Relationships between Nutrient Uptake and Nitrogen Fixation with Aflatoxin Contamination in Peanut under Terminal Drought

Wunna Htoon 1, Wanwipa Kaewpradit 1,*, Nimitr Vorasoot 1, Banyong Toomsan 1, Chutipong Akkasaeng 1, Naveen Puppala 2, Sopone Wongkaew 3 and Sanun Jogloy 1

1 Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
2 Agricultural Science Center, New Mexico State University, Clovis, NM 88101, USA
3 Suranaree University of Technology, Nakhon Ratchasima 3000, Thailand
* Correspondence: wanwka@gmail.com; Tel.: +66-62-5939-141; Fax: +66-043-364-636

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Abstract: Terminal drought greatly enhanced Aspergillus flavus Link infection (AI) and aflatoxin contamination (AC) in peanut. Identification of new surrogate traits which have an association with AC may be effective to improve peanut varieties with reduced AI and AC. The objective of this work was to examine the relationships of nutrient uptake and N2-fixation (NF) with AC in peanut in a terminal drought condition. Five peanut varieties were tested in well-watered (WW) and terminal drought (TD) conditions (1/3 available water (AW) from R7 (7th reproductive growth stage; beginning of pod maturity stage)). Data were collected for nutrient uptake (nitrogen, phosphorus, potassium, calcium, magnesium), nodule dry weight (NDW), and NF. AI and AC were also examined. Nutrient uptake, NDW, and NF had negative and significant correlations with AI and AC in the TD condition. Negative and significant correlations of the drought tolerance index of nutrient uptake, NDW, and NF with AI and AC were also observed in the TD condition. The results showed that the ability to maintain nutrient uptake and NF in TD might be a mechanism of tolerance to AI and AC. Moreover, due to their negative impacts on AI and AC, nutrient uptake and NF could be used as selection traits for resistance to AI and AC in peanut in TD.

Keywords: Aspergillus flavus kernel infection; end-season drought; soil nutrients; symbiotic N2-fixation; correlation

1. Introduction

Aflatoxin contamination infested by Aspergillus flavus particularly in terminal drought (TD) conditions is a serious problem of peanut (Arachis hypogaea L.) production in the tropics. TD is a drought during the pod and seed forming stages has been shown to reduce pod yield of peanut. TD is a major cause of Aspergillus flavus infection (AI) and aflatoxin contamination (AC) in peanut [1]. Drought resistant varieties could reduce aflatoxin production [2–4] and such drought tolerance could minimize the aflatoxin contamination in peanut. Some physiological and morphological traits are closely related to drought adaptation. Indirect traits related to drought resistance are also associated with AI and AC and these traits can be used as surrogate traits for resistance to AI and AC [1].

Some effective physiological traits for drought tolerance may be useful for breeding of aflatoxin resistance. Drought reduced nutrient uptake and therefore reduced nutrient concentrations in plant tissues [5]. As drought is the main cause of the reduction in nutrient uptake [6], low reduction in nutrient uptake and high nutrient use efficiency should be the promising criteria for drought resistance because the traits might involve drought tolerance mechanisms [7]. Therefore, the ability to maintain nutrient uptake and use efficiency in TD could be a trait for selection of peanut varieties for resistance to AI and AC in peanut in TD.
high nutrient uptake during terminal drought periods may presumably be used as a drought surrogate trait and such ability may be promising as indirect selection for improving resistance to AI and AC in peanut.

Peanut is a nitrogen-fixing legume through symbiotic biological N$_2$-fixation (NF). In fact, peanut varieties with high NF in drought stress could obtain high yields in drought stress and consequently, NF and related traits were used as complementary tools for drought tolerance and yield [8–10]. Moreover, the expression of specific genes (RT-PCR), which plays an important role in featuring nitrogen fixation pathway and nutrient uptake, could be a next step to substantiate this finding.

A recent study reported that high nitrogen fixation under long term drought was associated with low AC in peanut [11]. However, there is still a lack of information on the relationship between NF and AC especially under terminal drought, a condition which apparently induced AI and subsequently caused high AC.

