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Table: 1, Figs: 11

Allelopathic effects of harmala (*Peganum harmala* L.) seeds on germination and seedlings growth of barley (*Hordeum vulgare* L.)

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ABSTRACT

We investigated the allelopathic inhibitory effects of harmala (*Peganum harmala* L.) seeds, seed powder and water extracts on barley (*Hordeum vulgare* L.) seed germination and seedling growth. The harmala seeds inhibited the barley germination and seedling growth and also significant effected the on root growth than on shoot growth. Ground harmala seed (0.06-0.72 g) had highly significant inhibitory effects on barley germination, except at the lowest concentrations (0.06 and 0.12 g/dish). Water extracts of harmala seeds concentrations (0.6-7.2 %) inhibited the barley germination and seedling growth by 2.4 %. There were clear inhibitory effects on root elongation and plumule growth. Additionally, the harmala extract caused radicle decay in barley seedlings. In the field, 1 g of ground harmala seeds inhibited the germination and growth of barley seeds and monthly irrigation with 5 g/L inhibited the fresh and dry weight of barley plants. The inhibitory effects were also on spikes and roots, number and weight of seeds. The germination and growth of new seeds were also inhibited. Similarly, adding 5 g of ground harmala seeds significantly affected the fresh and dry weight of barley plants, with leaves, stems and roots were more affected than spikes. Overall, this study provides an explanation for the absence of barley growth in harmala-infested soil.

Key Words: Allelopathy effects, barley, inhibition, harmala, *Hordeum vulgare*, *Peganum harmala*, seed germination, seedlings growth.

INTRODUCTION

Allelopathy is a natural phenomenon describing the relationship between two or more plants, where one plant produces secondary metabolites that negatively affect the germination, growth, survival and reproduction of other organisms. The term allelopathy comes from two Greek words: "Allelon," meaning "each other," and "Pathos," meaning "harmful." Hans Molish first used the term in 1937 (12,20). Rice (24) defines allelopathy as a direct or indirect interaction between plants that results in stimulatory or inhibitory effects through the release of chemicals into the environment. Allelopathy is also known as allelochemical interactions and occurs in aquatic organisms, specifically between macrophytes and attached microbial assemblages (epiphytic), which influence several ecological processes (7). Allelopathy can affect individual plant performance, community structure and plant invasion (31), where one plant suppresses the other with specialized biomolecules. Allelopathy and autotoxicity frequently occur among living organisms. Plant and soil biospheres decrease or completely hide the phytotoxicity of allelochemicals (8,14). Various crops have been discovered to release allelopathic compounds that can either stimulate and/or inhibit plant growth at the same time (12).

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Barley (*Hordeum vulgare* L.) belongs to the Triticeae tribe in the family Poaceae or Gramineae (4), which includes all grasses. Gramineae is a large and nearly ubiquitous family of monocotyledonous flowering plants commonly known as grasses, including cereal grasses, bamboos, grasses of natural grasslands, as well as species cultivated in lawns and pastures. Barley is an economically important cereal crop worldwide, as its grains are used for food and fodder and its plant residues are animal feed during the dry season (26). It is the fourth most important crop.

Harmala (*Peganum harmala* L.) is a toxic plant (1) growing in semi-arid rangeland (Figure 1) and has many health benefits (15,17). A study based on the analysis of harmala alkaloids by liquid chromatography coupled to ion mobility spectrometry identified 13-alkaloids differing in their activities. For example, dipegine, harmalanine and harmalacinine have the highest potency in terms of both free energy of binding and similarity of ligand-receptor interactions (23,29). In Libya, the phenomenon of harmala invasion is common within barley fields. During the early 2000s, local farmers complained about the invasion of harmala plants in their barley fields, which had a significant negative impact on barley plants, leading to their weakness and death. This invasion caused the formation of barren areas (areas free of barley plants), as it affected the germination and growth of available seeds in the surrounding area, whether barley or harmala. The farmers complaints prompted the Botany department researchers to study the inhibitory role of harmala in barley crops on these farms and investigate whether it causes a significant negative impact on barley plants.

