https://doi.org/10.26651/allelo.j/2022-56-1-1386 0971-4693/94 Euro 20.00
Allelopathy Journal 56 (1): 41-58 (May, 2022) *International Allelopathy Foundation* 2022
Table: 3, Figs : 8

Effects of aqueous extract of *Parthenium hysterophorus.* L and synthetic phenolics on maize, green gram and kidney bean

J.A. Okundi[1,2,3], S. Cherono[2,3], M. Gichua[3,4], Y. Xue[1,3], J. Chen[5], H.Y. Li[5]* and Q.F. Wang[1,3]
Key Laboratory of Biodiversity and Evolution Ecology,
Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China,
E. mail: okundi2003@yahoo.com, jokundi@gmail.com

(Received in revised form: October 10, 2021)

ABSTRACT

We studied the allelochemicals stress induced by aqueous extract of *Parthenium hysterophorus* L. and synthetic phenolic compounds on the physiological responses of selected varieties of Kenyan *Zea mays* L. (H625, H614D, DH02, H513, H6213), *Phaseolus vulgaris L.* (GLP2, GLP92) and *Vigna radiata* (L.) R. Wilczek (KS20). The aqueous extracts significantly inhibited the growth of test crop varieties than control. However, the effects were variable depending on whether the phenolic compounds had hydroxy (-OH) or methoxy (-OCH_3) ring substituents. Aqueous extracts of *P. hysterophorus* and phenolic compounds with -OCH_3 groups (M-Anisic and P-Anisic acid) decreased the physiological responses of test plant, while, phenolic compound with -OH groups (Coumaric acid and Hydroxybenzoic acid) were stimulatory.

Key words: Antioxidant enzyme, chlorophyll content, cytomorphological behavior, green gram, kidney bean, maize, hydroxy -OH, methoxy -OCH_3, *Parthenium hysterophorus, Phaseolus vulgaris,* physiological responses, *Vigna radiata, Zea mays*

INTRODUCTION

Allelopathy is the inhibition of one plant by another (18,28) and the interactions can be positive or negative (1,5,7). The allelochemicals have different allelopathic effects, including the modification of plant community i.e. species inclusion or exclusion (9,32). It determines the species succession, dominance, diversity and climax of natural vegetation (33,50) and at the ecosystem level, it influences the abiotic factors. Allelochemicals are released through leachates, root exudates, volatilization, decomposition of plant residue in soil (1,5,7). The allelopathic effects depends on if the allelopathic compounds have the hydroxy -OH or methoxy -OCH_3 ring substituents (38). The phenols with -OH ring stimulates the germination and seedling growth, as they increase the isocitrate lyase activity of major enzymes involved in gluconeogenesis (16,31). While the -OCH_3 inhibits the germination and seedling growth, by decreasing their enzyme activities directly effecting the on enzyme catalysis (18). The inhibitory or stimulatory effects of phenols depends on the position of hydroxy and methoxy ring substituents (1).

When allelochemicals are released into the environment they affect the seed germination and early growth of plants (39,41), impairs the metabolic activities, which influences the growth, survival, development and reproduction of non-target and target

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*Correspondence author: Huanyong Li, [1]University of Chinese Academy of Sciences, International College, Yuquan Road, Shijingshan District, Beijing 100049, China, [2]Sino-Africa Joint Research Center, Chinese Academy of Sciences, Wuhan 430074, China, [3]Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya, P.O. Box 62000, 00200, Nairobi. [5]Binzhou Vocational College, Binzhou 256603, China.

plant species (18). The symptoms on the non-target and target plants include; loss of chlorophylls and carotenoids (18,44), roots dysfunction (8), reduction in water absorption (10), dehydrogenase activity in the roots and damage the cellular membranes of roots leading to loss of biomass due to desiccation (2,8,10).

The amount of phytochemicals in the plants depends on water availability, light, nutrients availability, disease and pesticides used (33). The impact on cultivated crops and weeds in the field often depends on the behavior of allelopathic secondary metabolites in soil (3,26), which determines the contact between the allelochemicals and the target plants (33). The allelopathic effects occur through mass flow, diffusion and root interception (5,9) and reduces the crop yields. The allelochemical's phenolic compounds affects the presence of microorganisms and pathogens in soil (5,33).

Parthenium hysterophorus L as invasive weed is found in most ecosystems (2,39,40) decreases the crop yield upto 40 % and forage production in grass lands upto 90 % (45,48). Its invasiveness success is due to the presence of allelopathic secondary metabolites (10,39), high reproductive potential and adaptive nature to wide range of environmental conditions (6,8,23,39,47). The plant's every part contain allelopathic secondary metabolites (19,39). *P. hysterophorus* L. contains several compounds [phenolic, pseudoguaianolides, flavonoids, oils and alkaloids (41)], many of which play an important role in its allelopathy (2,8,10), invasion, naturalization and establishment of monoculture (2,6,40,41) and dominate the natural plant communities (13).

Figure 1. *P. hysterophorus L* infestation of cultivated fields in kenya

In Kenya, maize (*Zea mays* L.), green gram (*Vigna radiata* (L.) R. Wilczek) and kidney bean (*Phaseolus vulgaris* L.*)* are main crops. Maize is staple food grown in 90 % agricultural land, green gram and kidney bean are major legumes (22,27). Hence, we determined the effects of *P. hysterophorus* aqueous extracts and phenolic compounds in Sandwich Bioassay and Pot culture on the morphological and physiological effects [shoot and root length, enzymatic antioxidants: Ascorbate Peroxidase (APX), Catalase (CAT), Peroxidase (POD), total protein content, relative water content, chlorophyll levels, electrolyte leakage, leaf and root C%)] of selected varieties of Kenyan *Zea mays* L., *Phaseolus vulgaris* L. and *Vigna radiata* (L.) R. Wilczek. This study aimed to determine the effects of *P. hysterophorus* aqueous extracts and phenolic compounds on the seedlings growth of maize (*Zea mays*), green gram (*Vigna radiata*) and kidney bean (*Phaseolus vulgaris*).

