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Dynamic changes in carotenoid and flavonoid content and relative water content (RWC) by corn leaf aphid infestation on sorghum

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Abstract
Sorghum is truly a versatile crop that can be grown as a grain, forage or sweet crop. Sorghum is one of the top five cereal crops in the world. Small bluish green colored, corn leaf aphids (*Rhopalosiphum maidis* (Fitch)) (Aphididae: Hemiptera) begin feasting early in a plant's development, feeding on the whorls of corn. The quantification of leaf pigment carotenoid indicated a significant loss in medium (200 aphids/plant) and high (300 aphids/plant) infestation compared to control. The per cent reduction of these pigment ranged between 10 to 40, respectively. On the contrary, the flavonoid content increased significantly in medium (200 aphids/plant) and high (300 aphids/plant) infestation. The relative water content (RWC) ranged between 89.3±1.20 to 87.2±1.21%, the per cent of reduction varying between 5.03 to 2.31 at different levels of infestation. The pigment loss was greater at high densities than the water loss.

Keywords: Carotenoid, Flavonoid, Relative water content, *Rhopalosiphum maidis*

Introduction
Sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal grain for human and animal consumption throughout the world, and world's fifth most important cereal crop after maize, rice, wheat and barley. It is the dietary staple of more than 500 million people in more than 30 countries with adequate crude protein (8-12%) and high amount of carbohydrates (65-80%). Nearly 150 insect species have been reported to damage the crop worldwide, causing an estimated loss of over US$ 1000 million annually. Aphids are an important group of insects with worldwide distribution. They are highly specialized phloem-feeding hemipterans, coevolved multi-level adaptations facilitating exploitation of the resources provided by their host plants (Czerniewicz *et al*., 2011) [10]; (Chrzanowski *et al*., 2012) [8]. These insects cause a wide spectrum of detrimental effects in infested plant parts, including mechanical disruption of penetrated tissues, depletion of photo assimilates and intensification of many intracellular processes (Sempruch *et al*., 2010) [26] ; (Sytykiewicz *et al*., 2011) [28]. Aphid salivary secretions contain a variety of hydrolytic enzymes and biologically active substances that modulate metabolic reactions within the host. In some cases, aphid infestation contributes to the activation of premature senescing process and programmed cell death in plant organs (Carolan *et al*., 2009) [7]; (Anstead *et al*., 2010) [3]. Colonization of the different host systems may lead to transmission of a wide range of pathogenic plant viruses (Makkouk and Kumari, 2009) [20]. Aphid feeding decreases the moisture content of infested leaves. Plants under the stress of early-season infestation allocated more resources for leaf growth, but stem growth was severely retarded (Petitt and Smilowitz, 1982) [22]. Herbivorous insects in soybean resulted in water loss in the infested leaves (Aldea *et al*., 2005) [2].
The mathematical relationship established between aphid infestation density and leaf pigments in this study could be very useful for creating pest subroutines in crop modeling studies in future.

Materials and methods
Plants and aphids: Sorghum was raised in plastic pots (10 × 10 cm dia.) in greenhouse. The aphids collected from CRIDA field were released on 30 days old potted sorghum plants @ 100-200 aphids/plant. When yellowing/chlorosis of the top leaves was noticed, fresh, uninfested 30 days old potted sorghum plants were offered. Later, these aphids served as a source of population for conducting various physiological experiments.
Sorghum SPV-462 seedlings were raised in pots (10 × 10 cm dia.) in green house. The pots were filled with one kg soil and four seeds/pot were dibbled and watered.

When the seedlings were 3, 13 and 23 days old, water soluble fertilizer POORNA-19 was applied @ 0.5 g/plant. The five day old seedlings were isolated with mylar tubes to prevent the infestation of insect pests. The leaves of 25 days old potted seedlings of sorghum cultivar (SPV-462) were infested with a mixed population of nymphs and adult aphids @ 100, 200 and 300 individuals per plant and graded as given below (Table 1). Uninfested seedlings were kept as control.