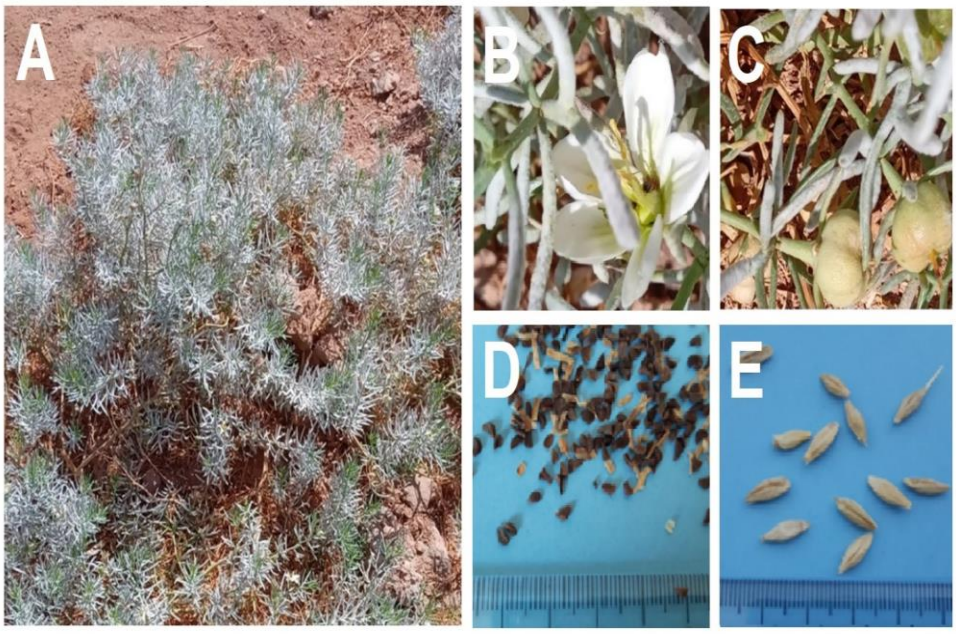

Figure 1. A) Harmala plants growing in desert. B) Harmala flower. C) Harmala fruits. D) Harmala seeds. E) Barley seeds. Scale: Millimeter gridded ruler.

The main goal of this research was to study the allelopathic effects of different numbers of harmala seeds, various weights of ground harmala seeds and different concentrations of water aqueous extract of harmala seeds on the germination and seedling growth of barley in the laboratory. Additionally, this research aims to investigate the allelopathy effect of the ground seeds and aqueous extract of *P. harmala* on the growth and productivity of barley plants in the field.

MATERIALS AND METHODS

The experiments were conducted at the Botany Department Laboratory, University of Tripoli, Libya during 2020-2021. The field experiments were done in the Botanical Garden, Botany Department, College of Science in the University of Tripoli. The coordinates of the location are 32°53′14″N 13°11′29″E and the elevation is 81m.

The barley (*Hordeum vulgare* L.) cultivar accession 'Aksad 179' was used for the experiments. It was obtained from the Libyan Agricultural Research Center. The harmala seeds (*Peganum harmala* L.) were collected from their natural habitat in the semi-arid area, south of Tripoli (Libya) during the summer of 2020. The collected harmala plant was dried at room temperature in the laboratory and the seeds were prepared for the experiments.

I. Bioassay : Petri dishes with double-layer filter papers wetted with distilled water were prepared and 100-barley seeds were sown in each dish. Four harmala treatments were applied to dishes with barley seeds as listed in Table 1.

(i). Harmala seeds (0, 25, 50, 100, 200 and 300 seeds/plate) were added to barley dishes, (ii). Ground harmala seeds (0.0, 0.06, 0.12, 0.24, 0.48 and 0.72 g/plate) were added to barley dishes, (iii). Aqueous extract of harmala seeds (0.0, 0.6, 1.2, 2.4, 4.8 and 7.2 %) in cold water (25ºC±1) and then added to barley dishes and(iv). Aqueous extract of harmala seeds (0.0, 0.6, 1.2, 2.4, 4.8 and 7.2 %) in warm water (50 ºC±1) were added to barley dishes.

Table 1. Harmala treatments of seed germination bioassays on barley seeds.

#	Treatment	Rate/Dose
	Harmala seeds	
1	Whole seeds Numbers	0, 25, 50, 100, 200 and 300
2	Powdered seeds (g/Plant)	0.0, 0.06, 0.12, 0.24, 0.48 and 0.72 g
	Aqueous Extracts of Harmala seeds	
3	**Cold water (%)** (25 ºC±1))	0.0, 0.6, 1.2, 2.4, 4.8 and 7.2%
4	**Warm water (%)** (50 ºC±1)	0.0, 0.6, 1.2, 2.4, 4.8 and 7.2%

Each treatment was replicated thrice in completely randomized design (CRD). The dishes were incubated in dark conditions at 25 ± 1 ºC and the germinated barley seeds were counted every day. Elongation measurements were taken after 5-days after incubation.

II. Field Experiment: It was done in plastic bags (5 kg semi-dry soil), to test the effects of harmala seeds on germination, growth and productivity of barley plants. There were 2-treatments: (i). Ground harmala seeds (1 or 5 g) added per plastic bag and (ii). Ground harmala seeds (1 or 5 g) added per plastic bag + monthly irrigation with 5 g/l of aqueous harmala seeds. The treatments were replicated 10-times in complete randonised design.

The plants were irrigated with tap water when needed and seedling emergence was determined after 10-days. All barley seedlings were removed but one was left to continue the growth and productivity measurements. The following parameters were measures in the field:

(i). **Plant Height**: Barley shoot was measured from the point, where the plant meets the soil till the spike or flag leaf by tape weekly till the experiment ended. The final plant height was at harvest time, which was taken.

(ii). **Tiller and spikes numbers**: Date of first tiller and spike production and their final number per plant.

(iii). **Fresh weight**: Leaves, stems, spikes and roots, fresh weight was taken immediately after the plant harvest. The plant organs were kept separately in envelopes.

(iv). **Dry weight**: The envelopes containing barley plant organs were dried in oven at 85 ºC for 24 h, thereafter the dry weight was recorded.

(v). **Number and weight of seeds/per plant**: The barley seeds/plant were counted and weighted.

(vi). **Germination of new seeds**: The germination of new barley seeds was determined.

(vii). **Seedlings Elongation of new seeds**: The Petri dishes of germinated barley seeds were incubated for 72 h to determine the seedlings growth.

Statistical Analysis

All plants were included in measurement and analysis. Significance among means was established by using Tukey's HSD (honestly significant difference) test. Hierarchical clustering of all phenotypic data (log2 transformed) collected in the field using Euclidean distance with average linkage. The clustering was illustrated as heat map.