MATERIALS AND METHODS

Fresh samples of *P. hysterophorus* L. were uprooted and collected in August 2018, in vegetative stage from infested areas in Kiambu county (1°10'29.3"S 36°49'49.4"E), 1679 m above sea level. The climate is warm, mean temperature: 18.8 °C and mean annual rainfall: 962 mm. These samples were washed in lab to remove dirt etc , thereafter, laboratory studies and Pot culture studies were done in our Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya. The studies were done between August 2018 to December 2019. The seeds of Kenyan *Zea mays* L. (H625, H614D, DH02, H513, H6213), *Phaseolus vulgaris* L. (GLP2, GLP92) and *Vigna radiata* (L.) R. Wilczek (KS20) varieties used for the study, were bought from Kenya Seed company (Table 1).

Table 1. Kenyan test crops (*Zea mays, Phaseolus vulgaris* and *Vigna radiata*) varieties used

Monocot	Variety
Zea mays	
Low land	DH02
Medium Altitude	H513
Highland	H6213, H 614
Transitional Zone	H625
Dicots	Variety
Phaseolus vulgaris	GLP2, GLP92
Vigna radiata	KS20

Plant leachates and synthetic phenolic compounds preparation

The leaves were separated, then oven dried at 45° C for 12 h and grinded into powder form, 50 g powder was soaked in 1000 ml distilled water in dark at room temperature (25° C) for 24 h. The solution was filtered through a double layer of muslin cloth, followed by Whatman No. 1 filter paper. For the synthetic phenols 1 mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA) and Hydroxybenzoic acid (HB) (Table 2), were prepared, by mixing the powders in 1 % methanol and then added autoclaved distilled water. The pH was adjusted to 6.0 using 0.5 mM $CaSO_4$ to avoid the strong acidifying effect.

Table 2. Test synthetic phenols used.

Acronym	Ring1	Ring 2	Ring 3	Ring 4	Ring 5	MW
HB	H	OH	H	H	H	138
PA	H	OCH_3	OH	H	H	164
mA	H	H	OCH_3	H	H	152
pA	H	H	H	H	H	152

H: Hydrogen, OH: Hydroxy, OCH_3: Methoxy, M: Anisic (mA), P: Anisic (pA), Coumaric (PA) and Hydroxybenzoic acid (HB), R: Ring, MW: Molecular weight.

Sandwich Bioassay

A method similar to that used by (40), was used with few modifications. Agar (7.5 L^{-1} w/v, Sigma-Aldrich) was mixed in distilled water and then autoclaved at 121° C for 20 min and allowed to cool at 45° C. 5 mL aliquots of agar solution was added to the sterilized culture plates (60 mm dia) under sterile conditions in laminar air flow hood and the agar was allowed to cool. The agar plates were then used for different treatments. Using a 5 ml syringe, 2 mL of each treatment solution was evenly spread on the surface of agar plates. Then further added 5 mL aliquot of liquid agar to plates to form a sandwich. Ten seeds of each variety were surface sterilized for 15 min using 5 % v/v Sodium hypochlorite solution and rinsed thrice in sterile water. These seeds were soaked in sterile water in dark for 12 h. The surface sterilized seeds of *Z. mays*, *P. vulgaris* and *V. radiata* varieties were sown equidistant on top of each culture plate. The plates were kept in the germination incubator (Ruihua, China) at 25/20° C (day/night) thermoperiod, with 12/12h day/night photoperiod (light intensity 100µmol $m^{-2}s^{-1}$) and relative humidity of 80 %. The locations of culture plates were randomly changed daily. After 4 days, the plates were opened and seedlings were thinned to 5 uniform seedlings per plate. The plates were covered and placed in the germination incubator for 3 days under similar conditions. After 7 days, the seedlings were gently removed from the agar and washed in sterile water. The shoot and root length of 5-seedlings per plate were measured by scale.

Pot culture

The test crops seeds were sterilized (treated with 5 % v/v Sodium hypochlorite solution for 15 min, rinsed thrice in sterile water and then soaked in sterile water in dark for 12 h). Five seeds of *Zea mays*, *Phaseolus vulgaris* and *Vigna radiata* were sown per pot (10 cm × 10 cm dia and depth). The pots were filled with 750 g mixture of loam soil + sand (1:2, w/w). Seedlings were grown in a BOD Incubator under 18/25° C (night/day), 14 h/10 h photoperiod and 60-85 % relative humidity. In each pot, 500 ml treatment solution was added and pots were covered with clear film to prevent evaporation of solutions. Afterwards 500-mL/pots were applied on alternate days for 8 days. The treatments were replicated thrice in Randomized Complete Block Design.

I. Physiological parameters

(i). Leaf Electrolyte Leakage (EL): It was measured using 0.1 g fresh samples. The fresh leaf samples were harvested 10-days after treatment. The leaf segments were then immersed in 50 ml tube that was filled with 15 ml deionized water and placed in shaker for 24 h at room temperature. The initial conductance (Ci) was then recorded using a conductivity meter. Then leaf segments in 50 ml tube were autoclaved at 120˚ C for 30 min. After the solution had cooled to room temperature, the maximum conductance (Cmax) of the incubation solution with dead tissues was recorded. The relative EL of the samples was calculated as (Ci/Cmax) ×100 similar to method used by (52).

$$EL = (Ci/Cmax) \times 100$$

(ii). Relative Water Content: After 10 days, Fresh leaves of test plants of *Zea mays, Phaseolus vulgaris* and *Vigna radiata* were harvested and fresh weight was recorded. The leaves were placed in aluminum foil and dried in oven for 48 h at 45° C to record Dry weight. Fresh and dry weight were used to calculate the relative water content:

$$RWC = FW - (DW/ FW)$$

Where, FW: Fresh weight, DW: Dry weight

(iii). Total Chlorophyll Content: Fresh leaf samples were harvested 10 days after treatment and 0.5 g fresh leaf tissue was taken and placed in 10 mL of 95 % ethanol and grounded. It was centrifuged at 5000 rpm for 10 min at 4 °C the supernatant was collected. Its absorbance was recorded at 647 nm and 664 nm against blank. Total chlorophyll content was calculated using the formulae (36):

Chlorophyll a = 13.19 (ABS at 664nm) − 2.57 (ABS at 647nm) μg/g dry weight,
Chlorophyll b= 22.10 (ABS at 647nm) − 5.26 (ABS at 664nm) μg/g dry weight,
Total chlorophyll= 7.93 (ABS at 664nm) + 19.53 (ABS at 647nm) μg/g dry weight.