Table 1: Grading of sorghum plants infested by aphids

No. of nymphs and adult aphids released/plant	Infestation
100	Low
200	Medium
300	High
Nil	Control

Leaf carotenoid concentration: 100 mg of the third leaf was weighed and placed in a vial with 25 ml dimethyl sulphoxide (DMSO) for carotene extraction. The sample vials (three replications) were incubated at room temperature in the dark for 48 h to allow for complete extraction of chlorophyll into the solution. Absorbance of the clear extracts was measured using Genesys UV/VIS spectrophotometer (Thermospectronic, Rochester, USA) at 480 nm. Concentration of leaf carotenoid as mg/g was calculated by using the following formula (Hiscox and Israelstam, 1979) [12].

$$\text{Carotenoid} = \frac{[(1000 \text{ x } A_{480nm}) - 1.29 \text{ x Chl } a - 53.78 \text{ x chl } b]}{420}$$

Leaf flavonoid content: One gram of the third leaf was weighed and added to a mixture of acetone: methanol: water at 1:1:1 ratio (3.33ml each). The leaf was crushed by using mortar and pestle, the extract was centrifuged at 1000 rpm for 10 min and filtered by using filter paper. From the filtrate, 2 ml of extract solution was mixed with 2 ml of 2% Aluminum chloride ($AlCl_3$) in methanol. The mixture was incubated for 10 min at room temperature, and the absorbance was measured at 420 nm in Genesys UV/VIS spectrophotometer (Thermospectronic, Rochester, USA) against blank samples. The total concentration of flavonoid in the extract was determined as microgram of Rutin Equivalent (RE) according to the formula that was obtained from standard rutin graph.
Absorbance = 0.0144 x Total Flavonoid (µg Rutin Equivalent) + 0.0556

Estimation of relative water content (rwc) in sorghum due to aphid feeding
500 mg of leaf sample (3^{rd} leaf from top) was weighed for RWC analysis. After recording the fresh weight (FW), the leaves were cut into small pieces and were soaked in water for 4 h for recording turgid weight (TW). Thereafter, the samples were dried at 80°C for about 48 h to determine the dry weight (DW). RWC was calculated as described by Smart and Bingham (1976) [27].

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}$$

Statistical analysis
Significant effects of aphid damage on leaf pigments concentration (carotenoid and flavonoid), and RWC were determined by analysis of variance (ANOVA). The software package SPSS (Superior Performance Software System, USA, version 16.0) was used for performing statistical analysis.

Results and discussion
Effect of aphid infestation on carotenoid content
The leaves of sorghum infested with *R. maidis* showed a tendency in decrease of carotenoid level compared to control. The lowest level of the analysed parameter was noted in high infestation *i.e.,* 0.6±0.09 mg/g of the leaf (Fig 1). The aphid infested leaves showed significant (F= 6.25, df = 3, 25 and P = 0.0026) differences in carotenoid content between the infested (medium and high) and un-infested leaves (Table 2).
From the results obtained it is evident that the carotenoid content decreased significantly in medium (200 aphids/plant) and high infestation (300 aphids/plant) compared to low infestation (100 aphids/plant).
The chemical composition of plants is not only affected by abiotic factors (Germ *et al*., 2010) [11] but may also be influenced considerably by biotic factors such as herbivory. The changes in pigment content due to feeding by *R. maidis* suggested a feeding induced response at different levels of infestation. Stress under aphid feeding led to lower carotenoid content in sorghum plants. Huang *et al*. (2014) [15] reported that the relative chlorophyll loss was related to the amount of feeding damage caused by insects and confirmed that this damage measured on individual host plants varied significantly depending on the insect density and stage of plant growth.
Photosynthetic pigment degradation is a complex phenomenon which often accompanies insect feeding damage to plants (Ni *et al*., 2002) [21]. According to Wilkaniec (1990) [31], aphid feeding causes changes in the metabolism of host plants, which in turn disturbs photosynthesis, speeds tissue aging, and causes morphological deformations.
This is connected with the direct harm done by aphids inserting saliva into plant tissues and blocking stomata with the honeydew they produce.

Table 2: Effect of different levels of aphid infestation on leaf pigments in sorghum.