RESULTS AND DISCUSSION

The alkaloids accumulate in the seed's tissues and fresh fruit, hence, harmala seeds and roots inhibits plant germination and growth (28). The inhibitory compounds may exist in the seed coat or in all plant tissues (19). The harmala plants inhibits seed germination and plant growth of nearby plants such as wheat, barley and ryegrass (10,34).

1. LABORATORY BIOASSAYS

Laboratory experiments were done to determine the germination rate and duration of barley seeds and harmala seeds. Barley seeds had 98 % germination rate and fully germinated in 3-days. In contrast, harmala seeds sprouted within 48 h and showed 100 % germination after one week of incubation. However, when harmala seeds were placed in plates alone (without barley seeds) and with seed numbers of 25, 50, 100, 200 and 300 seeds, it was found that the increasing number of harmala seeds had self-inhibitory effect on its own seed germination. The effect was non-significant up to 100 seeds ($P > 0.05$), but highly significant for 200 and 300 seeds ($P < 0.001$).

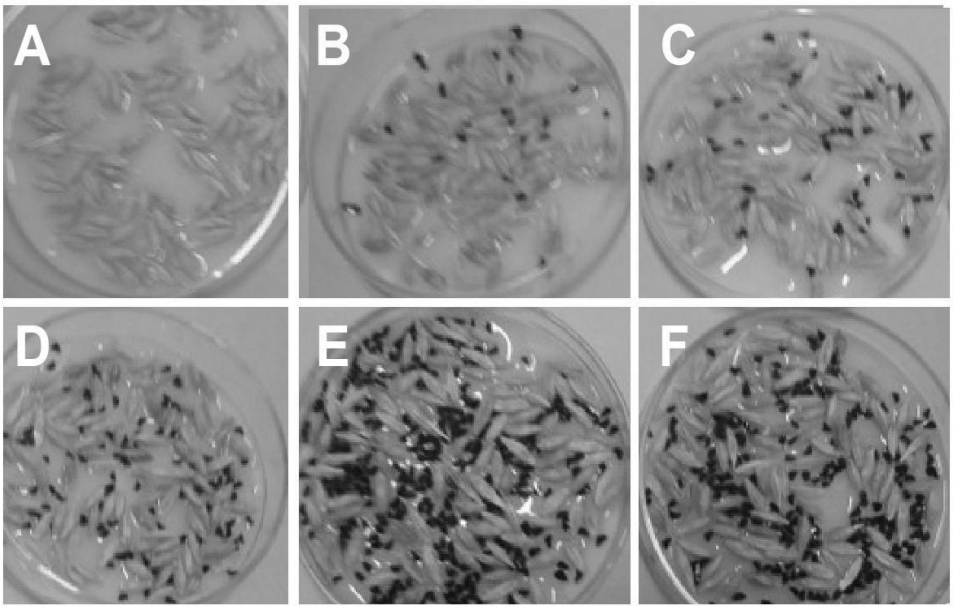

Figure 2. Hamala seed treatment at the start of experiment. A) control. B) 25 Harmala seeds. C) 50 Harmala seeds. D) 100 Harmala seeds. E) 200 Harmala seeds. F) 300 Harmala seeds.

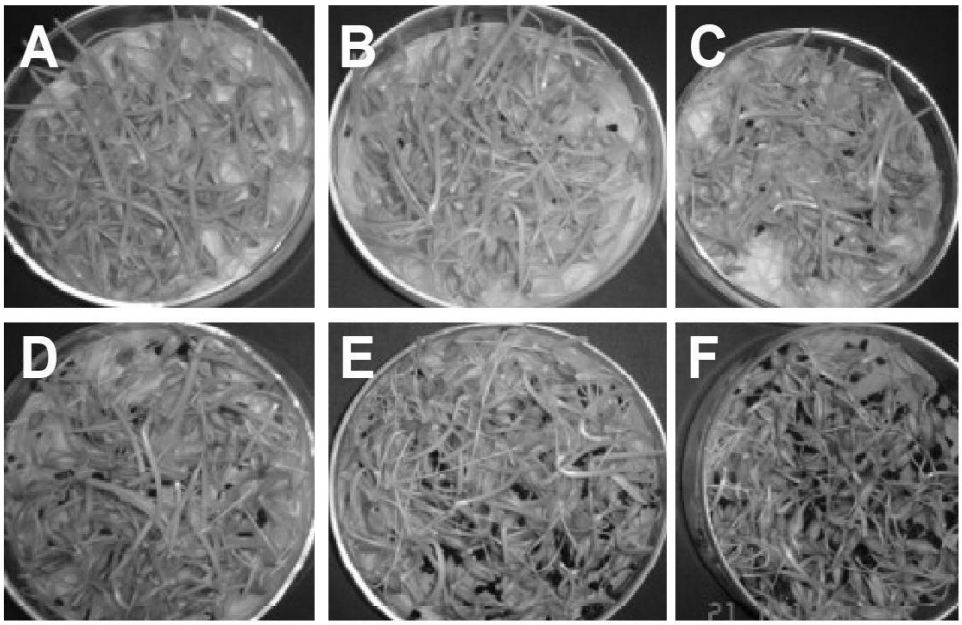

Figure 3. Hamala seed treatment at the end of experiment after one week. A) control. B) 25 Harmala seeds. C) 50 Harmala seeds. D) 100 Harmala seeds. E) 200 Harmala seeds. F) 300 Harmala seeds.