AI is a serious worldwide problem for peanut quality because of its potential major health hazards and huge economic losses. A better understanding of new surrogate traits and their contributions to AI in TD might be effective to select and improve peanut varieties with high drought tolerance and low pre-harvest AC. The objective of this work was to examine the associations of nutrient uptake and NF with AI and AC in peanut under TD conditions.

2. Materials and Methods

2.1. Experimental Design and Plant Materials

Four replications of a split-plot design were set up for two years in 2010–2012. Main plots were well-watered (WW) and TD. WW was maintained at field capacity (FC) level from planting to harvest. TD was commenced at R7 growth stage until harvest by maintaining soil moisture at 1/3 available water (AW). Sub plots consisted of five peanut varieties, including ICGV (ICRISAT groundnut varieties)’s 98,308, 98,324, 98,348, Tainan 9, and Tifton 8, and they were selected because of their differences in drought resistance in a previous study [1]. A non-modulating line was also used in the experiment as a non-nitrogen fixing line for observation only.

2.2. Crop Management

The experiment was conducted at the experimental plot of Khon Kaen University, Thailand, for two years in 2010–2011 and 2011–2012. The soil type at the experiment site was Yasothon soil series (loamy sand, Oxic Paleustults). The detailed soil properties are available in our parallel study [12].

Standard soil tillage was practiced. Phosphorus and potassium at the rate of 24.7 and 31.1 kg ha$^{-1}$, respectively, were incorporated into the soil during soil preparation (Chia Tai company limited, Phra Nakhon Si Ayutthaya, Thailand). The plots were inoculated with Rhyzobium soon after planting for optimum nitrogen fixation, and the crop was applied with gypsum (CaSO$_4$) at 40 days after emergence to provide sufficient calcium to the developing pods.

2.3. Irrigation

Irrigated water was supplied to the crop through a subsurface drip irrigation system prior to planting. The irrigated water was controlled at sub plot level by installing a sub valve for each sub plot. For the WW treatment, peanut plants were daily irrigated from planting to harvest. For the TD treatment, the initiation of water withholding for each peanut variety was dependent on the growth stage of each variety, which was not the same as other varieties, and the growth stages were carefully monitored until the crop reached R7. After irrigation withholding, soil moisture content was reduced until it reached 1/3 AW and was maintained at this level until harvest. Rainout shelters were available to control unpredictable rainfall and the crop relied solely on irrigated water. Water was replenished to the respective plots based on crop water requirements according to the calculation method described previously [13] and water loss from the soil surface [14].
2.4. Aspergillus flavus Inoculation

*Aspergillus flavus*, a virulent strain, kindly provided by Suraneree University of Technology, Thailand, was multiplied on peanut meal and incorporated into experimental plots to ensure the presence of sufficient aflatoxin-producing fungi in the peanut pod zone. At 20 days after emergence (DAE), *A. flavus* inoculum was broadcasted to peanut plots at the rate of 375 kg ha$^{-1}$.

2.5. Data Collection

2.5.1. Soil and Plant Water Status

Soil moisture content was monitored at 0–5, 10–15, 25–30, 40–45, 55–60, 70–75, and 85–90 cm below the soil surface 4 times; at planting, drought initiation, R7 growth stage, and at harvest. Relative water content (RWC) was also taken from 5 plants of each plot to determine plant water status according to the method described by Kramer [15]. RWC was taken on one leaflet of a second fully expanded leaf from the top of the main stem at 10:00 am–12:00 am [16]. Leaflet samples were carefully taken to the laboratory and leaf fresh weight was recorded immediately. The leaf samples were immersed in water for 8 h and turgid weights were determined. The leaf samples were further oven dried at 80 °C for 48 h and lead dry weight was recorded. RWC was determined as follows:

\[
\text{RWC} (%) = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100
\]  

where:
\[
\begin{align*}
\text{FW} &= \text{sample field weight}, \\
\text{TW} &= \text{sample turgid weight (saturated weight)}, \text{and} \\
\text{DW} &= \text{sample dry weight}.
\end{align*}
\]

2.5.2. Plant Nutrient Analysis

At final harvest, the bordered plants in the area of 8 m$^2$ in each plot were harvested. Pods were threshed, and leaves were separated from the stems. Leaves, stems, and pods were oven-dried at 80 °C for 48 h. The plant samples were analyzed for plant nutrients in shoot, shell, and seed. Nitrogen content was measured using the automated indophenol method [8] and was read on a flow injection analyzer (model 5012, Tecator Inc., Hoganas, Sweden).

