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Differential allelopathic effect of nine *Haplophyllum tuberculatum* growth forms through germination bioassay

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Key words: Allelopathy, *Lepidium sativum*, *Raphanus sativus*, Germination efficiency, Growth parameters.

Abstract

The main objective of the present study was to analyze and discriminate among nine growth forms (GF) of *Haplophyllum tuberculatum* collected from six natural sites along the Mediterranean coastal desert of Egypt by applying the phenomenon of allelopathy as a chemical marker. *H. tuberculatum* aqueous extracts (HTAE) were tested on germination efficiency and growth parameters of *Lepidium sativum* and *Raphanus sativus* seeds. At the full-strength concentration (100%) the highest effect on the germination (GP) and inhibition (IP) percentages, the time taken for 50% germination (T_{50}), mean germination time (MGT), germination energy (GE) and seed germination index (SGI) was exhibited by GF3 and GF5 on *Lepidium sativum* and *Raphanus sativus* seeds, respectively. The hypocotyl length (HL) was more sensitive than radicle length under HTAE. It was obvious that the allelopathic effect was prominent in *L. sativum* compared with *R. sativus* indicating the resistance of the latter to the allelochemicals extracted from HTAE. The study indicated that germination bioassay differentiated among three different growth forms (GF3, GF5 and the rest of the studied growth forms) which may need further studies using more highly specific techniques for separation and isolation of different allelochemicals.

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Introduction

The family Rutaceae in the northwestern Mediterranean desert of Egypt is monogeneric and represented by only one wild specific epithet *Haplophyllum tuberculatum* (Täckholm, 1974 and Boulos, 2000). The species exhibited an ecological and biochemical diverse, occupied a wide range of habitats, and generally showed striking interspecific differences in many morphological, chemical, anatomical and molecular characteristics (Salvo *et al*., 2011). The most difficult problem in the *Haplophyllum* is what the limits of *H. tuberculatum*. Field observations showed evidence of different morphological appearance such as size and shape of leaves, flower color, flowering time and others (Townsend, 1986).

Allelopathy may be defined as the beneficial or harmful effect that is caused by one plant on other thus releasing chemicals from plant parts by leaching, root exudates, volatilization, residue decomposition and other processes in both natural and agricultural systems (Ferguson and Rathinasabapathi, 2003). Allelopathy indirectly reflects the information coded in DNA respecting the chemical print and is thus considered amenable technique to provide a chemical basis for molecular phylogeny (Junaedi *et al*., 2010).

Chemotaxonomy of plants may be defined as a scientific investigation of the potentialities of chemical characters for the study of problems of plant taxonomy and plant phylogeny (Shoreland, 1963). The variation in color of flowers, size and shape of leaves and stems alongside another morphological criteria and oil chemical characteristics among the same species may be used as an indicator of different chemical constituents present in that species (Cronquist, 1981; El-Naggar *et al*., 2014).

The main objective of the present study is to report the application of allelopathy approach as a biochemical marker to the study of diversity within nine growth forms of *H. tuberculatum* collected from six natural sites along the Mediterranean coastal desert of Egypt (from Alexandria to Matruh).

Materials and methods

Collection of Specimens

Nine growth forms (GF1-GF9) of *H. tuberculatum* were collected from six natural sites along the Mediterranean coastal desert of Egypt (from Alexandria to Matruh). The collected specimens were selected in order to cover the most dominant habitats within the range of distribution of the genus **Fig. 1.** then identified according to Täckholm (1974) and Boulos (2009).

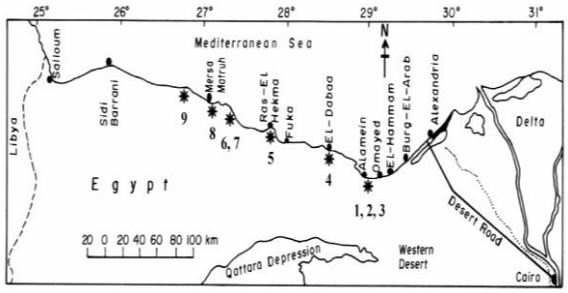

Fig. 1. Map of the western Mediterranean desert of Egypt indicating the sites (*) of the present study. Studied growth forms: 1-3 from El-Karma village (155 km Alexandria-Matruh road), 4 from El-Gophera village (95 km Alexandria-Matruh road), 5 from Ras El-Hekma (64 km Alexandria-Matruh road), 6-7 from Abo-Tamr village (41 km Alexandria- Matruh road), 8 from 22 km Alexandria-Matruh road and 9 from 5 km Matruh-Salloum road.