The results indicated a clear effect of harmala on germination and growth of barley, through secretion of allelopathic compounds in rhizosphere of harmala plants or their seeds (Figs 2-3). Those allelopathic compounds spread in the surrounding area of harmala plant material and inhibited seed germination and seedling growth of barley. This agrees with related studies that confirmed plants use alkaloids naturally against insects and microbial attack (8). The presence of these compounds surrounding the root environment affects the biosphere and other plants roots (18,28). These also, inhibited the germination of many plant seeds and seedling growth in their vicinity as well, while the alkaloids' effect varies with their type and concentration (6).

Effects of harmala seeds on germination and seedlings growth of barley seeds

Harmala was sown at 25 to 300 seeds per plate, while barley was sown at 100 seeds per plate. Germination of barley seeds was inhibited, when harmala seeds densities were > 100 seeds and there was 40 % decrease with 300 harmala seeds/dish (Figure 2). The germination (%) of harmala seeds with barley dishes significantly decreased than harmala seeds alone (Figure 3). Apart from inhibiting barley germination, harmala seeds also reduced the number of barley seedlings in Petri dishes and also inhibited their elongation. For instance, 100 harmala seeds in barley dishes changed the colour of barley root tips to dark brown, along with inhibition in their elongation. Moreover, root tips strongly decayed at harmala seed densities of 200 and 300, with vesicles forming at the seedling root tips, followed by vesicle burst. Additionally, harmala seeds significantly reduced the number of seedling roots in barley. The different numbers of harmala seeds in barley dishes also inhibited barley seedlings plumule elongation. One possible explanation for the inhibitory effects of harmala seeds was the presence of a sticky substance in their seed coat that caused them to stick together when wet. This feature increases autotoxicity within harmala seeds to each other, further contributing to their inhibitory effects. Additionally, harmala seeds stick to barley seedling roots, which further increased their inhibitory effects. All responses mentioned before were significant at $P < 0.001$.

The presence of (25 harmala seeds) in barley petri dishes did not decrease the barley seed germination. Increasing of harmala seeds in barley dishes significantly inhibited the barley seeds germination. A sticky substance was released from the harmala seeds coat that helps them to stick to barley roots grown with them in the same dish to increase the inhibitory effect. In addition, they cause necrosis and decomposition of barley roots, which caused the seedling death. Harmala components inhibits a variety of microorganisms by inducing the accumulation of ROS, damaging cell membranes, thickening cell walls, disturbing cytoplasm and interfering with DNA synthesis (34). Inhibition was extended in barley seedlings by preventing their growth and reducing roots number. Harmala also inhibits the seeds germination of tomato, carrot and turnip (13). In addition, harmala leads to the formation of vesicles on barley seedlings' root tips and inhibit their length compared to non-treated barley. The harmful effects of harmala seeds occurs through allelopathic compounds, these reduced the barley root length, as the added harmala seeds increased their inhibitory effect increased on root elongation than on root number. This effect is similar to that reported in Arabidopsis seedlings (3). Likewise, the length of barley seedlings' plumules was significantly inhibited due to increasing number of harmala seeds. The laboratory experiment showed that germinated barley did not fully develop due to harmala stress.

The effect of the ground harmala seeds differs from whole harmala seeds on barley germination and growth. This effect strongly increases as the weight of ground harmala seeds increases. This effect is similar to toxicity effect of three different weed powders and their inhibitory effects on germination and growth in maize (11). This effect was also recorded in leaves aqueous extract of (*Parthenium hysterophorus* L.), which inhibited germination and growth of rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) (16). These results are consistent with earlier study showing that different organ extracts had different negative effects on seed germination and seedling growth of (*Portulaca oleracea* L.) and (*Chenopodium album* L.) (2). This result explains the failure of barley seedlings' growth in the field where harmala plants grew earlier in the Gharyan district.

Effects of ground harmala seeds and their aqueous seed extracts on germination and seedlings growth of barley seeds

Ground harmala seeds released allelochemicals that inhibited barley germination at doses > 0.12 g/dish, with a maximum inhibition of 40 % at 0.72 g/dish (Figure 4). Aqueous harmala seed extracts at all concentrations significantly inhibited ($p < 0.001$) barley germination (%) in both cold and warm water (Figure 5). Harmala extract exerted a stronger inhibition in warm water than in cold water. Warm water extract of ground harmala seeds in concentration from (0.6 to 7.2 %) inhibited barley seeds germination to 50 %. Harmala extract negatively affected the elongation of barley seedlings and inhibited their root and plumule elongation, with a highly significant inhibitory effects on root and plumule number (Figure 5). Harmala seeds extract also decomposed the barley seedling roots, resulting in seedling death. The barley seedlings treated with 4.8 % harmala seed extract improved their plumule elongation but decomposed their roots.

