then resuspended in 2 mL of MeCl, and transferred to a 2 mL tube, allowed to dry once again and the residue was resuspended in acetone and spotted on a TLC plate (Si250F, J.T. Baker). The plate was then developed in toluene/ethyl acetate/formic acid (5:4:1, v/v/v), and air-dried. The TLC plates were sprayed with aluminum chloride (15% in ethanol) to intensify AF fluorescence upon exposure to long-wave (365-nm) UV light and baked for 10 min at 80°C prior to being viewed. The approximate sensitivity of the assay was 25 ng. AF purchased from Sigma-Aldrich (St. Louis) was used as a standard.

### Ergosterol Analysis

The ergosterol content of peanut seeds (cotyledons) was analyzed as a quantitative measure of fungal growth in the colonized plant material (in addition to the micrographs shown). Ground seeds (1g) were extracted overnight at room temperature in 4 mL of 2:1 chloroform:methanol, filtered through sterile miracloth, dried and resuspended in 3 mL of the same extraction mixture. Ergosterol analysis was performed by injecting 25 µl of the suspension into a Waters 1525 HPLC system equipped with a binary pump and a Waters 717 Autosampler. HPLC separation was performed at 50°C on a Phenomenex C18 4.6×25 mm, 5 micron analytical column with a column guard. UV detection was at 282 nm (Waters 2487 Dual λ Absorbance Detector). Compounds were eluted using an isocratic mobile phase of 100% HPLC-grade methanol with a flow rate of 1.0 ml/min. Ergosterol quantification of the samples was determined by comparing peak areas of samples to a standard curve created using HPLC-grade ergosterol (Sigma-Aldrich, St. Louis).

### Statistical Analysis

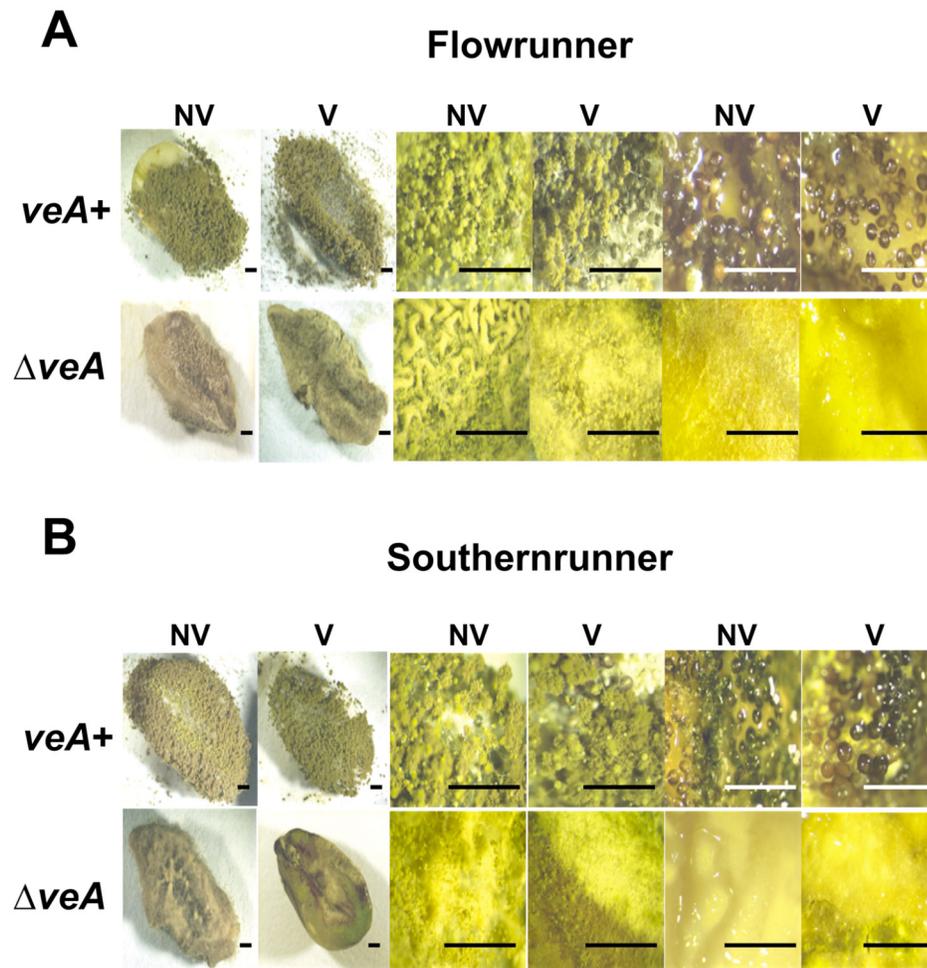
Quantitative data was evaluated by analysis of variance (ANOVA), in conjunction with the Tukey's test for multiple comparisons using the Statistical Package for the Social Sciences (SPSS 15.0 for windows SPSS Inc. Chicago, IL). Fungal discoloration and damage to intercarpellary membranes flanking the infected locules of 10 bolls were assessed and analyzed by Student t-test using GraphPad Prism software. Differences among mean comparisons between the  $\Delta veA$  and *veA+* values were significant ( $P < 0.05$ ) according to the Wilcoxin signed-rank test.