II. Antioxidant Enzyme Activities

(i). Ascorbate Peroxidase (APX) Activity: The APX activity was determined as per (20). 0.2 g fresh leaves of test crops *Zea mays, Phaseolus vulgaris* and *Vigna radiata* were grinded in liquid nitrogen and 2 ml buffer was used for extraction (20). Two hundred mM potassium phosphate buffer (pH 7.0), 10 mM ascorbic acid and 0.5 M EDTA were mixed to prepare the assay buffer. The leaf samples were placed into tubes containing the assay buffer and centrifuged at 12000 rpm for 20 min at 4 °C and the supernatant was collected. An activity assay solution of 10 mM ascorbic acid, 0.5 M EDTA and 200 mM potassium phosphate buffer, H_2O_2 (1 ml) and supernatant 50 µl was used to measure the APX at 290 nm.

(ii). Catalase (CAT) Activity: The CAT activity was determined as per (12). 0.2 g of fresh leaves of test crops *Zea mays, Phaseolus vulgaris* and *Vigna radiata* were dry grinded in liquid nitrogen. It was homogenized at 12000 rpm for 20 min at 4 ºC in buffer solution [50 mM potassium phosphate buffer (pH 7.0) and 1 mM dithiothreitol (DTT)] to collect the supernatant to determine CAT. An assay solution containing 50 mM phosphate buffer (pH 7.0), 59 mM H_2O_2 and 0.1 ml enzyme extract was used to measure the CAT activity. The decrease in absorbance at 240 nm was measured after every minute. CAT activity was expressed on seed weight basis, as absorbance change of 0.01 $min^{-}1$ was defined as 1 Unit of CAT activity.

(iii). Peroxidase (POD) Activity: The POD activity was determined as per (17). 0.2 g of fresh leaves of test crops *Zea mays, Phaseolus vulgaris* and *Vigna radiata* were dry grinded in liquid nitrogen then homogenized in assay buffer [50 mM potassium phosphate buffer (pH 7.0), 0.1 M EDTA and 1 mM DTT (17)]. An assay solution of distilled water (545 µl), 200 mM phosphate buffer (pH 7.0), 200 mM guaiacol, 400 Mm H_2O_2 and 15 µl enzyme extract was used to measure POD activity. The enzyme extract was added to the assay solution to initiate the reaction. The increase in absorbance at 470 nm of the reaction solution was recorded. POD activity was expressed on seed weight basis with one unit of POD activity being defined as an absorbance change of 0.01min^{-}1.

IV. Total Protein Content

The total protein was determined as per (15). 0.2 g of fresh leaves of test crops *Zea mays, Phaseolus vulgaris* and *Vigna radiata* were dry grinded in liquid nitrogen then homogenized in assay buffer [50 mM potassium phosphate buffer (pH 7.0)]. An assay solution [5 µl of supernatant and 0.1 N NaCl were mixed with 1.0 ml of Bradford dye] and the mixture was allowed to stand for 5 min to form a protein dye complex. The absorbance was measured at 595 nm.

V. Leaf and Root Total Carbon

The leaf and root total carbon % was determined as per (25). The leaf and root samples of test *Zea mays, Phaseolus vulgaris* and *Vigna radiata* varieties were collected and dried in oven at 45 ˚C for 24 h and ground into fine powder. Then encapsulated leaf samples (0.002-0.0035 g) and root samples (0.0009-0.0015 g) were subjected to isotope analysis using Isotope Ratio Mass Spectrometer (Finnegan: Thermo Fisher Scientific, model MAT-253, Germany) at Public Stable Isotope lab, Wuhan Botanical Garden.

Statistical Analysis

All the data was reported as mean ± SD. Descriptive statistics was applied to analyze and organize the resulting data. Data were analyzed using one-way ANOVA. Significance of data was tested by analysis of variance and Tukey (HSD) Test at $p < 0.05$ and where applicable at $p < 0.01$ using SPSS 20.0 software (SPSS Inc., Chicago, USA). HPLC data were acquired and analyzed by the Analyst software version 1.6.1 (AB SCIEX, Foster City, CA).

RESULTS AND DISCUSSION

Shoot and Root lengths

The *Z. mays* varieties H614D, DH 02 and GLP 92 were most resistant to the allelochemicals, while *Vigna radiata* was least resistant (Figure 2 & 3). Application of synthetic phenols M-Anisic (mA) and P-Anisic (pA) were most inhibitory to shoot and root length. But the Hydroxybenzoic acid (HB) and Coumaric (PA increased the root length, than aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH3 ring substituents (Fig 2 and 3). The root length of *Z. mays* was reduced, by 35.04-51.72 % PA, by 60.14-76.44 % mA, by 66.56-83.40 % pA and by 0.64-19.68 % HB i.e. pA and mA were most inhibitory The root length of *P. vulgaris L.* and *V. radiata* was reduced 28.33-48.70 % (PA), 44.65-58.62 % (mA), 51.76-62.49 % (pA) and 3.70-4.75 % (HB) (Fig 1).

Shoot length of *Z. mays* was reduced by 32.34-49.79 % (PA), 54.64-65. 61% (mA), 60.44-74. 09% (pA) and 3.72-10.89 % (HB), while the inhibition in shoot length of *P. vulgaris* and *V. radiata* was 25.72-49.94 % (PA), 40.78-62.83 % (mA), 50.34-69.40 % (pA) and 3.80-10.58 % (HB). The pA and mA caused maximum inhibition (%).