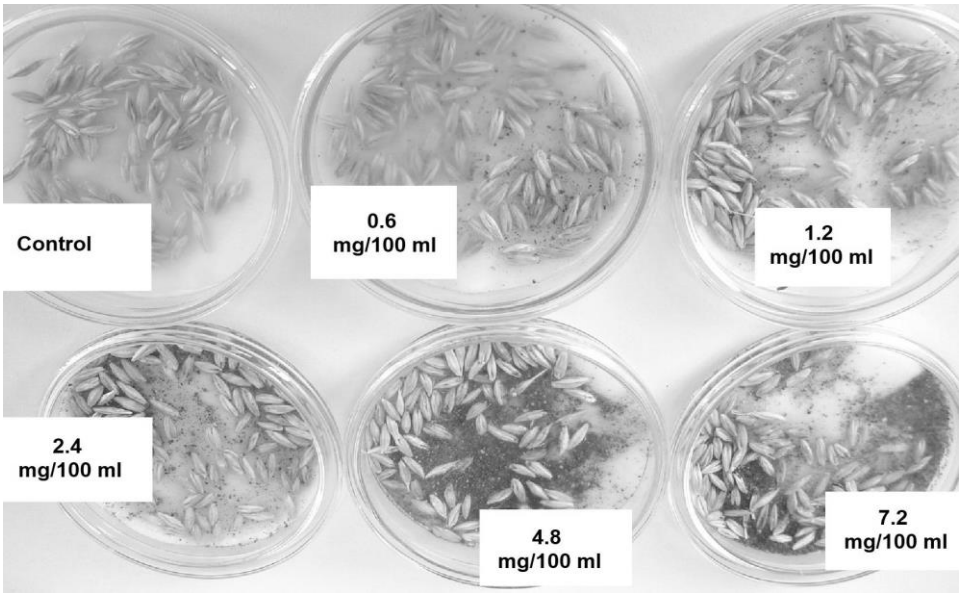

Figure 4. Hamala ground seeds treatment at the start of experiment, beside control, treatments are weight of ground harmala seeds in 100 ml.

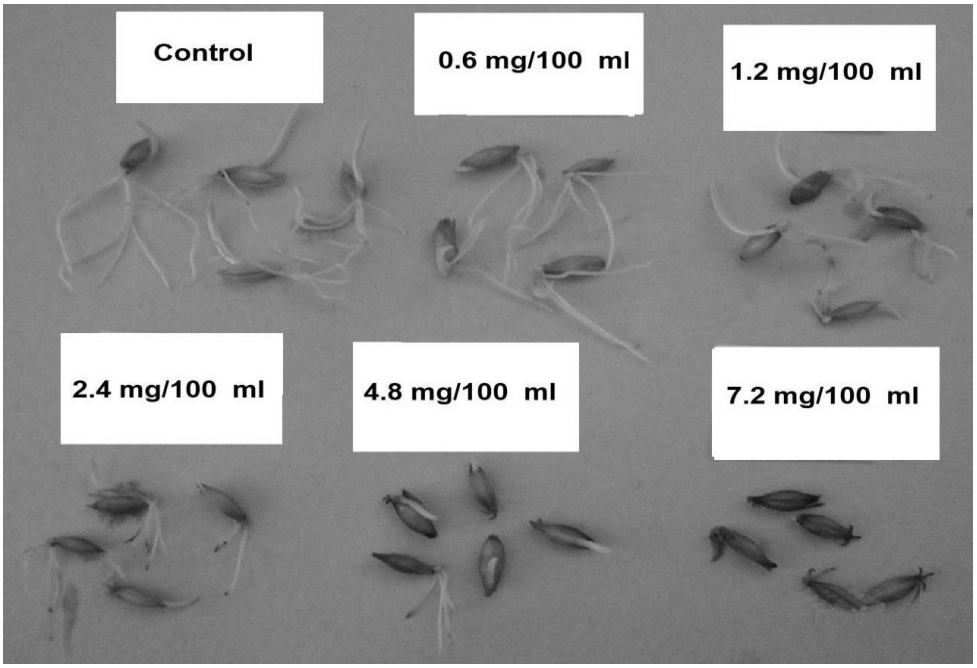

Figure 5. Hamala ground seed treatment at the end of the experiment after one week, beside control, treatments are weight of ground harmala seeds in 100 ml.

2. FIELD STUDIES

Effects of ground harmala seeds (1 or 5 g) added per plastic bag

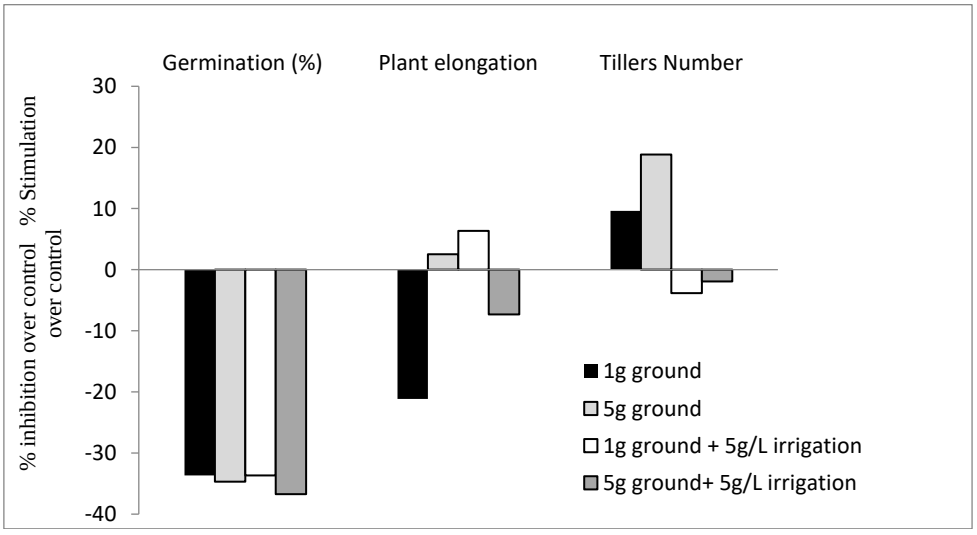

Figure 6. Inhibition/stimulation effects of harmala seeds treatment on germination and growth of barley plants in field.