## RESULTS AND DISCUSSION

It is well known that asexual spores are an efficient means of fungal dissemination [33, 34], causing infestation of field crops, as is the case for AF-producing isolates of *A. flavus*. In addition, this fungus produces resistant structures termed sclerotia that are able to survive adverse environmental conditions over several years [18-20] and can contain high levels of AF [35]. When environmental conditions are again favorable, sclerotia will produce mycelia and conidia thus reestablishing infections and AF contamination of food commodities. In this study we investigated the importance of the global regulatory gene *veA* in developmental differentiation, AF production and virulence of *A. flavus* when growing on a variety of oil seeds that are common hosts for this organism. The *veA* gene product is unique to fungi and conserved in *Aspergilli* and across fungal genera (Calvo *et al.*, unpublished). We are evaluating the suitability of *veA* as a possible target for future control strategies to reduce fungal infection and toxin contamination of important agricultural commodities.

### Peanut Infection

All strains were able to grow on peanut seeds, however, we found that peanut seed infection by the *veA+* strain was significantly different when compared to seed infected with the  $\Delta veA$  strain. Conidial formation was reduced in the case of peanut seeds inoculated with *A. flavus*  $\Delta veA$  in comparison with seeds infected with the *veA+* strain. This was true when the strains were growing on Flowrunner or Southernrunner peanut lines, with conidiation reduced to 42% and 3% on viable seeds and to 67% and 57% on non-viable seeds, respectively (Figs. 1, 2A, D). Fungal colonization of the seeds was reduced in the absence of the *veA* gene in either viable or non-viable seeds as determined by micrographs as well as ergosterol levels measured in the infected seeds (Figs. 1, 2B, E). The presence of fungi in commodities such as corn and peanut can be determined by measuring ergosterol which is the predominant sterol in most fungi and not found in plants [36]. Fungal ergosterol content in viable Flowrunner and Southernrunner peanut seeds infected with the  $\Delta veA$  mutant were reduced to 19% and 29% (respectively) as compared to levels obtained in seeds infected with the *veA+* strain. In  $\Delta veA$ -infected non-viable seed the percentage of ergosterol detected was reduced to 45% and 73% in Flowrunner and Southernrunner respectively, compared to the levels observed in *veA+*-infected seeds (Fig. 2B, E). Our results indicate that the presence of a wild-type allele of *A. flavus* *veA* is necessary to establish normal virulence levels in peanut, since its absence results in reduced growth and impaired developmental processes in this agronomically important plant pathogen.



**Fig. (1)** Peanut seed lines contaminated by *A. flavus* wild-type *veA*<sup>+</sup> strain (WT) or deletion *veA* ( $\Delta veA$ ) strain. (A) Flowrunner seed line; (B) Southernrunner seed line. Non-viable (NV) or viable (V) peanut cotyledons. Micrographs were captured with a Leica MZ75 dissecting microscope. The last four panels on the right side (in both A and B) correspond to samples that were sprayed with 70% ethanol to flatten conidiophores for better visualization of sclerotia. Bars represent 2 mm.