From this study we found that allelochemical stress greatly affected the cytomorphological behavior of the target crop varieties. The effects depended on the variety of target species and the chemical structure of the tested phenolic compounds. The phenolic compounds with the –OCH_3 (mA, pA) ring substituents decreased the morphology and cytomorphological behavior, while, those with –OH (HB, PA) ring substituents increased these parameters. The results are similar to (16) who found that the effects depended on –OH or –OCH_3 ring substituents on *Cucumis sativus* L. The inhibitory effects of phenols are dependent on the position of their hydroxy and methoxy ring substituents (31,38).

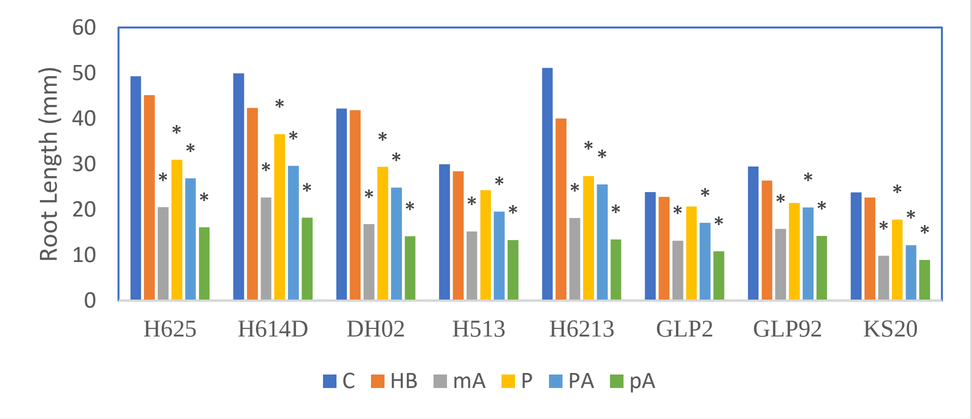

Figure 2. Effects of Allelochemical stress on the root length of selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

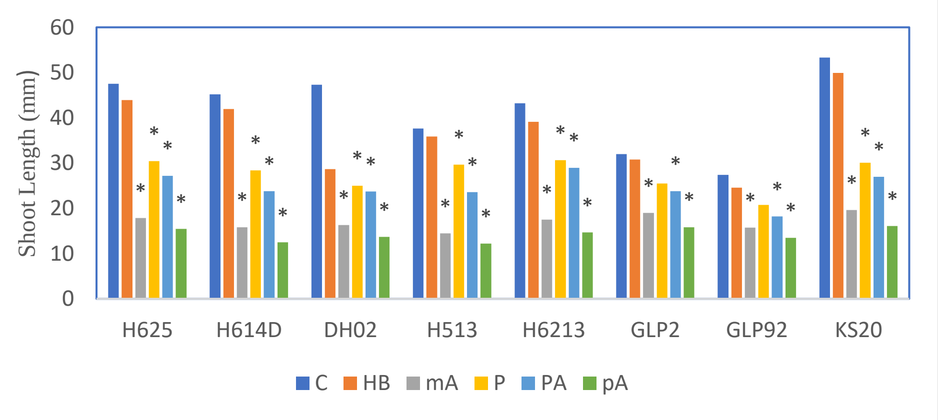

Figure 3. Effects of Allelochemical stress on the shoot length of selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

Membrane integrity

The allelochemical stress increased the electrolyte leakage (EL) ($P < 0.05$) (Figure 4). The induced inhibitory effects on target species varied with the treatments. The applied aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents (pA and mA) increased the EL ($P < 0.05$), while those treated with phenolic compound with –OH ring substituent PA and HB) decreased the EL ($P < 0.05$). The effects on membrane integrity among the target species ranged from 7-47 % (HB), 43-76 % (mA), 38-64 % (P), 39-68 % (PA) and 56-81 % (pA). The membrane integrity of *Z. mays* varieties H614D and DH02 was very resistant to allelochemical stress, while among the dicots, *P.vulgaris* cv. GLP92 showed the greatest resistance. While, maize varieties (H6213, H513), *P. vulgaris* cv. GLP2 and v radiata Cv KS20 were least resistant to allelochemical stress on membrane integrity.

Allelochemicals drastically increased the electrolyte leakage (EL) of target species grown in the aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents, compared to those treated with phenolic compound with –OH ring substituent and control (Figure 4). The increase in EL showed increased membrane damage, thereby, affecting the membrane activity. Allelochemical stress alters the membrane proteins through oxidation or cross linking the sulfhydryl groups and also modifies the membrane permeability (38). Besides the causes the rapid depolarization membrane due to the effects of phenolic compounds on the diffusion potential or electrogenic potential of plant cells (31,37). The effects on the membrane depends on

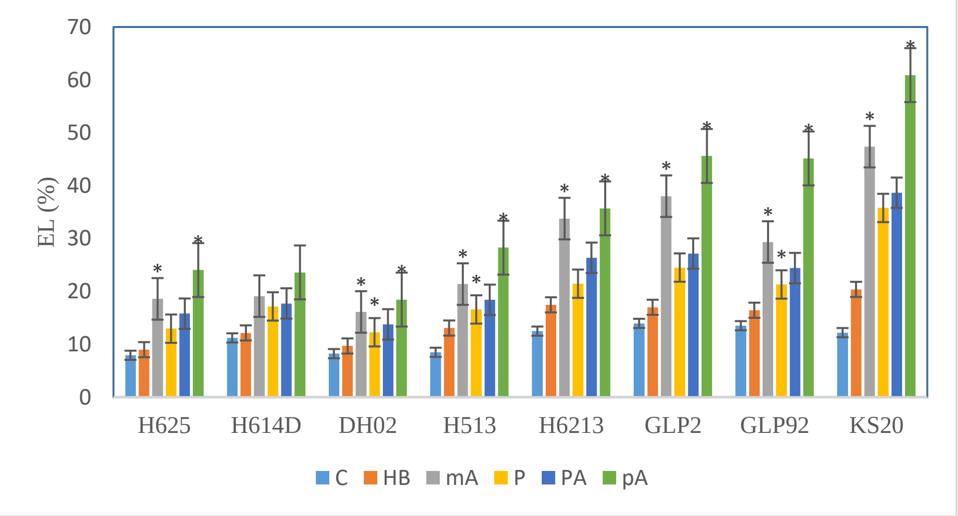

Figure 4. Effects of Allelochemical stress on the leaf electrolyte leakage (EL) content in selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

whether the phenolic compounds have an –OH or –OCH_3 ring substituents (31). Those with –OCH_3 ring substituents causes depolarization due to low lipophilicity, while those with –OH ring substituents leads to hyperpolarization as a result of high lipophilicity (31). This explains the reason for the increase in EL for the target varieties treated with aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH_3 ring substituents compared to those treated with phenolic compound with –OH ring substituent and control that decreased EL.