Germination Bioassay

Petri-dish experiment was applied to investigate the potential allelopathic effects of *Haplophyllum tuberculatum* aqueous extract (HTAE) of nine growth forms on germination percentage (GP), inhibition percentage (IP), the time to get 50% germination (T_{50}), mean germination time (MGT), germination energy (GE), seed germination index (SGI), and hypocotyl (HL) and radicle (RL) lengths. To accomplish this experiment, 20 seeds of each of *Lepidium sativum* and *Raphanus sativus* (target species) were arranged in 9 cm diameter Petri-dishes on 2 discs of whatman No.1 filter paper under normal

laboratory conditions with day temperature range of 25-30°C and night temperature range 20-25°C. Five ml of HTAE were added daily to three replicates. GP, HL and RL were recorded daily for successive seven days.

Calculations

Inhibition Percentage (IP)

Likewise, Inhibition Percentage (IP) was calculated according to the general equations:

Relative reduction = [1- (allelopathic/control) 100]

The Time to Get 50% Germination (T50)

The time to get 50% germination (T_{50}) was calculated according to the formula of **Coolbear *et al.* (1984)** which modified by **Farooq *et al.* (2005).**

$$T_{50} = t_i\ (N/2\text{-}ni)\ (t_j - t_i)/n_j\text{-}n_i$$

Where N is the final number of germinated seeds and n_j, n_i are the cumulative number of seeds germinated by adjacent counts at times t_j, t_i when $n_i < N/2 < n_j$.

The Mean Germination Time (MGT)

The mean germination time (MGT) was calculated according to the equation of **Battle and Whittington (1969).**

$$MGT = \Sigma\ (G \times T) / F$$

Where,

T = the day on which germination count was made

G = the number of seeds germinated on the day of the count

F = final number of seeds which germinated in each replicate

Energy of Germination (GE)

Energy of germination (GE) was recorded according to **Farooq *et al.* (2005)** at the 4th day after sowing. It is the percentage of germinating seeds (GP) four days after sowing relative to the total number of seeds tested (TNST).

GE= GP (4th day)/TNST

Seed Germination Index (SGI)

Seed germination index (SGI) was calculated according to the equation of **Scott *et al.* (1984).**

SGI= Σ Ti Ni/S

Where,

Ti= is the number of days after sowing

Ni= is the number of seeds germinated on day i

S= is the total number of seeds planted

Data Analysis and Computer Programmes

Data concerning the effect of different concentrations of *Haplophyllum tuberculatum* aqueous extract (HTAE) growth forms on germination percentage (GP), The time to get 50 % germination (T_{50}) , The mean germination time (MGT), hypocotyl (HL) and radicle length (RL) were subjected to standard analysis of variance (ANOVA) (Zar, 1984) using the COSTAT 2.00 statistical analysis software manufactured by Cohort software. The results are presented as the means ± standard deviation (SDM) of three replications.

Agglomerative cluster analysis of the 27 OTU's was conducted using PAST program (Hammer *et al.*, 2001) to compute the genetic distances and generate the dendrogram that showed the relationships among the nine growth forms of studied *Haplophyllum tuberculatum*.

Results

Germination Efficiency and Growth Parameters

Germination Efficiency

Data concerning the germination percentage (GP) of *Lepidium sativum* and *Raphanus sativus* are illustrated and statistically represented in **Fig. 2.** Petri-dish experiment demonstrated that the GP of the two recipient species was significantly ($p \leq 0.05$) affected upon applying different concentration levels of the nine growth forms of HTAE. Commonly, GP decreased with the increase in HTAE concentration. After seven days from sowing, the percentage attended a value of about 100 % at control level for the nine growth forms. However, at the subsequent concentrations (25, 50, and 100%) the values were

significantly decreased and prominent in *L. sativum* relative to *R. sativus*.