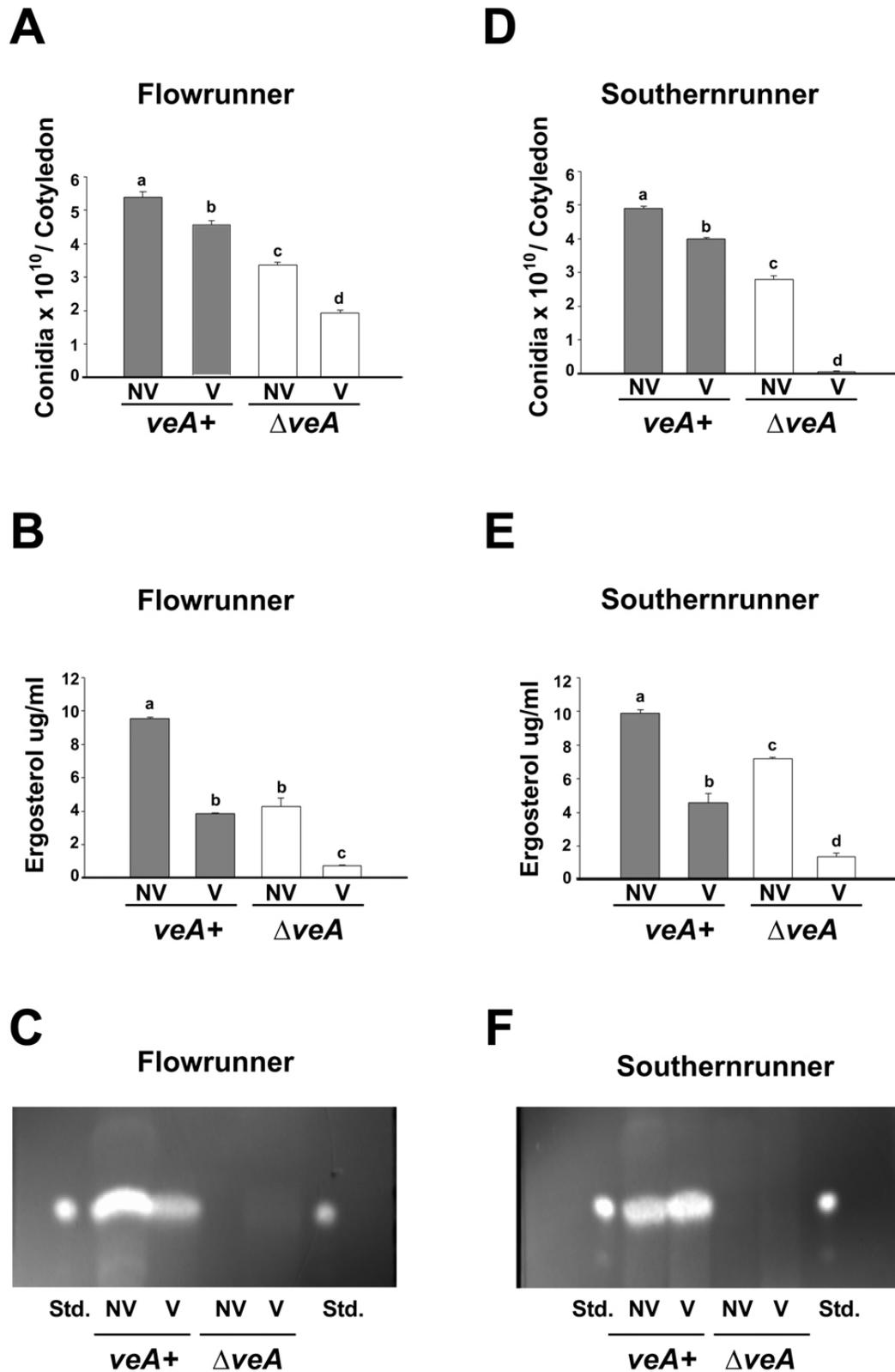
The  $\Delta veA$  mutant is compromised in its capacity to colonize seeds as determined by micrographs, ergosterol levels and reduced conidiation (Figs. 1, 2). This is particularly notable in viable seed assays, exemplified in this peanut seed experiment. In general, viable seeds were more resistant to *A. flavus* infection than non-viable seeds in both peanut lines (Fig. 2). Plants have stress-response defense mechanisms that can be activated by different factors, including fungal attack (*i.e.* [37, 38]). As in our *A. flavus* study, differences between viable and non-viable peanut seeds infected with *A. parasiticus* have been previously interpreted to be a consequence of differences in the effectiveness of the plant defensive response when infected by the fungus [30]. Our results also indicate that *veA*<sup>+</sup> in *A. flavus* is especially relevant for colonization of viable seeds, as shown in both peanut lines tested (Figs. 1, 2).