Attributes	Carotenoid*(mg/g)	% reduction in Carotenoid
No Infestation (Control)	1.0 ± 0.04^{a}	-
Low Infestation	0.9 ± 0.05^{ab}	10
Medium Infestation	0.7 ± 0.04^{b}	30
High Infestation	0.6 ± 0.09^{b}	40
df	3, 25	-
F value	6.25	-
P value	0.0026	-

Means within a column followed by the same letter are not significantly different using Tukey's HSD test (α=0.05); df: degrees of freedom.
*(Mean±SEM)

The phloem-feeding aphid continually controls and/or modifies the metabolic substances levels of the surrounding tissues. It was reported that strong and persistent flow of host assimilates created by the continual removal of metabolites and breakdown of insoluble reserves by insects (Kattab, 2005) [17]. John *et al.* (2007) [16] explained that *A. glycines* removes phloem sap, which can result in a reduction of chlorophyll content. Hemmat Khattab (2007) [13] showed the defence mechanism of cabbage plant against phloem sucking aphid. The levels of antioxidant compounds (like carotenoids) were changed in response to aphid feeding.

Effect of aphid infestation on flavonoid content

Aphid infestation also altered the flavonoid levels, which significantly increased under the stress of *R. maidis* infestation in tissues of sorghum leaves (Table 3 and Fig 2). The plants with high infestation of aphids showed higher amount of flavonoid content *i.e.* 77.37±3.82 µg Rutin Equivalent (RE) compared to the control (38.24±3.32 µg RE). The aphid infested leaves showed significant (F= 27.87, df = 8, 11 and P = 0.0001) differences between the infested and un-infested leaves. The % increase in flavonoid content over no- infestation was 24.21, 46.02 and 102.32 for various levels of infestation (low, medium and high infestation, respectively).

In our study, an increase of flavonoid content in tissues of the sorghum leaves *vis-a-vis* aphid density, under the stress of aphid feeding was clearly evident. Flavonoids are a large class of secondary metabolites encompassingmore than 10,000 structures and are of great interest for their bioactivities, basically related to their antioxidant properties (Cao *et al.,* 2013) [6]. Several lines of evidence demonstrated that they have antioxidant functions in higher plants challenged with a range of environmental stresses (Winkel-Shirley, 2002) [32]; (Agati *et al.,* 2012) [1]. The stress reactions of the sorghum plants point to the negative effect of aphid infestation and to activation of protective mechanisms such as an increase of flavonoid content. Tevini *et al.* (1991) [29] showed that accumulation of these pigments in rice lessened the damage to the photosynthetic activity of mesophyll chloroplasts. Flavonoids are generally involved in plant resistance to herbivores (Bennett and Wallsgrove, 1994) [4]; (Wu *et al.,* 2007) [33]. Leiss *et al.* (2009) [19] showed that resistant hybrids contained higher amounts of the flavonoid kaempferol glucoside. Kaempferol glucosides also have a negative effect on aphids. Aphid-resistant cow pea lines contained significantly higher amounts of flavonoids, including kaempferol, than susceptible lines (Lattanzio *et al.,* 2000) [18].

This study provided essential information on the effect of *R. maidis* feeding on carotenoid and flavonoid contents in sorghum leaves. The extracted carotenoid contents in medium and high infestation showed significant difference over no infestation while, in low infestation both the pigments were on par with no infestation. The flavonoid content increased as infestation level increased. Thus losses of carotenoid concentrations and increased in flavonoid content in response to *R. maidis* infestation suggest a feeding-induced stress response in sorghum depending on the aphid density.

Table 3: Effect of different levels of aphid infestation on flavonoid content in sorghum.