Concentration of phosphorous was analyzed by a spectrophotometer (U-2900UV/VIS, Hitachi, Tokyo, Japan) and concentration of potassium was determined by a flame photometer (M410 flame photometer, Sherwood, Cambridge, England) [17]. Concentrations of Ca and Mg were recorded by atomic absorption spectroscopy (novAA 350, Analytik Jena, Münster, Germany). Nutrient uptake (g plant$^{-1}$) was individually calculated by multiplying dry weight and nutrient concentration.

The drought tolerance index (DTI) of nutrient uptake of each variety was calculated as the ratio of nutrient uptake at TD to nutrient uptake under WW conditions.

2.5.3. Nodule Dry Weight (NDW) and Nitrogen Fixation (NF)

Five plants were uprooted from each sub-plot and the roots were washed to remove soil on a 0.5 mm mesh screen at harvest. Nodules were removed from roots and oven-dried at 80 °C for 48 h and NDW was recorded for each variety.

Fixed nitrogen was examined at harvest by the same reliable N-difference method following Pimratch et al. [8]. NF content was calculated as follows:

\[
\text{NF of genotype} = \text{Total N of genotype} - \text{Total N of non-nodulating line at respective main plot} \quad (2)
\]

DTI of NDW and NF as suggested by Nautiyal et al. [18], were calculated as the ratio of each parameter at TD to WW conditions.
2.5.4. Aspergillus flavus and Aflatoxin Measurements

AI and AC were measured according to the methods described by Girdthai et al. [1] and Arunyanark et al. [2]. At harvest, the pods were air-dried until the moisture content reached approximately 8% and hand shelled. One hundred seeds in each plot were surface-sterilized and placed on moistened sterilized paper in a sterilized box which was disinfected by 70% ethanol. The seeds were incubated at room temperature for five days. After 5 days of incubation, green conidial heads of Aspergillus flavus were counted on the seeds to determine the percentage of colonization.

Seed samples of 100 g each were used for determination of AC. The seed samples were ground in a blender. For each assay, a sub-sample of 20 g meal for each of four replications was used for aflatoxin extraction and the supernatants were used for aflatoxin B1 analysis using ELISA (enzyme linked immunosorbent assay) [2].

2.5.5. Statistical Analysis

Simple correlations were used to determine the associations of nutrient uptake and NF with AI and AC. All statistical analyses were carried out using MSTAT-C version 1.42 developed by the Crop and Soil Science Division, Michigan State University, Michigan, USA.

3. Results

3.1. Soil and Plant Water Stress

The data for soil moisture percentage for the crop grown under WW and TD conditions across two years are shown in Table 1. Pooled data across years showed that soil moisture content at 0–30 cm and 0–60 cm in the WW condition ranged from 9.78%–10.29%. Soil moisture content at 0–30 cm was similar to those measured by the pressure plate method, especially to predetermined 1/3 AW (6.35%), and they were 6.14% at R7 stage and 6.15% at harvest. Nevertheless, soil moisture content measured at 60 cm in TD were similar to those of the WW control at all evaluation times.

Table 1. Soil moisture content (%) at sowing, the last day of irrigation, R7 growth stage, and harvest at 0–30 cm and 0–60 cm under well-watered and terminal drought conditions across 2010/2011 and 2011/2012.

| Treatments          | Soil Depth (cm) | Soil Moisture Content (%) |   |   |   |   |
|---------------------|-----------------|---------------------------|---|---|---|---|
|                     |                 | Sowing                    | Last Day of Irrigation | R7 Stage | Harvest |
| Well-watered        | 0–30            | 10.20                     | 10.28                     | 10.27     | 10.29   |
| Terminal drought    | 0–30            | 10.28                     | 10.21                     | 6.14      | 6.15    |
| Well-watered        | 0–60            | 10.27                     | 9.78                      | 9.84      | 10.19   |
| Terminal drought    | 0–60            | 10.24                     | 9.75                      | 6.02      | 6.44    |

The data for RWC are shown in Figure 1. TD reduced RWC at R7 (94%) and harvest (94%) in 2010/2011. Similar reductions of RWC at R7 and final harvest were shown in 2011/2012 and they were 94% and 95%, respectively.