Sclerotial production was completely blocked in the  $\Delta veA$ -inoculated Flowrunner or Southernrunner peanut seeds, while numerous sclerotia, tightly adhered to the seed surface, were found in seeds inoculated with the *veA*<sup>+</sup> strain. This demonstrates that the *veA* gene is necessary for sclerotial formation in *A. flavus* when growing on peanut seeds (Fig. 1).

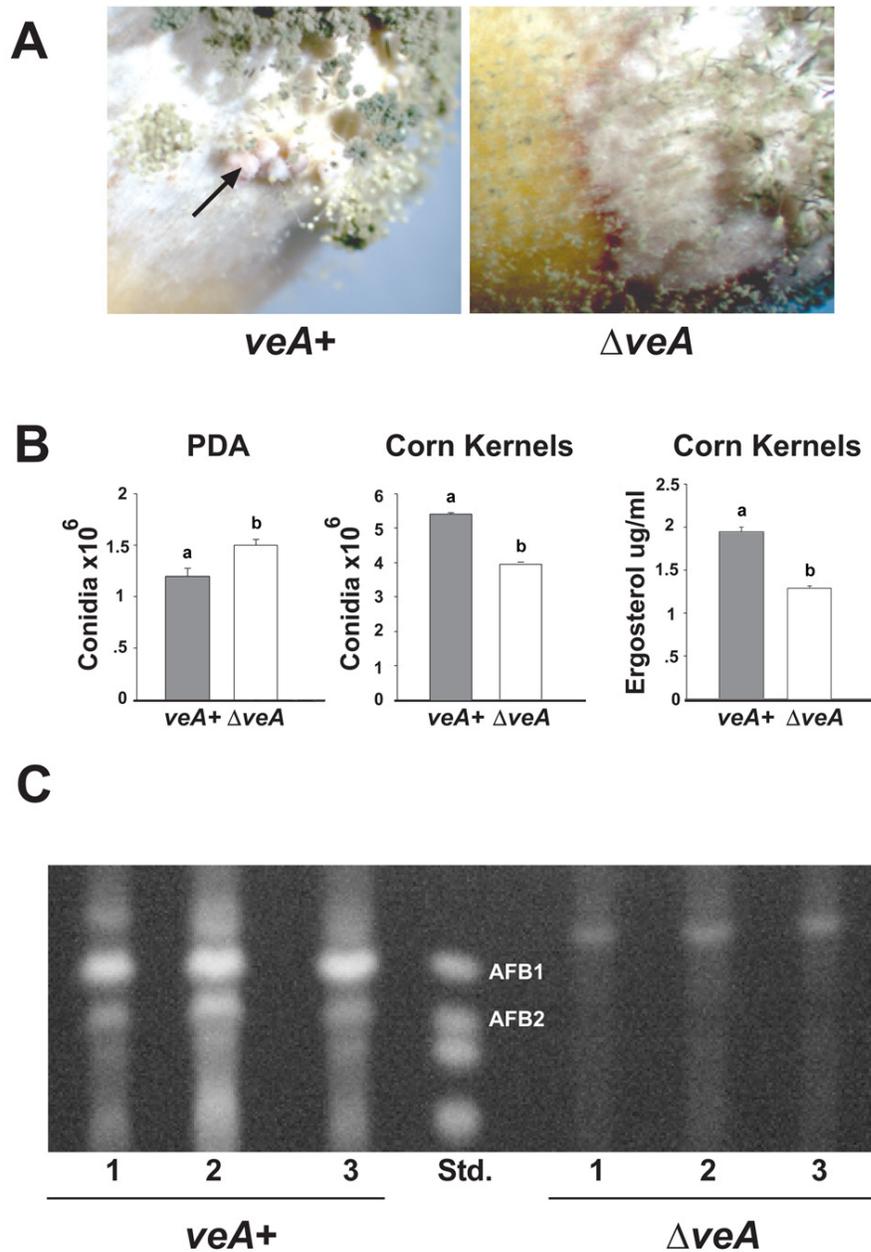
Importantly, our AF analysis of peanut seeds indicated that this mycotoxin was absent from all seeds, viable or non-viable, when infected with the *A. flavus*  $\Delta veA$  mutant, while AF was detected when seeds were infected with the *veA*<sup>+</sup> strain (Figs. 2C, F). This indicates that a functional *veA* gene is necessary for AF production during growth on a natural substrate such as peanut seed.