	Flavonoid content* (µg Rutin Equivalent)	% increase in flavonoid content
No Infestation (control)	38.24 ± 3.32^{a}	-
Low Infestation	47.95 ± 3.41^{ab}	24.21
Medium Infestation	55.84 ± 1.57^{b}	46.02
High Infestation	77.37 ± 3.82^{c}	102.32
df	8, 11	-
F value	27.87	-
P value	0.0001	-

Means within a column followed by the same letter are not significantly different using Tukey's HSD test (α=0.05); df: degrees of freedom.
*(Mean±SEM)

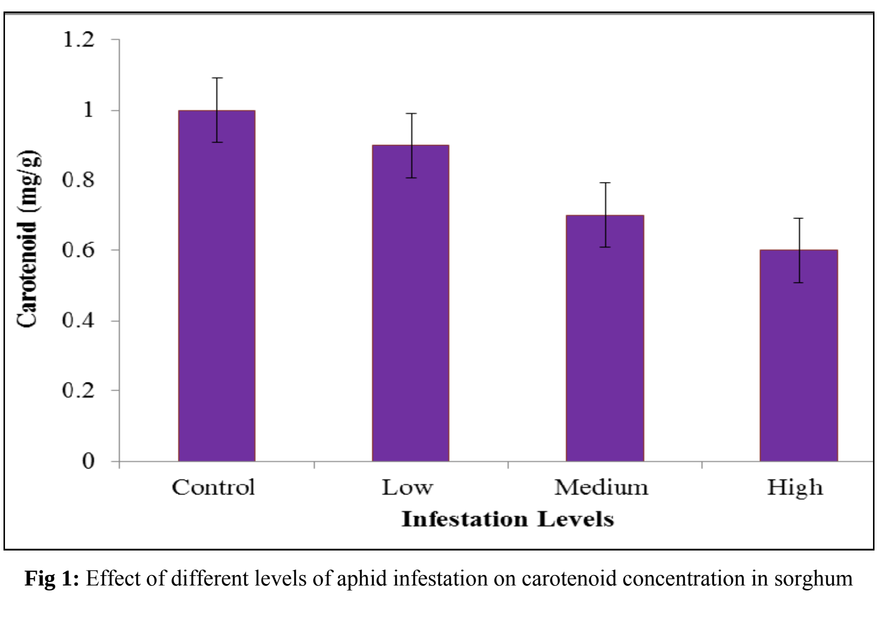

Fig 1: Effect of different levels of aphid infestation on carotenoid concentration in sorghum

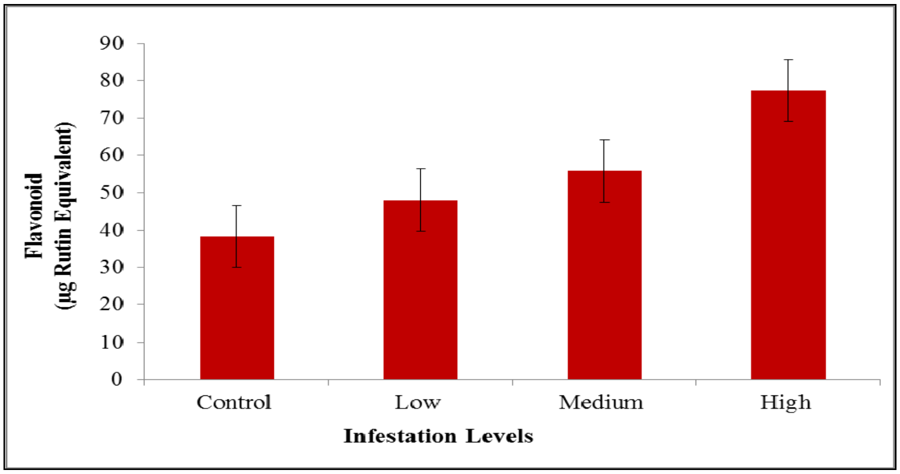

Fig 2: Effect of different levels of aphid infestation on flavonoid concentration in sorghum

Relative water content (rwc) in sorghum due to aphid feeding

Relative water content (RWC) decreased significantly in the affected plants compared to no infestation. The RWC in low and medium infestation (84.8±1.20% and 84.1±0.77%, respectively) showed lower RWC compared to high infestation (87.2±1.21%). However, the relative water content of low and medium infested plants showed significant difference with no infestation (89.3±1.20%) although the latter was on par with high infestation plants (Table 4).

Walter (1989) [30] stated that leaves of barley plant infested with wheat aphid, *D. noxia* had lower relative water content than corresponding leaves from un-infested plants. This is similar to our findings where corn leaf aphid *R. maidis* infestation reduced RWC (from 89.3 % to 84.1%) in sorghum. Sanjay *et al.* (1984) [24] showed that feeding injury caused by *E. dodecastigma* onbitter gourd leavesdecreased the RWC varying from 96.5% to 78.0%.