FC field capacity) = 10.16%; PWP (permanent wilting point) = 4.48; 1/3 AW = 6.35 using the pressure plate method; FC: field capacity; PWP: permanent wilting point; AW: available water; well-watered = full irrigation since sowing until final harvest; terminal drought = 1/3 available water (AW) at R7 until final harvest.
3.2. Relationships between Nutrient Uptake with A. flavus Seed Infection and Aflatoxin Contamination

The correlations of nutrient uptake and DTI for A1 in the seed and AC were evaluated across years in the WW and TD conditions, as shown in Table 2. The correlation coefficient of Ca uptake with AI in the WW condition was negative and significant \((r = -0.45, p \leq 0.01)\). The correlation coefficients of uptake of N \((r = -0.50, p \leq 0.01)\), P \((r = -0.33, p \leq 0.05)\), K \((r = -0.51, p \leq 0.01)\), Ca \((r = -0.52, p \leq 0.01)\), and Mg \((r = -0.32, p \leq 0.05)\) with AI in TD were also negative and significant. Similarly, the correlation coefficients of AI with DTI of N uptake \((r = -0.78, p \leq 0.01)\), DTI of P uptake \((r = -0.66, p \leq 0.01)\), DTI of K uptake \((r = -0.72, p \leq 0.01)\), DTI of Ca uptake \((r = -0.65, p \leq 0.01)\), and DTI of Mg uptake \((r = -0.75, p \leq 0.01)\) were negative and significant.

Table 2. Correlation coefficients between Aspergillus flavus seed infection and aflatoxin contamination with nutrient uptake and drought tolerance index (DTI) of N, P, K, Ca, and Mg under well-watered and terminal drought conditions across 2010/2011 and 2011/2012.

| Nutrient Uptake (g plant\(^{-1}\)) | A. flavus Seed Infection (%) | Aflatoxin Contamination (ppb) |
|-----------------------------------|-----------------------------|-------------------------------|
|                                   | Well-Watered | Terminal Drought | DTI | Well-Watered | Terminal Drought | DTI |
| N                                 | -0.02       | -0.50 **        | -0.78 ** | -0.30       | -0.66 **        | -0.82 ** |
| P                                 | -0.05       | -0.33 *         | -0.66 ** | -0.15       | -0.43 **        | -0.60 ** |
| K                                 | 0.08        | -0.51 **        | -0.72 ** | -0.18       | -0.60 **        | -0.68 ** |
| Ca                                | -0.45 **    | -0.52 **        | -0.65 ** | -0.21       | -0.75 **        | -0.73 ** |
| Mg                                | 0.11        | -0.32 *         | -0.75 ** | -0.22       | -0.50 **        | -0.72 ** |

* * = significant at \(p \leq 0.05\) and \(p \leq 0.01\), respectively; well-watered = full irrigation since sowing until final harvest; terminal drought = 1/3 available water at R7 until final harvest; DTI = drought tolerance index of nutrient uptake was calculated by the ratio of nutrient uptake at terminal drought/nutrient uptake as well-watered conditions; N: nitrogen; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; ppb: parts-per-billion.

The correlation coefficients of nutrient uptake and AC in the WW condition were not significant. Correlations of nutrient uptake with AC in TD were negative and significant (ranged from \(r = -0.43, p \leq 0.01\) to \(r = -0.75, p \leq 0.01\)). The reverse correlations of DTI of uptake of N, P, K, Ca, and Mg with AI were also significant and they ranged from \(r = -0.60, p \leq 0.01\) to \(r = -0.82, p \leq 0.01\).