Relative Water Content

Relative water content differed with treatments ($P < 0.05$) (Figure 5). The applied aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH_3 ring substituents (pA and mA) decreased the relative water content by 10-60 % (mA), 20-76 % (P) and 6-57 % (pA). While Relative water content of those treated with phenolic compound with –OH ring substituent (HB and PA) was increased by 26-85 % (HB), 17-70 % (PA). Maize varieties (H614D and DH02) showed greatest resistance to allelochemical stress from the aqueous extracts of *P. hysterophorus* and phenolic compounds; while among the dicots, *P. vulgaris* variety GLP92 showed the greatest resistance. Maize variety H6213, *P. vulgaris* cv. GLP2 and *V. radiata* Cv KS20 relative water content (%) was least resistant to allelochemicals stress from the aqueous extracts of *P. hysterophorus* and phenolic compounds.

The increase in EL, indicates an increase in membrane damage, which leads to a drastical reduction on the relative water content (Figure 5). The membrane is the initial site of action for the phenolic compounds as they act at the plasma membrane level (31,38), in which after perfusion they changes the membrane permeability (6). This was as a result of imbalance in the electroneutral equilibrium due to the production of negative surface potential or increase in the anionic field (16), which reduced the relative water

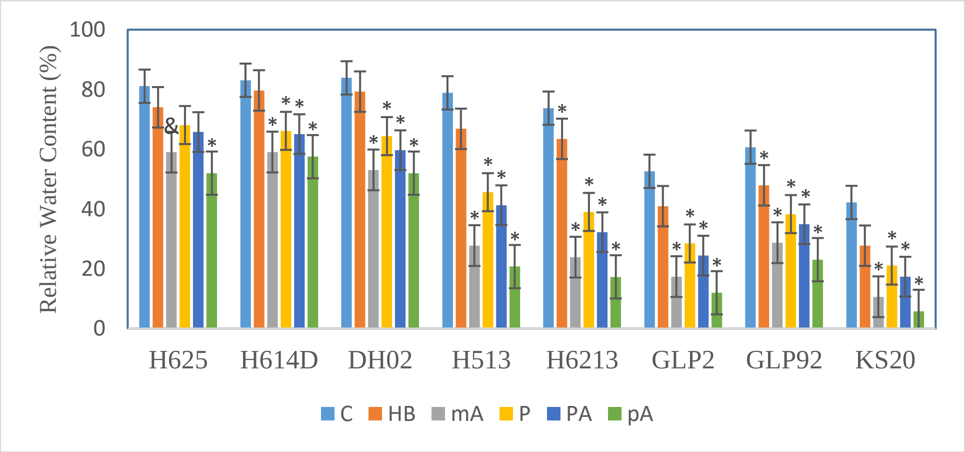

Figure 5. Effects of Allelochemical stress on the Relative Water Content % in selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

content, in target species treated with aqueous extracts of *P. hysterophorus* L. and phenolic compounds with –OCH_3 ring substituents (mA, pA) than those treated with phenolic compound with –OH ring substituent and control. This leads to symptoms that includes; plant dysfunction (8), reduction in water absorption and use (10), dehydrogenase activity in the roots, loss of chlorophylls and carotenoids (44) and damage of cellular membranes of plants leading to loss of biomass due to desiccation (16,39,40,42). This can be detrimental to the crops, as it affects the communication between the plants and the environment and in turn affects the biophysical, biological and biochemical interactions of the crops and their environment leading to lower yields (5,10,14). Our results are similar to those reported by (46) on musk melon and cucumber and (35) on wheat, rice, millet and sorghum.

Leaf Chlorophyll Content

The allelochemicals present in aqueous extracts of *P. hysterophorus* decreased the leaf chlorophyll content ($P < 0.05$) and caused the leaf necrosis and senescence (Figure 6). The effects depended on whether the phenolic compounds had an –OH (HB, PA) or –OCH_3 (mA, pA) ring substituents. The applied aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH_3 ring substituents decreased the leaf chlorophyll (Chla, Chlb and total Chl) content ($P < 0.05$), while, those treated with phenolic compound with –OH ring substituent increased the leaf chlorophyll content ($P < 0.05$). Maize variety H614D showed the highest total Chl, while, legume *P.vulgaris* cv. GLP2 showed the highest total Chl levels among the dicotyledons.

Allelochemical stress causes leaf necrosis and senescence, as evident from the reduction in leaf chlorophyll content in target varieties treated with aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH_3 ring substituents. This is due to allelochemicals stress resulting in Chl degradation. The Chl degradation may be owing to allelochemical stress leading to the over production of O_2 and H_2O_2 that causes destruction of Chl and chloroplast (44). The degradation of Chl in the plant leads to decrease in photosynthesis levels (34) as H_2O_2 inhibits CO_2 fixation due to the oxidation of the thiolmedulated enzymes of the Calvin cycle due to the buildup of H_2O_2 (44) (Figure 6).