In our study, in the aphid infested sorghum leaves, 2.35 to 5.82% reduction in RWC was noticed (Fig. 3). The significant reduction in RWC in cotton plants was 1.93 to 23.49% due to mealybug, *P. solenopsis* (Prabhakar *et al.*, 2013) [23], 17% due to spider mite, *T. urticae* (Schmidt *et al.* 2009). Water-stress caused by greenbug infestation induces several metabolic changes that may be at nutritional-physiological level or behavioural responses, such as the length of time for feeding and preference for specific plants or location on plants (Zuniga *et al.*, 1989) [34] ; (Holtzer *et al.*, 1988) [14]. Cornish and Zeevaart (1985) [9] suggested that greenbug infestation could affect the regulation of water balance in leaves by affecting stomatal physiology. Cabrera *et al*. (1995) [5] stated that barley seedlings infested with *S. graminum* have drought-stress symptoms, such as lower water potentials and lower relative water contents, even in the presence of ample root moisture.

Table 4: Effect of different levels of aphid infestation on relative water content (RWC) in sorghum.

Treatment	Relative water content* (%)	% reduction in relative water content
No Infestation	89.3 ± 1.20^{a}	-
Low Infestation	84.8 ± 0.77^{b}	5.03
Medium Infestation	84.1 ± 0.86^{b}	5.82
High Infestation	87.2 ± 1.21^{ab}	2.35
Df	3, 25	-
F value	5.64	-
P value	0.0043	-

Means within a column followed by the same letter are not significantly different using Tukey's HSD test (α=0.05); df: degrees of freedom. *(Mean±SEM)

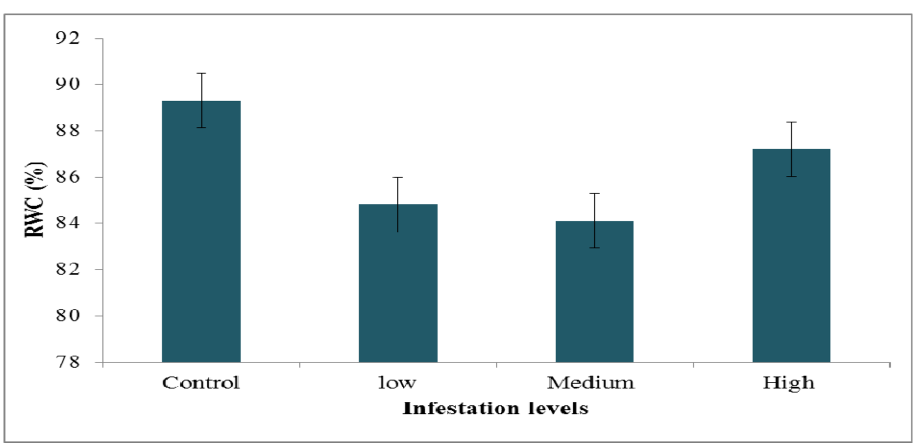

Fig 3: Effect of different levels of aphid infestation on relative water content (RWC) in sorghum.

In the present findings the per cent reduction in RWC was greater in low and medium infestation plants compared to high infestation. The probable reason could be that feeding by aphids in high infestation (300 aphids/plant) might have been effected due to overcrowding and competition as compared to low (100 aphids/plant) and medium infestation (200 aphids/plant). However, at high infestation there was a greater loss of leaf pigments, probably due to the compensation of pigments from the unaffected part of the leaf.

Conclusion

Sorghum is attacked by many insect-pests which are the principal limiting factor for its productivity throughout the country. Leaf corn aphid, *Rhopalosiphum maidis* (Fitch) is one of the major insect pests which widely distributed and prevalent throughout India. To manage the aphids, adoption of ecologically safe strategy like Integrated Pest Management (IPM) is recommended.

The present study focuses on need to understand the relationship between insect densities and there effect on various physiological parameters before formulating sound strategies for pest management.

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