3.3. Relationships between N\(_2\)-Fixation (NF) and Nodule Dry Weight (NDW) with A. flavus Seed Infection and Aflatoxin Contamination

The correlation coefficients of NF with AI \((r = -0.41, p \leq 0.01\) and \(r = -0.50, p \leq 0.01\), respectively) were negative and significant under the WW and TD conditions, as shown in Table 3. There was a reverse and significant correlation of DTI (NF) with AI in seed \((r = -0.65, p \leq 0.01\).
Table 3. Correlation coefficients between *A. flavus* seed infection, aflatoxin contamination with N$_2$-fixation (NF), nodule dry weight (NDW), drought tolerance index (DTI) N and DTI (NDW) under well-watered and terminal drought conditions across 2010/2011 and 2011/2012.

| NF and Related Trait (g plant$^{-1}$) | *A. flavus* Seed Infection (%) | Aflatoxin Contamination (ppb) |
|--------------------------------------|-------------------------------|-------------------------------|
|                                      | Well-Watered | Terminal Drought | DTI       | Well-Watered | Terminal Drought | DTI       |
| NF                                   |              |                  | N         |              |                  | N         |
|                                      | −0.41 **     | −0.50 **         | −0.58 **  | −0.62 **     | −0.38 *          |           |
| NDW                                  | −0.58 **     | −0.62 **         | −0.71 **  | −0.33 *      | −0.35 *          | −0.33 *   |

*, ** = significant at *p* ≤ 0.05 and *p* ≤ 0.01, respectively; well-watered = full irrigation since sowing until final harvest; terminal drought = 1/3 available water at R7 until final harvest; DTI = drought tolerance index of NF and NDW were calculated by the ratio of NF or NDW at terminal drought/NF or NDW at well-watered condition; ppb: parts-per-billion.

The correlations of NDW with AI were negative and significant in the WW and TD conditions, as shown in Table 3. The correlation of DTI (NDW) and AI was also reversed and significant (*r* = −0.71, *p* ≤ 0.01).

The correlation coefficients of NF with AC provided similar information and they were negative and significant in the WW (*r* = −0.58, *p* ≤ 0.01) and TD (*r* = −0.62, *p* ≤ 0.01) conditions. In addition, there were reversed and significant correlations.

The correlation coefficients of NDW with AC in the WW and TD conditions (*r* = −0.33, *p* ≤ 0.05) and (*r* = −0.35, *p* ≤ 0.05, respectively) were negative and significant. A similar negative and significant correlation coefficient (*r* = −0.33, *p* ≤ 0.05) was found of DTI (NDW) with AC.

4. Discussion

The traits related to drought resistance are useful as indirect selection tools for improving drought resistance to pre-harvest AC in peanut. Holbrook et al. [3] previously reported that peanut varieties with drought resistance had higher reduction in AC than did the drought sensitive varieties. According to McMillian et al. [19], drought tolerant mechanisms may be responsible for the reduction in AC. Early works showed that AC was related to drought tolerance, such as leaf temperature and visual stress rating [6]. Specific leaf area, root length density, and AI are potentially selection tools for reduced AC in peanut [2]. Identification of new traits which potentially relate to AC might lead to achieve the peanut varieties with reduced AC.

To the best of our knowledge, TD causes the most severe AC in peanut [1]. This study showed that TD reduced nutrient uptake, but it increased AI and AC. In fact, their relationships in TD were stronger than the WW conditions. In a parallel study, TD reduced the uptake of N, P, K, Ca, and Mg and the responses of peanut varieties for nutrient uptake varied greatly [20]. Evidence presented in their study indicated that ICGV’s 98,324 and 98,348 were the best varieties for the nutrient uptake and nutrient use efficiency. According to Koolachart et al. [21], TD increased AI and AC, and ICGV’s 98,348 and 98,324 were the best varieties for low AI and AC. These peanut varieties are promising for selection of high nutrient uptake and low AI and AC. Our results indicated the ability of peanut varieties for high nutrient uptake in the TD condition might involve a drought tolerant mechanism to reduce AI and, consequently, AC later on. Our results supported previous findings that varieties showing a low reduction in nutrient uptake are more resistant to drought [20,22] and such drought tolerance could minimize the AC in peanut [2]. Uppala [23], who studied pre-harvest AC of peanut, also noted that the relationship of calcium content in peanut leaves, shells, and kernels with AC were negative and significant. Therefore, maintaining high nutrient uptake in TD meant lower AI and AC.