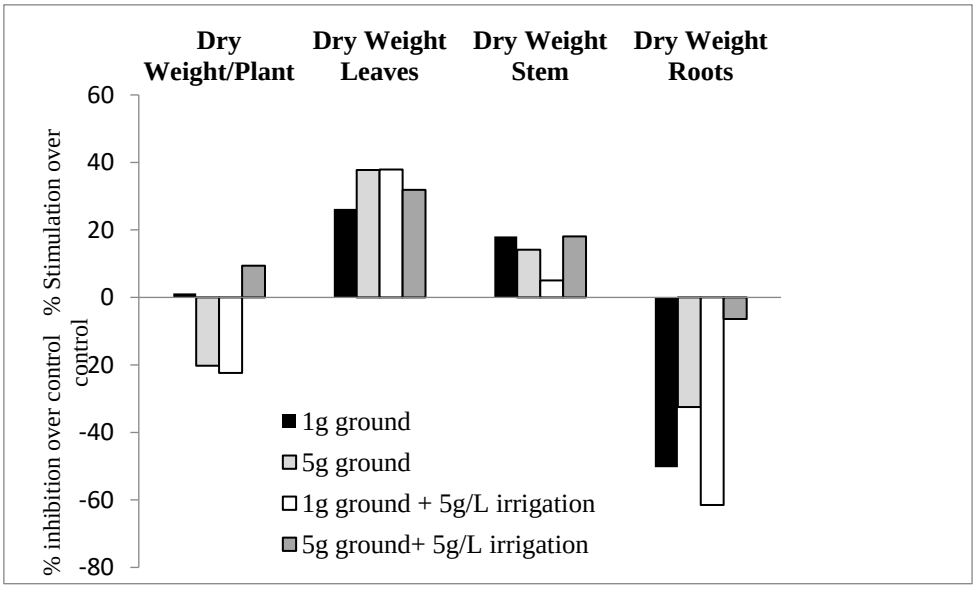

Figure 7. Inhibition / stimulation effects of harmala seeds treatment on dry weight of barley plants in field.

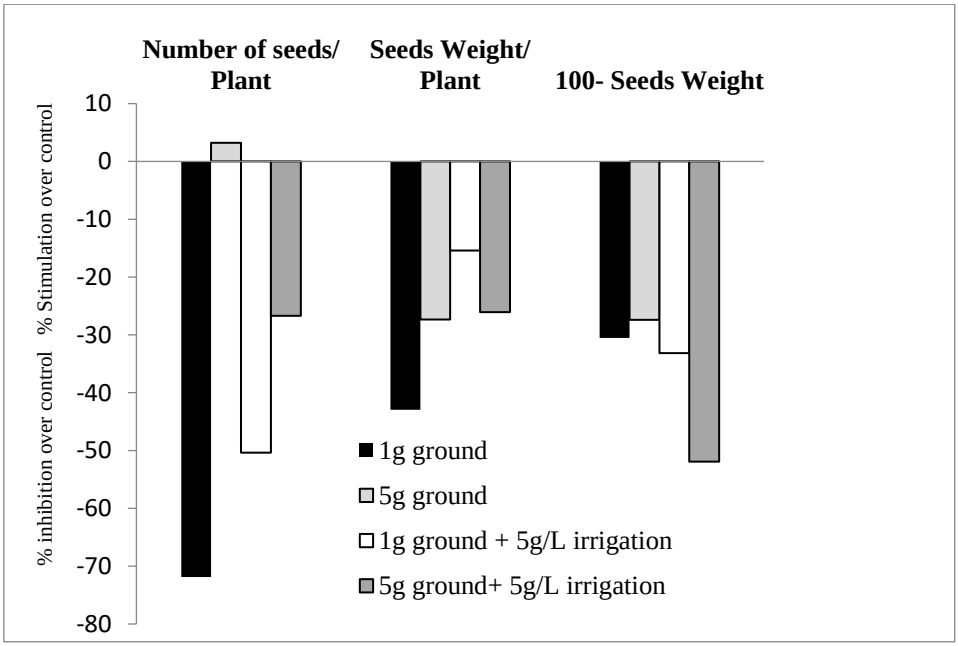

Figure 8. Inhibition / stimulation effects of harmala seeds treatment on yield attributes of barley plants in field.

The effects of adding ground harmala seeds to plant pots immediately before sowing of barley seeds were examined (Figure 8). Adding 1 g of harmala seeds inhibited seed germination and plant height, as well as significantly decreased fresh weight of leaves, and roots, but increased the dry weight of leaves and stems. Barley seed production and weight were significantly inhibited, as was the germination (%) of new seeds. Adding 5 g of harmala seeds significantly inhibited barley seed germination, with no effect on plant height but significantly increased the number of tillers and immature spikes. The dry weight of leaves and stems increased. Barley seed production was not significantly affected, but the weight of 100-seeds was significantly decreased.

Barley seedling root length was strongly inhibited by harmala extract in high concentration (7.2 %) down to 1 mm (compared to 50 mm in control).. As indicated in a study of the harmala effect on (*Cynodon dactylon* L.) seedlings growth the result showed different toxicity degrees of different parts of (*P. harmala*), as under : seeds > mixture > stems (6). Moreover, harmala seeds exhibited the strongest inhibitory activities than other plant parts (22). The extract of ground harmala seeds also has similar effect on barley seedlings root in addition to changing root tip into dark colour and causing seedling death, in addition, plumule length was also inhibited.