In addition, the time to get 50% germination (T_{50}), the mean germination time (MGT), energy of germination (GE) and seed germination index (SGI) of *L. sativum* and *R. sativus* are illustrated and statistically represented in **Fig. 3 and 4.**

In two recipient species (T_{50}) started with a value of about 1 day in all growth forms at control level. At 25% HTAE concentration, T_{50} values ranged from a minimum of 1 day in GF1 to a maximum of 4 days in GF3 in *L. sativum* while T_{50} values of *R. sativus* was about only 1 day in all growth forms. T_{50} values of *L. sativum* ranged from a minimum of 3 days in GF6 and GF7 to a maximum of 6 days in GF2, GF3, GF5, and GF9 at 50% HTAE concentration but T_{50} values of *R. sativus* ranged from a minimum of 1 day in GF3, GF4, GF5, and GF6 and GF7 to a maximum of 3 days in GF9. In *L. sativum* a great imperative decrease was attained along the higher HTAE concentration (100%); it was null values in all growth forms except GF6 was (3 days) while it was ranged from a minimum of 1 day in GF4, GF6 and GF7 to maximum of 4 days in GF5 and GF9.

The value of the mean germination time (MGT) decreased markedly as HTAE concentration increased in two recipient species. This reduction was statistically significant at ($p \leq 0.05$) as evaluated by ANOVA test. At control level, MGT of *L. sativum* started with a minimum value in GF4 to a maximum in both GF1 and GF9 while values of *R. sativus* ranged from a minimum value in GF1 to a maximum in GF9. In *L. sativum* MGT values ranged from a minimum in GF3 to a maximum in GF8 were attained at 25% HTAE concentration while in *R. sativus* the values ranged from a minimum in GF2 and a maximum in GF6. At 50% HTAE concentration, values of *L. sativum* ranged from a minimum in GF9 to a maximum in GF6 but values of *R. sativus* ranged from a minimum in GF9 to a maximum in GF6. In two recipient species a notable reduction was attained along the higher HTAE concentration (100%), in *L. sativum* the values were reduced in GF5 and GF6 while in *R. sativus* the values were reduced in GF5 and GF6.

Regarding GE, the value decreased specifically as HTAE concentration increased. This reduction was statistically significant at ($p \leq 0.05$) as evaluated by ANOVA test. In *L. sativum* GE starts with a value ranged from a minimum in GF5 to a maximum in GF6 at control level while In *R. sativus* a value ranged from a minimum in GF1 to a maximum in GF6. At 25% HTAE concentration GE values of *L. sativum* ranged from a minimum in GF3 to a maximum in GF4 but in *R. sativus* the values ranged from a minimum in GF1 to a maximum in GF6. At 50% HTAE concentration, values of *L. sativum* ranged from a minimum in GF5 and GF8 to a maximum in GF6 while in *R. sativus* values ranged from a minimum in GF9 to a maximum in GF6. In two recipient species a great striking reduction was attained along the higher HTAE concentration (100%), it ranged from a minimum value in GF2 to maximum in GF6 in *L. sativum* while in *R. sativus* the values ranged from a minimum in GF5 to maximum in GF6.

The value of SGI, decreased distinctly as HTAE concentration increased. This reduction was statistically significant at ($p \leq 0.05$) as evaluated by ANOVA test. In *L. sativum* at control level, SGI attained a value of about 7 in all growth forms in two recipient species. At 25 % HTAE concentration, SGI attained a value ranged from a minimum in GF5 to a maximum in GF2 while in *R. sativus* values of SGI were the same in all growth forms. With respect to 50% HTAE concentration, in *L. sativum* the minimum and maximum value was achieved by GF5 and GF6 respectively while in *R. sativus* the minimum and maximum value was achieved by GF9 and GF6, respectively. Finally, a significant reduction was attained along the higher HTAE concentration (100%); in *L. sativum* SGI ranged from a minimum value in GF3 to maximum value in GF6 but in *R.*

sativus it the value ranged from a minimum GF5 to a maximum in GF7.