Enzymatic Antioxidants

The applied aqueous extracts of *P. hysterophorus* L. and phenolic compounds with –OCH_3 ring substituents, reduced the levels of antioxidant Enzymes (APX, CAT and POD) ($P < 0.05$) in all test plants (Figure 7). The application of phenolic compounds with –OH ring substituent increased the level of antioxidant enzymes ($P < 0.05$) (Figure 6) than those treated with –OCH_3 ring substituents. For APX, the effects ranged from 6-28 % (HB), 64-90 % (mA), 31-71 % (P), 47-74 % (PA) and 71-95 % (pA). Maize variety H6213, *P.vulgaris* cv. GLP2 were most affected, but the maize variety DH02 was resistant to the inhibitory effects of allelochemicals. For CAT the effects of the treatments ranged from 9-33 % (HB), 53-72 % (mA), 37-52 % (P), 43-65 % (PA) and 70-81 % (pA). POD was most inhibited by the allelochemicals in aqueous extracts of *P. hysterophorus* and phenolic compounds with –OCH_3 ring substituents (Figure 6) and the effects ranged from 11-34 % (HB), 52-95 % (mA), 37-83 % (P), 45-80 % (PA) and 65-97 % (pA). The greatest effect was observed in *V. radiata* variety KS20 and the least effect was observed in *P.vulgaris* cv. GLP2 and Maize varieties H625, H614D, DH02.

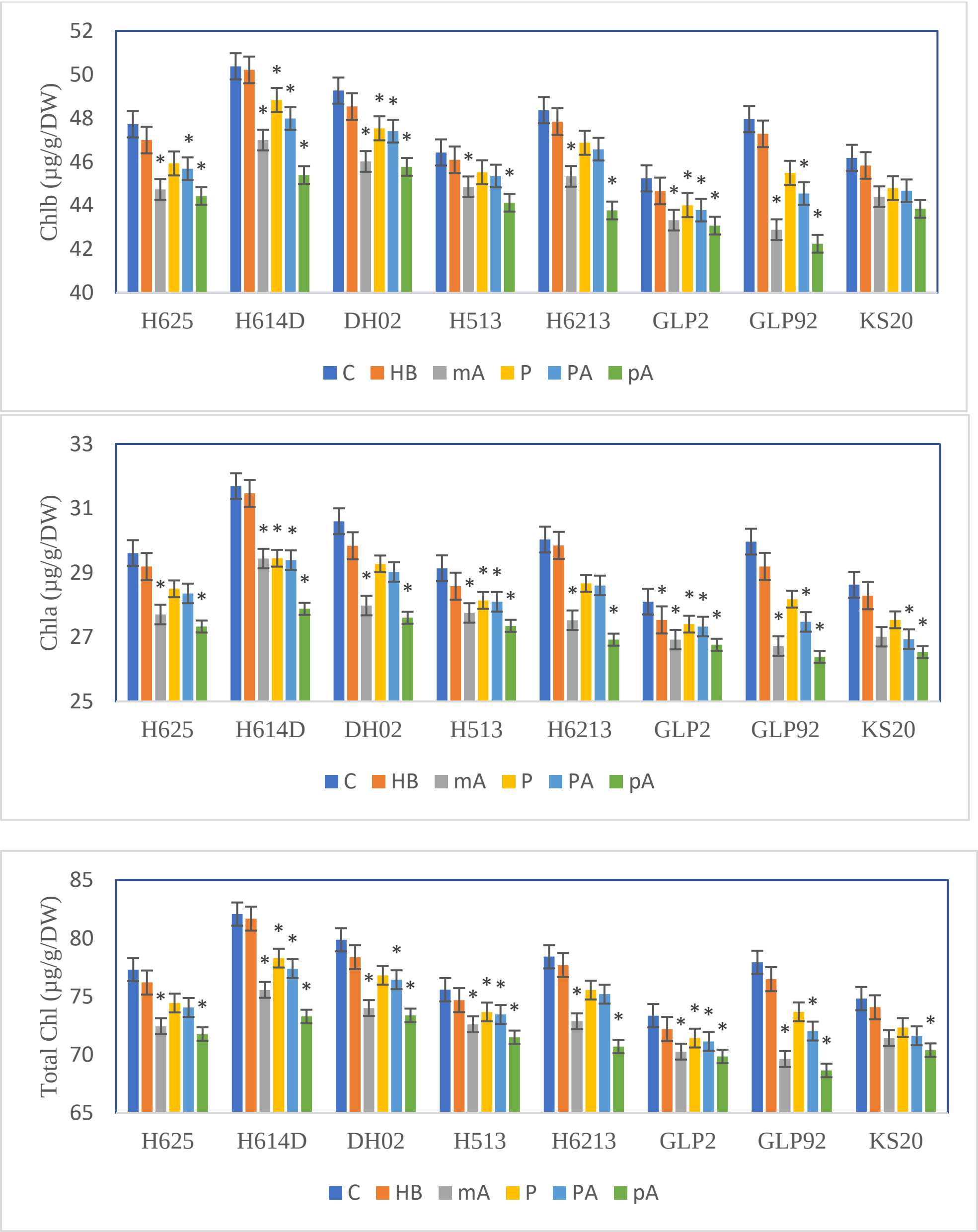

Figure 6. Effects of Allelochemical stress on Leaf Chlorophyll Content in selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

The antioxidant enzymes (APX, CAT and POD) (Figure 7), were significantly reduced by allelochemical stress. Ascorbate peroxidase (APX) enzyme which is crucial compound for the glutathione ascorbate cycle was significantly reduced by allelochemical stress. It is involved in detoxification of H_2O_2 and protection of plant cells from environmental stress (29). The reduction in APX can be seen in the reduction in Chl content levels (44) caused by the increase in H_2O_2 and increase in EL as a result in changes in membrane activity due to the reduction in enzymes involved in plant cell protection. Catalase (CAT) was also significantly reduced in the target varieties treated with aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents. CAT is important and critical against oxidative stress (49), induced by environmental stress (30). The reduction in CAT levels shows the lack of resistance to allelochemical stress by test crop varieties, which reduces the growth and increases the cytomorphological behavior of crops (10). Peroxidase (POD) was the enzyme most affected by allelochemical stress. It is responsible for scavenging the reactive O_2 (49) that is responsible for cell oxidative injury, control of growth by lignification, cross linking of pectin's and structural proteins in cell wall, catabolism of auxins (43,51). It regulates most of the important physiological processes in the plant. The aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents substantially lowered the enzyme activity showing a direct link to enzyme catalysis (24) . The results are similar to those observed by (30) in their study on the effect of leaf leachates of *Gmelina arborea* Roxb. on inhibition of essential seed germination enzymes in greengram, redgram, blackgram and chickpea. The reduction greatly affects the growth and development of crops, as allelochemical stress affects the release of amino acids, stored proteins and carbohydrates that are responsible for germination and plant establishment (4,42).