### Corn Infection

Visual examination corn kernels incubated with illumination revealed a higher level of colonization and conidiation present on the surface of kernels inoculated with the *veA*<sup>+</sup> strain than with the  $\Delta veA$  strain. This was confirmed by counts of conidia (Fig. 3B). Kernels inoculated with the  $\Delta veA$  strain showed a reduction in conidial production of about 11% with respect to the conidia produced by the *veA*<sup>+</sup> strain. Interestingly, the number of conidia increased in the  $\Delta veA$  strain compared to the *veA*<sup>+</sup> strain when grown on artificial medium ([23] and Fig. 3B). In previous studies in the model fungus *A. nidulans* we showed that conidiation levels in the  $\Delta veA$  strain were medium-dependent [28]. The results of our current study



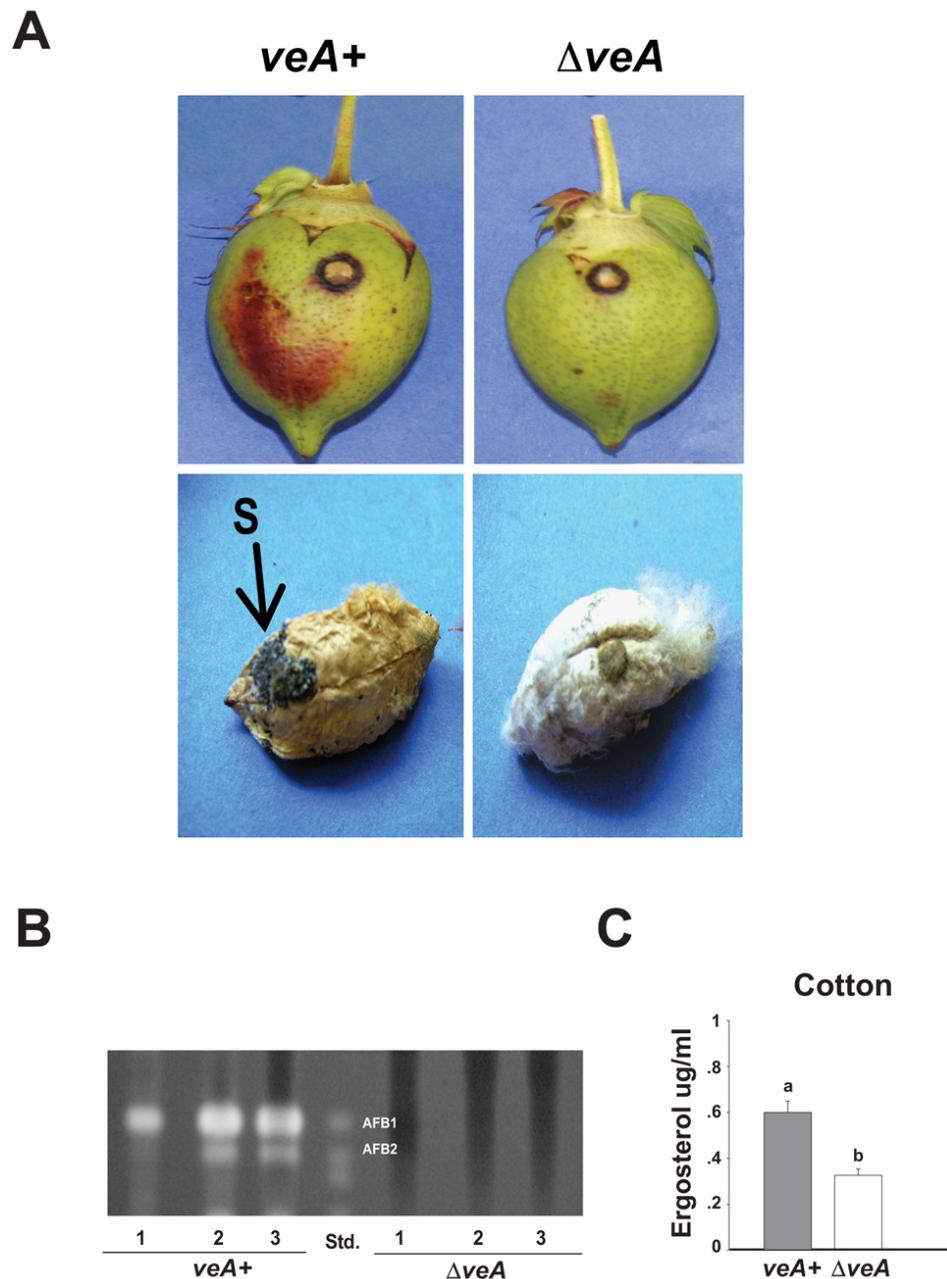
**Fig. (2).** Comparison of the effects of *A. flavus veA* deletion on the colonization between two peanut lines Flowrunner and Southernrunner. (A, D) Quantification of conidial production by *A. flavus* wild-type *veA* strain (WT) or deletion *veA* ( $\Delta veA$ ) strain when infecting Flowrunner peanut seeds (A) or Southernrunner seed line (D). (B, E) Quantification of fungal ergosterol content in infected peanut cotyledons of Flowrunner peanut line (B) and Southernrunner line (E). (C, F) TLC analysis of AF content in infected Flowrunner cotyledons (C) or Southernrunner cotyledons (F). non-viable (NV) or viable (V). Std.: AF standard. The values represent means of 4 replicates. Standard error is shown. Columns with different letters represent values that are statistically significantly different ( $P < 0.05$ ).