Effects of ground harmala seeds and their aqueous extract

Adding 1 g of ground harmala seeds per plastic bag significantly inhibited the barley seed germination plant growth (Figures 9-11). Irrigation with 5 g/L of aqueous harmala seed extract once a month had a similar effect. The treatment did not affect the number of tillers, but inhibited the number of spikes, fresh weight and roots of treated barley plants. The treated plants produced fewer seeds than non-treated plants, with a significant decrease in seed weight and weight of 100-seeds.

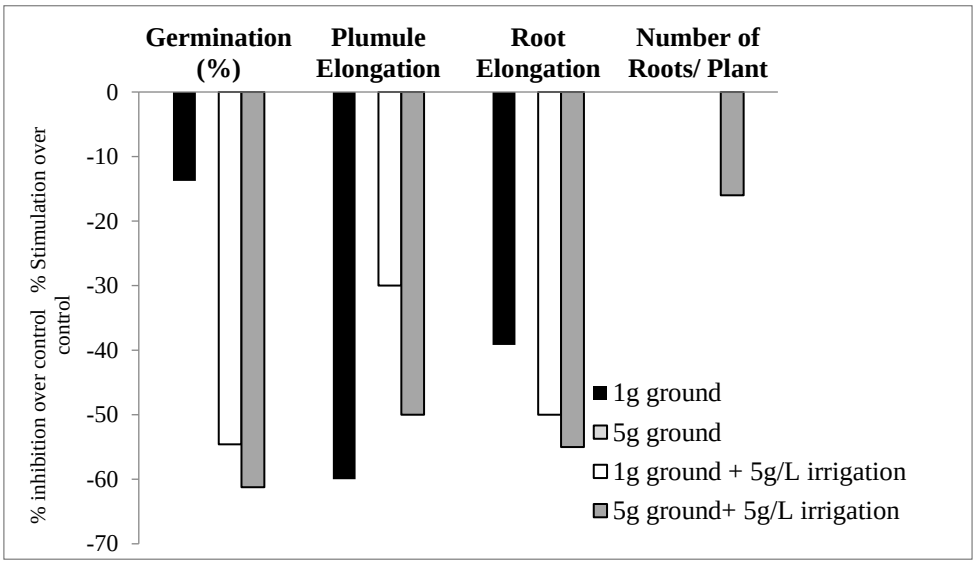

Figure 9. Inhibition / stimulation effects of harmala seeds treatment on newly harvested seeds germination and growth of barley plants in field (elongation in mm).

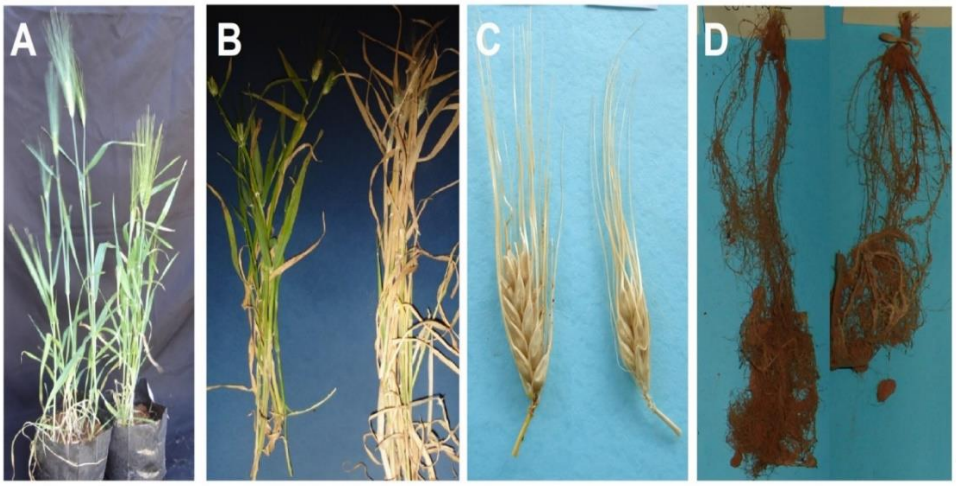

Figure 10. Adding 1 g of ground harmala seeds per plastic bag and monthly irrigation with 5 g/L of aqueous harmala seeds extract once a month. A) During spike development. B) After harvest. C) Spikes. D). Root system. For each panel, the left side is the control and the right side is the treatment.