Growth Parameters (HL and RL)

The allelopathic effect of HTAE concentration on hypocotyl (HL) length of *Lepidium sativum* L. and *Raphanus sativus* L. are illustrated and statistically represented in **Fig. 5.** HTAE extract concentration levels have statistically reduced HL. The applied concentrations are significant at $p \leq 0.05$. Usually, HL decreased with the increase of HTAE concentration. After seven days from sowing. The HL of *L. sativum* only attended a null value at 100% for the nine growth forms. However, at the subsequent concentrations (control, 25, and 50%) the values were significantly decreased and prominent in *L. sativum* relative to *R. sativus*.

The allelopathic effect of HTAE concentration on radicle (RL) length of *L. sativum* L. and *R. sativus* L. are illustrated and statistically represented in **Fig. 6.** HTAE extract concentration levels have statistically reduced RL. The applied concentrations are significant at $p \leq 0.05$. Usually, RL decreased with the increase of HTAE concentration. After seven days from sowing, RL of two recipient species attended a gradual reduction at (control level, 25%, 50% and 100%) for the nine growth forms. The values were significantly decreased and prominent in *L. sativum* relative to *R. sativus*.

Dendrogram of Raphanus sativus at seven day (GP, GE, SGI, HL, RL, T50, MGT, IP)

The agglomerative cluster analyses of 9 OTU's of *Haplophyllum tuberculatum* are exemplified by the different dendrograms resulting from the various methods of sorting (Paired group, Single linkage and Ward's method by using different coefficients). These different dendrograms are congruent with each other though using diverse methods of sorting **Fig. 7**. The average taxonomic distance of the studied OTU's is 0.975. At this level, two major groups (I and II) are found. The major group "I" comprises OTU 5 which is from Ras El-Hekma, while the rest of OTU's assemble under the major group "II". The latter group further divaricates into two subgroups, "A" and "B" at 0.98 similarity level. The subgroup "A" includes OTU's 1 and 9 which are from El-Karma village and5 km Matruh-Salloum road. However, at 0.982 similarity level subgroup "B" further distinguished into two clusters "1" and "2". The cluster "1" contains OTU's 4, 6 and 7 which from El-Gophera village and Abo-Tamr village, while the rest of OTU's assemble under the cluster "2". The cluster "2" is discriminated into two subclusters "a" and "b" at 0.993 similarity level. The subcluster "a" comprises OUT 2 from El-Karma village, while subcluster "b" encloses OTU's 3 and 8 from El-Karma village and 22 km Alexandria-Matruh road.

Dendrogram of Lepidium sativum at seven day (GP, GE, SGI, HL, RL, T50, MGT, IP)

The agglomerative cluster analyses of 9 OTU's of *Haplophyllum tuberculatum* are exemplified by the different dendrograms resulting from the various methods of sorting (Paired group, Single linkage and Ward's method by using different coefficients). These different dendrograms are congruent with each other though using diverse methods of sorting **Fig. 8**. The average taxonomic distance of the studied OTU's is 0.91. At this level, two major groups (I and II) are found. The major group "I" comprises OUT 6 which is from Abo-Tamr village, while the rest of OTU's assemble under the major group "II". The latter group further divaricates into two subgroups, "A" and "B" at 0.96 similarity level. The subgroup "A" includes OTU's 5 and 7 which are from Ras El-Hekma and Abo-Tamr village. However, at 0.97 similarity level subgroup "B" further distinguished into two clusters "1" and "2". The cluster "1" contains OUT 1 which is from El-Karma village, while the rest of OTU's assemble under the cluster "2". The cluster "2" is discriminated into two subclusters "a" and "b" at 0.985 similarity level. The subcluster "a" comprises OTU's 1 and 9 from El-Karma village and 5 km Matruh-Salloum road, while subcluster "b" encloses OTU's 2, 4 and 8 from El-Karma village, El-Gophera village and 22 km Alexandria-Matruh road.