**Fig. (3).** Effect of the *veA* deletion on *A. flavus* colonization of corn kernels. (A) Presence of non-melanized sclerotia on surface of *veA*<sup>+</sup> inoculated corn kernel (arrow denotes cluster of sclerotia) and absence of sclerotia on the surface of a  $\Delta veA$  inoculated kernel. (B) Conidial production of *A. flavus veA*<sup>+</sup> and  $\Delta veA$  strains on corn kernels (per 8 kernels) and on PDA medium (per 5 mm-diameter core). In (B) columns with different letters represent values that are statistically significantly different ( $P < 0.05$ ). (C) Thin Layer Chromatography analysis of AF production by *A. flavus veA*<sup>+</sup> and  $\Delta veA$  strains growing on corn kernels. The experiment included five replicates.

suggest that this is also the case in the *A. flavus*  $\Delta veA$  strain, in which conidiation is severely affected when growing on natural substrates such as peanut and corn seeds. This is further supported by a previous study of *A. parasiticus* growth on peanut seeds from our laboratory [24]. In the model fungus *A. nidulans* it is known that the role of *veA* in regulating conidiation is influenced by external stimuli (*i.e.* light)[25, 39]. The *A. nidulans veA* gene product (VeA) forms a nuclear protein complex with several proteins, including light-sensing proteins and proteins involved in regulating development [40]. We hypothesize that VeA

could act as a scaffold protein that integrates external stimuli with a genetic response by an orchestrated action with VeA-interacting proteins, leading to developmental adaptive responses [25]. Based on this and previous studies [24, 28], it is possible that VeA function in *Aspergilli* could be influenced by other environmental cues, in addition to light, for example, availability of different types of nutrients and/or in this case unknown factors generated during seed colonization that could lead to the observed increase in conidiation.



**Fig. (4).** Effect of *veA* deletion on *A. flavus* colonization of cotton bolls. **(A)** Top Panels: Outer surface of boll near inoculation site demonstrating significant discoloration following inoculation with the *veA*<sup>+</sup> strain and little to no discoloration on the  $\Delta$ *veA* inoculated boll. Lower Panels: Surface of locule from boll inoculated with the *veA*<sup>+</sup> strain demonstrating presence of melanized sclerotia (S; denoted by arrow) and a locule from a boll inoculated with the  $\Delta$ *veA* strain lacking sclerotia. **(B)** TLC analysis of AF production from extracts of cottonseed harvested from bolls inoculated with either the *veA*<sup>+</sup> or  $\Delta$ *veA* strain. Experiments were repeated twice with similar results. **(C)** Quantification of ergosterol content from seed harvested from locules adjacent to the inoculated locule. In **(C)** columns with different letters represent values that are statistically significantly different ( $P < 0.05$ ).