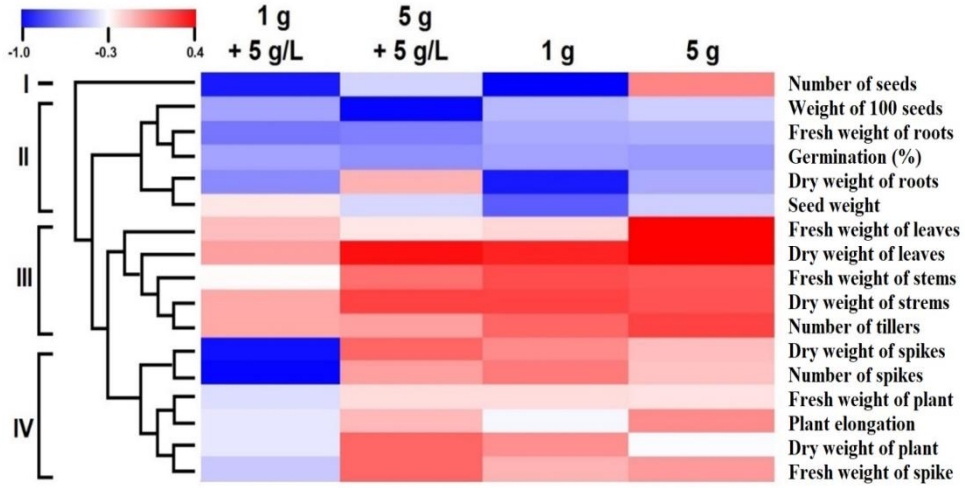

Figure 11. Hierarchical clustering and heat map major field treatments of ground harmala seeds (1 g followed by irrigation with 5 g/L, 5 g followed by irrigation with 5 g/L, one time 1 g and one time 5 g). Each raw represents a trait and each column represents a treatment. Four major clusters are evident (each with distinctive traits that differentiate it from other clusters).

Re-germinating the new barley seeds from the field experiment showed decreased germination than control seeds. The growth measurement of new barley seedlings showed significant inhibition of root and plumule elongation but did not affect the root numbers. Adding 5 g ground harmala seeds to the soil significantly inhibited barley seed germination.

Irrigating with 5 g/L of aqueous harmala seed extract significantly inhibited the elongation and final height of barley plants. Harmala treatment did not effect the tillers and spikes of barley plants, but significantly inhibited their fresh weight, with significant inhibited the effects on roots but not on leaves, stems and spikes. Treatment did not effect the barley dry weight, stimulated leaf, stem and spike production but inhibitory effects on root dry weight. The number of seeds produced was significantly inhibited, with highly significant differences in 100-seeds weight. Germination (%) of newly produced barley seeds was significantly inhibited in the laboratory. Treatment had no significant effect on number of roots of barley seedlings, but significantly inhibited the root and plumule elongation.

The inhibitory compound released from the harmala seeds gave a neutral solution after 24 h and pH 6 after 48 h of treating barley dishes. However, after 72 h, the pH was =10, which means that germination media in barley dishes changes into alkaline. The high pH of barley germination media would influence compounds released from harmala seeds (usually basic chemical compounds). This might result from the disintegration of harmala alkaloids released from harmala seeds powder after soaking. Alkaloids are organic compounds that contain linked nitrogen (5).

Adding 1 or 5 g of grounded harmala seeds to the soil and monthly irrigation with 5 g/L of harmala seed aqueous extraction, significantly inhibited the barley germination with a very high correlation. And this is consistent with the results obtained in the laboratory. It is worth noting that the inhibitory effects of ground harmala seeds on barley seeds in the field increased as the amount increases. Adding dry plant residues of various plants to different seeds in the field causes an inhibitory effect on seed germination and the effect depends on the type of treated seeds, some seeds resist the inhibition effect and give good germination and some seeds give weak germination, while others are severely affected and do not germinate. However the leaves extracts of horseweed trigger significantly higher allelopathic effects on seedlings' growth parameters of lettuce (31), while the dry residue of sunflower inhibited the germination and growth of radish seeds (21).

The aqueous extract of harmala seeds inhibited barley seeds germination in the field stronger than ground seeds. A similar result indicated that the aqueous extract of *Aritemisia capilaris* and *Stipa bungeana* at different concentrations significantly suppressed the seed imbibition, germination potential, germination rate, germination index, seedlings height, above and below-ground biomass of alfalfa seedlings (9). Moreover, the aqueous extract of Eucalyptus inhibits the germination of wheat and maize (26). Treated barley produced more tillers, which increased stem fresh and dry weight, but the number of spikes decreased and most of them were immature, these decreased the seed number and weight, and dry weight of root mass. A similar result was obtained when using aqueous methanol extracts of *Eleocharis atropurpurea* on different types of seeds showing inhibitory effect on seedling growth (32). Likewise, sorghum (*Sorghum bicolor* (L.) causes inhibitory effects on wheat (*Triticum aestivum* L.) when the crops are grown in the rotation that reduced grain yield of wheat (25).

The results of field study indicated that ground harmala seeds' aqueous extract added into the soil inhibited the germination, growth and yield of barley plants. The inhibitory effect was more on roots mass, shoots and seeds numbers and weight. However, to gain a holistic view of all measured barely phenotypic traits as affected by harmala treatment in the field, hierarchical clustering and heat map was generated (Figure 7). It was evident to see weight of seeds and weight of roots characteristics to cluster together (cluster II), which

showed the least affected traits among all other phenotypes. On the other hand, both weight of leaves and weight of stems were clustered in another major cluster (III) and were the most affected by the harmala treatments. An additionally affected group of traits include spike characteristics, plant elongation and weight of plant, which were clustered together in cluster IV, however, they were less affected as those traits in cluster III.

CONCLUSIONS

The laboratory and field experiments, showed that harmala plant affected the barley crop. This is likely to be caused by allelopathic compounds secreted by harmala seeds, whether they grow in the same area or nearby or their residue. This inhibited the germination and growth of barley, besides lowered daily rate of barley germination, leading to death of most seedlings as affected by allelopathic compounds. Moreover, seedlings that tolerate initial harmala effect were weak or became dwarf with multiple tillers, which bears immature spikes which decreased barley yield.

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who 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.

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