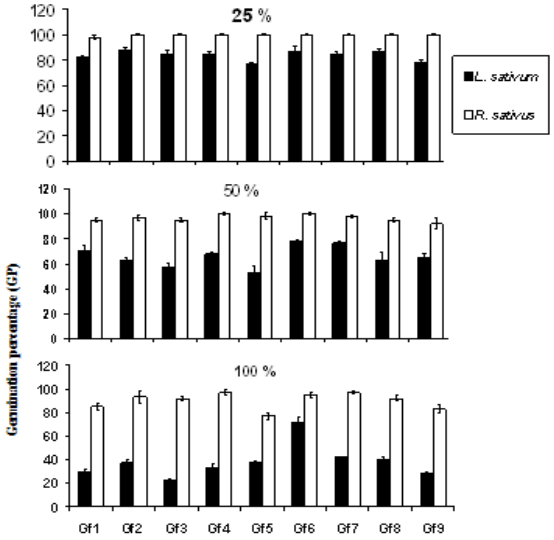

Fig. 2. Variation in the germination percentage (GP) of *Lepidium sativum* and *Raphanus sativus* seeds as affected by different concentration levels (%) of aqueous extract of *Haplophyllum tuberculatum* growth forms (GF1-GF9). Error bars indicate standard error of means.

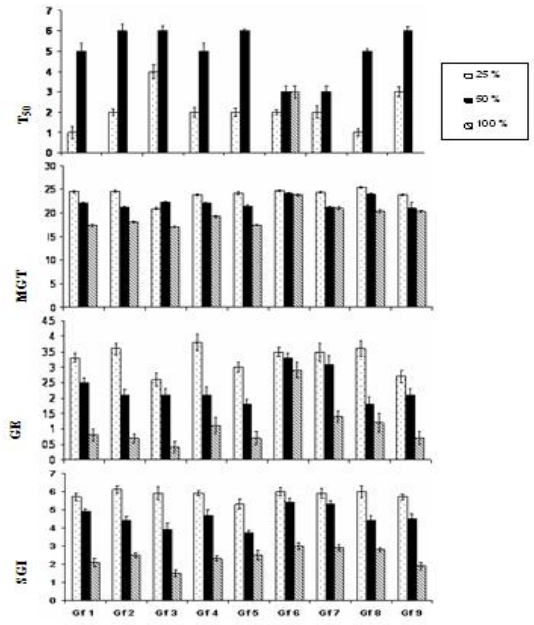

Fig. 3. Variation in the time taken for 50 % germination (T_{50}), mean germination time (MGT), germination energy (GE) and seed germination index (SGI) of *Lepidium sativum* seeds as affected by different concentration levels (%) of aqueous extract of *Haplophyllum tuberculatum* growth forms (GF1-GF9). Error bars indicate standard error of means.

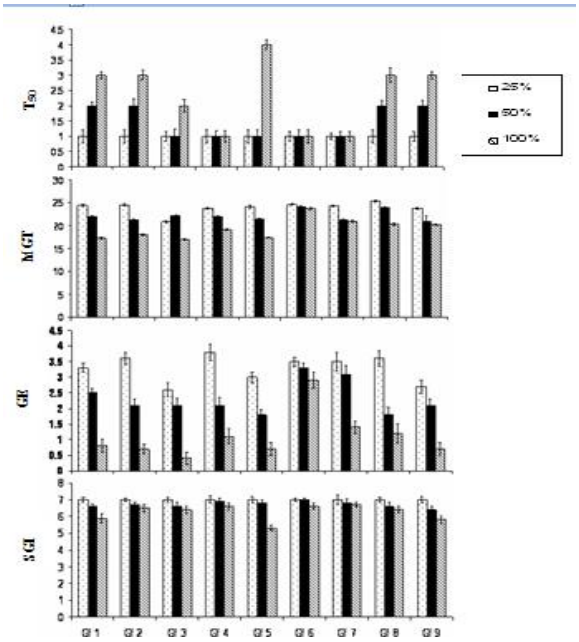

Fig. 4. Variation in the time taken for 50 % germination (T_{50}), mean germination time (MGT), germination energy (GE) and seed germination index (SGI) of *Raphanus sativus* seeds as affected by different concentration levels (%) of aqueous extract of *Haplophyllum tuberculatum* growth forms (GF1-GF9). Error bars indicate standard error of means.