As in the case of the peanut infection studies, ergosterol level were also significantly reduced when comparing kernels infected with  $\Delta$ *veA* and the *veA*<sup>+</sup> control (Fig. 3B), indicating a reduction in fungal colonization of seeds in the absence of *veA*.

As was observed with peanut seed (above), sclerotia were present on surfaces of corn kernels inoculated with the *veA*<sup>+</sup> strain while no sclerotia were detected on seeds inoculated with the  $\Delta$ *veA* strain (Fig. 3A). Our analyses also

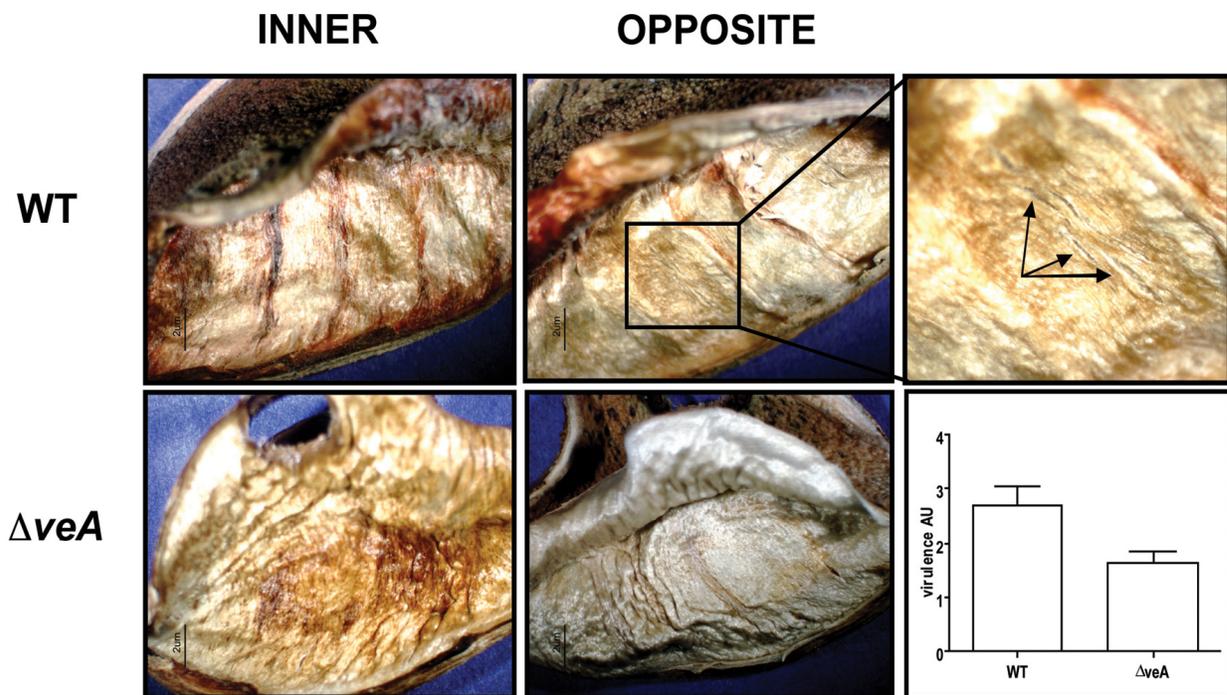
demonstrated that AF was produced in corn kernels inoculated with the *veA*<sup>+</sup> strain, while no AF was detected with the  $\Delta$ *veA* strain (Fig. 3C).

#### ***In Planta* Cotton Infection**

Visual examination of the cotton bolls showed that there was more discoloration on the boll surface for those bolls inoculated with the *veA*<sup>+</sup> strain compared to the  $\Delta$ *veA* strain (Fig. 4A). The significance of this discoloration is not clear