According to our results, the negative and strong relationships of DTI of nutrient uptake with AI and AC indicated that peanut varieties with a higher DTI, or a lower reduction for nutrient uptake, also had low AI which was resulting in low AC in TD. Gunes et al. [7] and Baligar et al. [24] reported that total nutrient uptake reduced in water-stressed conditions and Fageria et al. [6] also concluded that drought stress might be important for the nutrient content in plant tissues by affecting nutrient uptake. Our results were in accordance with the previous report. In chickpea, Gunes et al. [7] found that the
varieties showing a lower reduction in nutrient uptake and nutrient efficiency were more resistant to drought than the sensitive genotypes and some mechanisms of drought resistance might operate in these varieties. In the most recent study, Girdthai et al. [1] revealed that, in TD, drought-resistant varieties had lower AI and pre-harvest AC. Therefore, higher nutrient uptake in drought might lead to lower incidences of AI and AC.

NF was decreased by drought and this reduction led to low total nitrogen content [25]. NF in peanut under drought relied on the degree of stress, the period of stress, and the stage of crop development [25,26]. Previous works also reported that not only NF, but also its related traits such as nodule number, NDW, nitrogenase activity, biomass, and SPAD chlorophyll meter reading (SCMR) decreased when peanut varieties were exposed to drought [8–10]. It was interesting to note that, in our study, the relationships of NF with AI and AC were negative and significant under both WW and TD conditions. Their relationships were relatively stronger in TD indicating that peanut varieties which could maintain high NF in a water deficit condition could reduce the AI and AC. Our supposition was reasonable because Arunyanark et al. [2] and Holbrook et al. [6] pointed out that there would be mechanisms that conferred resistance to AC and these mechanisms may be related to drought resistance. Htoon et al. [12] reported that TD during the pod filling stage until harvest greatly reduced the NF and NDW, but peanut varieties did differ for NF and NDW in the TD condition. Htoon et al. [12] exposed peanut varieties to TD and they observed that ICGV 98,308 reduced 58% of NF in TD and Tainan 9 also had lower NF in both WW and TD conditions. Girdthai et al. [1] also revealed that Tainan 9 was supposed to be a drought sensitive variety due to low biomass and pod yield under TD. Koolachart et al. [21], who worked with peanut varieties in TD, reported that ICGV 98,308 and Tainan 9 had the highest AI in the TD condition (8.75% and 5.25%, respectively), and they also had the highest AI in TD conditions (22.50 and 18.93 ppb, respectively). From our study and the results of their experiments, it can be concluded that peanut varieties which could not maintain their NF in TD were supposed to be drought sensitive, resulting in high AI and AC. On the other hand, if peanut varieties had an ability to keep high NF in TD, such ability might help to reduce AI and AC.

Our results were in good agreement with Arunyanark et al. [11] who reported for the first time that the ability of peanut varieties to maintain high NF and its related traits in TD conditions might help to reduce AI and AC. They also showed the first evidence that total nitrogen content, NF, and its related traits had negative and significant effects on kernel AI and AC. The current study differs from Arunyanark et al. [11] in that they also found a strong and a negative correlation of NF and NDW with AI and AC under prolonged drought, because our results were observed in TD which enhanced AI and AC. In addition, this study is the first report showing new evidence of the role of nutrient uptake in peanut production in TD in order to maintain a high drought tolerance level and low AC. Although our study was new evidence, which clearly indicated the negative relationships of nutrient uptake and NF with AI and AC in TD, the basis of the relationships was still not very clear and needs further study.

5. Conclusions

In conclusion, the ability of peanut to maintain high nutrient uptake and NF might be a mechanism, or parts of a mechanism, of tolerance to TD. The results of these studies reinforce the significant roles of nutrient uptake and NF in predisposing peanuts to AC in TD. Due to their negative impacts on AI and AC, nutrient uptake and NF could be potentially used as indirect selection traits for resistance to AC.

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