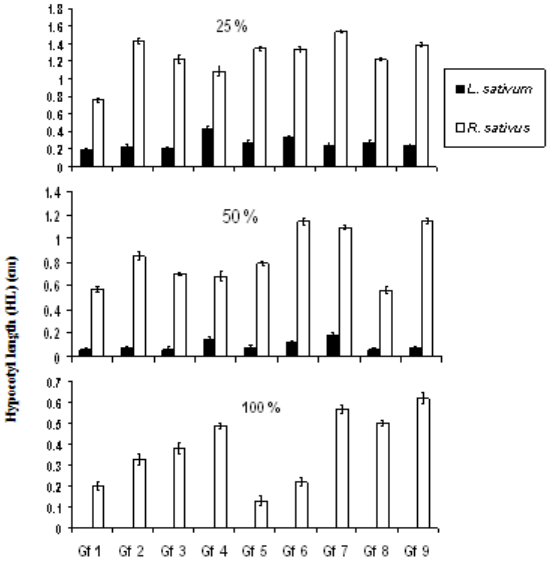

Fig. 5. Variation in the hypocotyl length (HL) of *Lepidium sativum Raphanus sativus* seeds as affected by different concentration levels (%) of aqueous extract of *Haplophyllum tuberculatum* growth forms (GF1-GF9). Error bars indicate standard error of means.

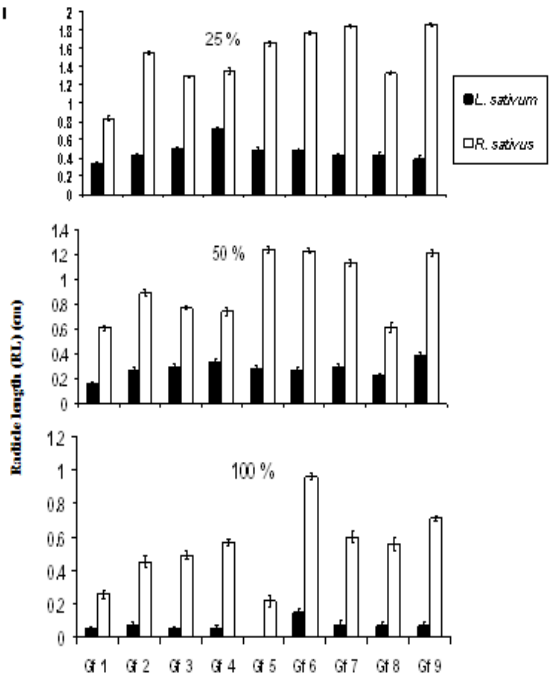

Fig. 6. Variation in the radicle length (RL) of *Lepidium sativum* and *Raphanus sativus* as affected by different concentration levels (%) of aqueous extract of *Haplophyllum tuberculatum* growth forms (GF1-GF9). Error bars indicate standard error of means.

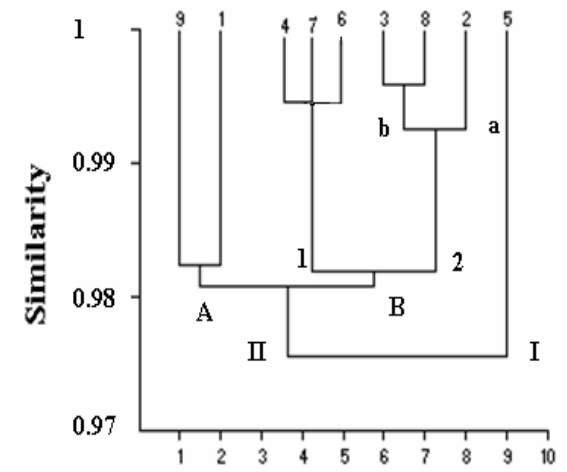

Fig. 7. Dendrogram resulting from UPGMA method of sorting of 9 OTU's of *Haplophyllum tuberculatum* collected from Mediterranean coastal desert of Egypt (from Alexandria to Matruh) using germination percentage (GP), inhibition percentage (IP), the time taken for 50 % germination (T_{50}), mean germination time (MGT), germination energy (GE) and seed germination index (SGI), hypocotyl (HL), Radical length (RL) characters of *Raphanus sativus* at seven day.