at this time but may be related to stress. There were more conidia present on locules inoculated with the *veA+* strain compared to the *ΔveA* strain (Fig. 4A). As expected, there were no sclerotia present on the locules of bolls inoculated with the *ΔveA* strain while sclerotia were evident in locules from bolls inoculated with the *veA+* strain (Fig. 4A). As in previous observations with peanut and corn seeds, *A. flavus* *ΔveA*-infected cotton seeds were free of AF contamination, while AF accumulated in those seeds infected with the *veA+* strain (Fig. 4B). A method for evaluating fungal spread between cotton boll locules is to look for the presence of bright greenish yellow fluorescence (BGYF) on cotton lint as this has been shown to be a reliable indicator of *A. flavus* spread [41]. However, this was not possible in this study as BGYF is a metabolite of the oxidation of kojic acid and the *ΔveA* strain does not appear to produce kojic acid during growth in cotton bolls. Copious amounts of BGYF was detected on the lint of locules adjacent to the locule inoculated with the *veA+* strain (data not shown). The degree of fungal spread was instead determined by analysis of ergosterol levels in the seed collected from locules adjacent to the inoculated locule. Ergosterol levels were significantly greater in the locules taken from bolls infected with the *veA+*

strain compared to those infected with the *ΔveA* strain (Fig. 4C). The degree of aggressiveness of the fungal strains was determined by microscopic examination of discoloration and damage to the intercarpellary membranes of the locules adjacent to the inoculated locule (Fig. 5). The incidence of greater discoloration and tears on the side of the intercarpellary membrane opposite the inoculated locule was consistently greater in the *veA+* inoculated bolls compared to those inoculated with the *ΔveA* strain. This was supported by statistical analysis that indicated the *veA+* strain produced a greater degree of membrane discoloration and tearing ( $P < 0.05$ ) than bolls inoculated with the *ΔveA* strain (Fig. 5). The increased level of spread and aggressiveness of the *veA+* strain would indicate that it is more efficient at penetrating the intercarpellary wall separating the locules. This requires the production of a number of different hydrolytic enzymes such as pectinases and proteases, suggesting that their production may be either directly or indirectly dependent on *VeA*. Future studies in our laboratory will be directed at elucidating the possible role of *veA* in the production of hydrolytic enzymes implicated in plant tissue colonization.



**Fig. (5).** Analysis of intercarpellary membrane damage from cotton bolls infected with either *A. flavus* *veA+* or *ΔveA* mutant strains. Upper left panel: Micrograph demonstrating discoloration on the side of the membrane facing the inoculated locule of a boll infected with the *veA+* strain. Upper middle panel: Micrograph demonstrating discoloration on the opposite side of the membrane adjacent to the inoculated locule of a boll infected with the *veA+* strain. Upper right panel: enlargement of area of membrane denoted in middle panel. Note tears in the membrane indicative of extensive damage by the fungus. Lower left panel: Micrograph demonstrating discoloration on the side of the membrane facing the inoculated locule of a boll infected with the *ΔveA* strain. Lower middle panel: Micrograph demonstrating lack of discoloration and damage on the opposite side of the membrane adjacent to the inoculated locule of a boll infected with the *ΔveA* strain. Lower right panel: Values represent a Student t-test analysis of the means and standard deviations of damage observations taken from the 2 intercarpellary membranes flanking the infected locules of 10 bolls. Differences among mean comparisons between the *ΔveA* and *veA+* samples were statistically significant ( $P < 0.05$ ) according to the Wilcoxon rank test. Membranes were rated as follows: 0, no discoloration; 1, discolored on surface of membrane facing inoculated locule; 2, same as 1 plus slightly discolored on opposite surface; 3, highly discolored on both surfaces; 4, same as 3 but presence of pin-hole tears; 5, same as 3 plus tears of 1 mm or larger. Experiments were repeated twice with similar results.

## CONCLUSIONS

This study has shown how a functional *veA* contributes to the virulence of *A. flavus* during *in vitro* infection of peanut and corn seed and *in planta* infection of cottonseed and boll tissues. *A. flavus* conidiation, sclerotial production, and AF biosynthesis are severely compromised when the fungus lacks a functional *veA* gene product. In this study it is particularly interesting that seed substrates, as opposed to laboratory media, promoted *A. flavus* conidiation in the presence of a *veA* wild-type allele. Furthermore, our study demonstrated that this holds true during the infection of a number of different oil seeds both *in vitro* and *in planta*. The observation that *veA* can play a role as a virulence factor during *A. flavus* invasion of crops, together with the fact that *veA* has only been found in fungi, suggest a high potential for the implementation of a control strategy targeting *veA* to inhibit *A. flavus* invasion of food and feed crops. Since *veA* is conserved in other fungi, such a strategy could also potentially be used to control contamination of crops by other fungal pathogens.

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