Total Protein Content

Allelochemicals stress decreased the total protein content ($P < 0.05$) (Figure 8) in test crops varieties than control. The effects depended on whether the phenolic compounds had an –OH (HB, PA) or $–OCH_3$ (mA, pA) ring substituents. The target species grown in the aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents decreased the total protein content 30-55 % (mA), 21-44 % (P) and 44-64 % (pA), while, those treated with phenolic compound with –OH ring substituent increased the total protein content 6-2 9% (HB), 25-5 0% (PA.

The effects of allelochemical stress on enzymatic antioxidants was further observed in the low protein content of the target species treated with the aqueous extracts of *P. hysterophorus* and phenolic compounds with $–OCH_3$ ring substituents. The target varieties treated with the phenolic compounds that had the –OH ring substituents increased the protein content compared than target species treated with phenolic compounds with $–OCH_3$ ring substituents. Allelochemical stress alters the protein content of crops by affecting the enzymes responsible for protein synthesis (10,24) and by oxidizing and cross linking the sulfhydryl groups (11). The effects on protein content shows that the phenolic compounds responsible for allelochemical stress are not limited only to the plasma membrane (where they alter the membrane activity), but they also affects the cytosol causing adverse effects in other metabolic processes (6,8). The decrease in protein content due to allelochemical stress may be attributed to decrease in vegetative growth parameters, photosynthesis and free amino acids found in the leaf tissue (10).

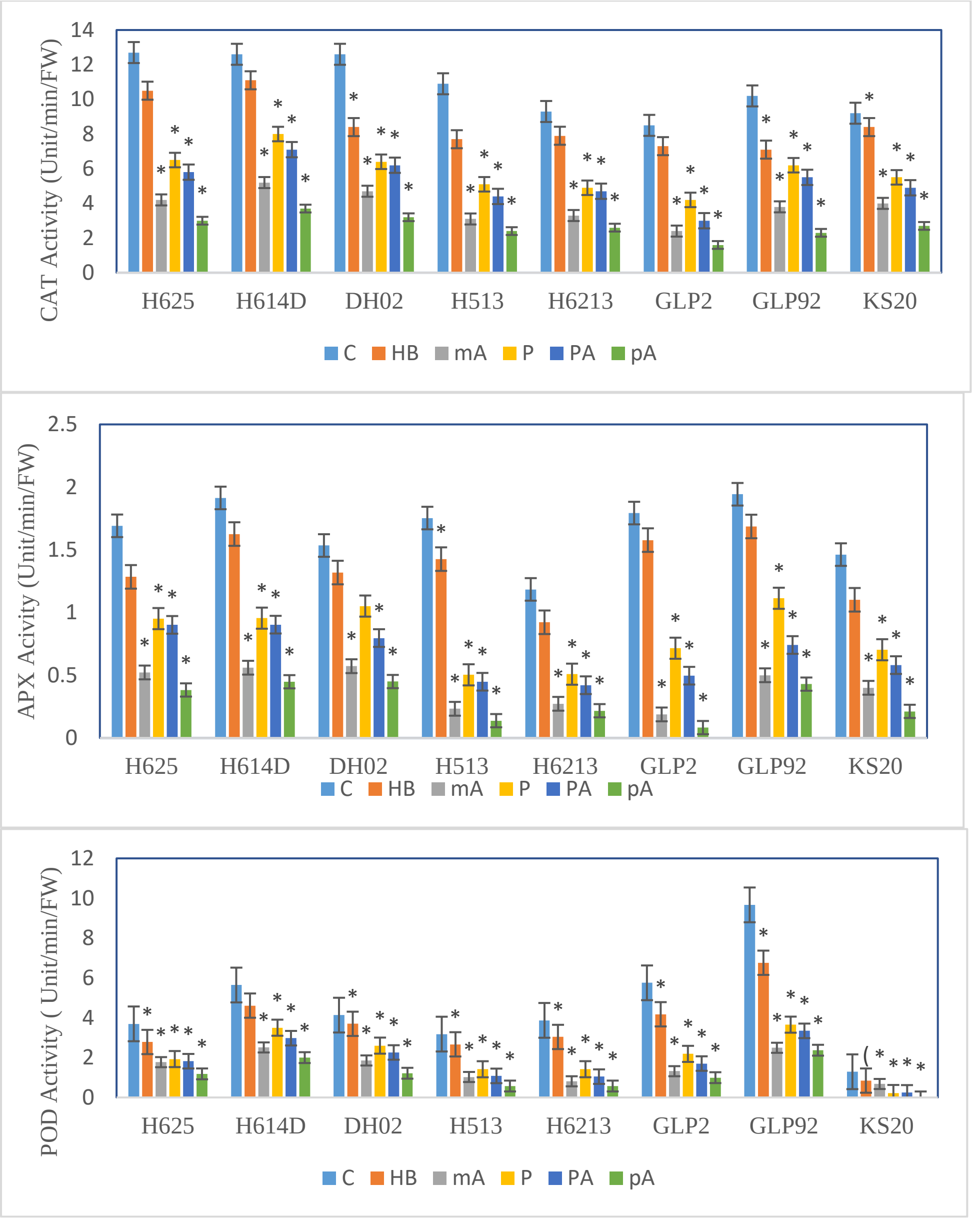

Figure 7. Effects of Allelochemical stress on Enzymatic antioxidants (APX, CAT and POD) in selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