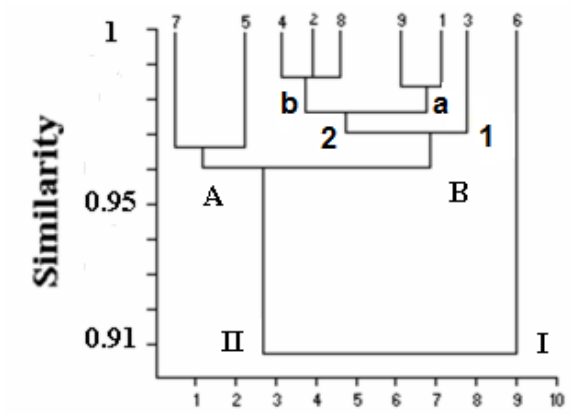

Fig. 8. Dendrogram resulting from UPGMA method of sorting of 27 OTU's of Haplophyllum tuberculatum collected from Mediterranean coastal desert of Egypt (from Alexandria to Matruh) using germination percentage (GP), inhibition percentage (IP), the time taken for 50 % germination (T50), mean germination time (MGT), germination energy (GE) and seed germination index (SGI), hypocotyl length (HL), Radical length (RL) characters of *Lepidium sativum* at seven day.

Discussion

Germination percentage (GP) of *Lepidium sativum* and *Raphanus sativus* seeds was differentially affected by the aqueous extract of *Haplophyllum tuberculatum* (HTAE) growth forms and the reduction was concentration dependent. Al-Zahrani and Al-Robai (2007) found a difference between the species germination percentages in response to allelopathic materials coming from *Calotropis gigantea* leaves which agrees with the findings of Patil (1994) on *Glyricidia maculata*. At the full-strength concentration (100%) GF3 and GF5 exert the highest allelopathic effect on the GP of the two tested species seeds. Reduction in GP of the two current studied species may be due to phenolics compound present in HTAE such as ferulic acid, P- coumaric, vanillic, caffeic, chlorogenic and others (Inderjit and Mallik, 2002; Chon *et al.*, 2005). These phenolics reduce the seed germination percentage (Williams and Hoagland, 1982; Al-Charchafchi *et al.*, 1987), due to their interference with indol acetic acid metabolism, or synthesis of protein and ion uptake by the plant (Hussain and Khan, 1988).

Results of seed germination index (SGI) indicated that a gradual reduction of SGI in the two investigated species as a response to the regular applying of higher HTAE concentration levels was attained. At the full-strength concentration (100%), GF3 and GF5 exert the highest allelopathic effect on the SGI of *L. sativum* and *R. sativus* seeds, respectively. These results are in consistence with those reported by El-Darier and Zein El-Dien (2011a) who found a gradual reduction in SGI of tomato seeds as a response to the higher concentration levels of *Medicago sativa* aqueous extract. Additionally, the same was obtained by Tanveer *et al.* (2010) on the effect of *Euphorbia helioscopia* aqueous extract on SGI of *Triticum aestivum*, *Cicer arietinum*, and *Lens culinaris*.

Inhibition percentage (IP) or relative reduction in the two studied species increased gradually with the increase of HTAE concentration levels. These results agree with El-Darier and Zein El-Dien (2011a) who found a gradual increase of IP of tomato seeds as a response to the higher concentration levels of *Medicago sativa* aqueous extract. Turk *et al.* (2003 and 2005) reported that degree of inhibition percentage of radish and alfalfa seeds increased with the increase of black mustard extract concentration. At the full strength concentration of HTAE (100%), GF3 and GF5 accomplished the highest allelopathic effect on the IP of *L. sativum* and *R. sativus* seeds, in that order. Alam and Islam (2002) suggested that the inhibition percentage (IP) of crop plants may be due to the disturbance in the activities of peroxidase, alpha-amylase and acid phosphataes.

A gradual reduction in the mean germination time (MGT) of the two investigated species as a response to the regular applying of higher HTAE concentration levels was attained. At the full-strength concentration (100%), GF3 and GF5 exert the highest allelopathic effect on the MGT of the two investigated species, respectively. This result is in agreement with Shanee *et al.* (2011) who studied the phytotoxic effects of *Euphorbia dracunculoides* on a rainfed chickpea-chickpea cropping system. The highest mean germination time increases when the aqueous extract of HTAE concentration increased which showed that increased HTAE concentration caused a decrease in germination velocity.

To go through with this, the energy of germination (GE) in *Raphanus sativus* seeds exhibited a gradual reduction as a response to the regular applying of higher HTAE concentration levels. These results was similar to that recorded by Jankowska *et al.* (2009) who found that the GE of *Lolium westerwoldicum* seeds inhibited as response to high concentration level of *Taraxacum officinale* extract. On contrarily, Farooq *et al.* (2011) found that the GE of *Oryza sativa* seeds was increased under high concentration levels of sunflower extract. At the full-strength concentration (100%), GF3 and GF5 exert the highest allelopathic effect on the GE of *L. sativum* and *R. sativus* seeds, respectively. A gradual increase of T_{50} in the two investigated species as a response to the regular applying of higher HTAE concentration levels was attained. These results are consistent with Maiti *et al.* (2010) who suggested that the T_{50} of *Vinga radiata* seeds increased progressively as response to the increase of *Lantana camara* L. aqueous extract concentration. Jabran *et al.* (2010) found that the T_{50} of *Avena fatua* and *Phalaris minor* seeds reduce gradually by increase the concentration levels of mulberry, barnyard grass and winter cherry extract. At the full-strength concentration (100%) GF3 and GF5 exert the highest allelopathic effect on the T_{50} of *L. sativum* and *R. sativus* seeds, respectively.

The current study inferred that the hypocotyl length (HL) of *L. sativum* and *R. sativus* was found more sensitive and responds more strongly to the increase in HTAE concentration than the radicle length (RL). The reduction may be due to phytotoxic activity of phytochemicals present in aqueous extracts of *Haplophyllum tuberculatum* like lignans (Sheriha and Abouamer, 1984; Sheriha *et al.*, 1987) and alkaloids (Sheriha *et al.*, 1985, 1987; Al-Yahya *et al.*, 1991; Al-Rehaily *et al.*, 2001). Monoterpenes and

sesquiterpenes have also been shown to be the major components of the essential oil of *H. tuberculatum* (Brunke *et al.,* 1991 and Yari *et al.*, 2000). Bora *et al.* (1999) found that the elongation of radicle and hypocotyl was reduced in all treatments of *Acacia auriculiformis* extract proportional to concentration levels. Effects of *A. auriculiformis* extract much more pronounced on hypocotyl than radicle elongation. Fag and Stewart (1994) suggested that the inhibitory effect was related to the presence of allelochemical including tannis, wax, flavonoides and phenolic acids.

The germination of some test species was inhibited and that of the others remained unaffected or less stimulated. It was interesting to note that the response of germination and seedling growth of test species towards the same extract was variable (*L. sativum* was more sensitive than *R. sativus*) and this agree with Hisashi *et al.* (2009).

At the end we conclude that all concentrations of HTAE of GF3 and GF5 reduced germination ability of *L. sativum* and *R. sativus*, which are considered to be an important visible and reliable index for the evaluation of allelopathic effect.

Conclusion

The allelopathic effect was prominent in *L. sativum* compared with *R. sativus* indicating the resistance of the latter to the allelochemicals extracted from *H. tuberculatum* aqueous extract (HTAE) growth forms. Commonly In both investigated species hypocotyl length was more sensitive than radicle length as affected by the aqueous extract of *H. tuberculatum* growth forms (HTAE). Obviously Three different growth forms (GF3, GF5 and the rest of the studied growth forms) are allelopathically distinguished which need further studies using more highly specific techniques (e.g. HPLC) for separation and isolation of a variety of allelochemicals.

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