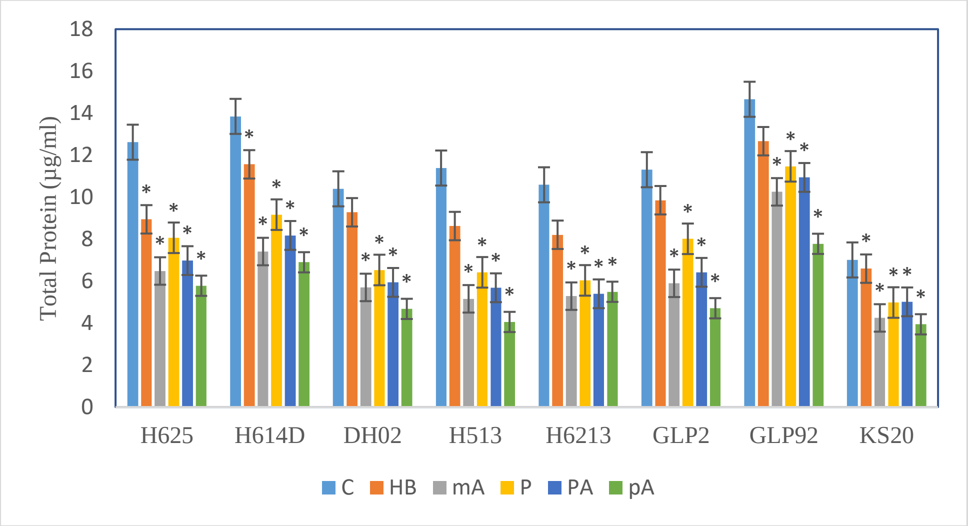

Figure 8. Effects of Allelochemical stress on Total Protein Content in selected Kenyan *Zea mays L. (H625, H614D, DH02, H513, H6213), Phaseolus vulgaris L. (GLP2, GLP92)* and *Vigna radiata (L.) R. Wilczek (KS20)* varieties pretreated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C). Data are expressed as mean _ SE. * mean difference is significant at 0.05 level.

Leaf and Root Carbon (%)

Allelochemicals stress decreased the leaf and root carbon (%) in test crops varieties compared to control (Table 3). The target species grown in the aqueous extracts of *P. hysterophorus* and phenolic compounds with $-OCH_3$ ring substituents decreased leaf and root carbon (%) (Table 3). The applied pA and mA treatments drastically decreased the leaf and root carbon (%). While, it was increased in those treated with phenolic

Table 3. Effects of allelochemicals stress on leaf and root carbon (%) content in selected *Zea mays, Phaseolus vulgaris* and *Vigna radiata* varieties pre-treated with aqueous extracts of *P. hysterophorus* (P) and 1mM of M-Anisic (mA), P-Anisic (pA), Coumaric (PA), Hydroxybenzoic acid (HB) and Control (C).

Crops	Varieties	Carbon (%)	
		Leaf	Root
Maize	DH02	36.49±1.465**	60.35±2.470**
	H513	35.03±1.772**	58.60±2.592**
	H625	33.89±1.747**	54.97±2.169**
	H614D	37.93±1.258**	68.42±2.800**
	H6213	39.07±3.021**	57.20±3.598**
Kidney bean	GLP2	14.89±0.424**	46.76±1.732**
	GLP92	15.18±0.537**	44.92±3.817**
Greengram	KS20	14.98±0.731**	40.28±2.078**

Data presented as mean ± S.E.** means significant from control at P< 0.01 after applying student *t*- test compound with –OH ring substituent similar to control. The effects on leaf carbon (%) ranged from 16-50 % (C), 16-46 % (HB), 14-53 % (mA), 15-38 % (P), 14-37 % (PA) and 13-35 % (pA). The effects on root carbon (%) ranged from 48-79 % (C), 44-74 % (HB), 36-64 % (mA), 41-66 % (P), 36-66 % (PA) and 36-61 % (pA). (Table 3).

Allelochemical stress as a result of release of phenolic compounds into the atmosphere is harmful to the plant, by effecting the membrane activity and water balance that affects the stomatal response in treated target varieties (6). The effects on stomatal response are observed in high diffusive resistance, transpiration rates and the less negative Carbon isotope values observed in plants (25), leading to low levels of C % observed in leaf and root. This can be attributed to the reduced diffusion of CO_2 through the stomatal aperture (34). Allelochemical stress causes closure of stomata and this leads to discrimination on different isotopes, it also interferes with the uptake and availability of crucial nutrients by other plants (16).

AUTHOR'S CONTRIBUTION

Data curation: Jeffrey Okundi and Sylvia Cherono; Formal analysis, Jeffrey Okundi and Sylvia Cherono; **Funding acquisition:** Yan Xue and Qing-Feng Wang; **Investigation:** Jeffrey Okundi; **Methodology:** jeffrey Okundi and Sylvia Cherono; **Supervision:** Moses Kirega, Yan Xue and Qing-Feng Wang; **Validation:** Moses Kirega, Yan Xue and Qing-Feng Wang; **Writing-original draft:** Jeffrey Okundi and Moses Kirega; **Writing- review & editing:** Jeffrey Okundi, Sylvia Cherono, Moses Kirega, Yan Xue and Qing-Feng Wang

COMPETING INTERESTS SECTION

JO declares that he has no competing interest, SC declares that she has no competing interest, MG declares that she has no competing interest XY declares that he has no competing interest and QF declares that he has no competing interest.

FUNDING

This study was funded by Sino-Africa Joint Research Centre, Chinese Academy of Sciences (SAJC201322, SAJ201330) and the National Science Foundation of PR China (31270372).

ACKNOWLEDGEMENTS

We acknowledge Kang Ji, Mo Belal, Dr. Flora, Elizabeth Gathoni, Millicent Oulu, Muchuku Kamau and Misganaw for assistance, experiments and development of this manuscript.

DECLARATION

We declare that all authors of this Ms have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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PUBLISHER NOTE