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Allelopathic effects of *Caragana intermedia* on monocot and dicot plant species and identification of allelochemicals

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ABSTRACT

We studied, the effects of aqueous extracts *Caragana intermedia* roots on germination and seedling growth of 4-crops: Dicots [*Sophora alopecuroides* L., *Melilotus officinalis* L], Monocots [*Elymus dahuricus* Turcz. and *Sorghum sudanense* Piper.]. The aqueous extract inhibited the germination of dicotyledonous species and the effect was concentration dependent. In monocots, aqeous extracts of high concentrations (2.0, 5.0 and 10.0%) reduced the germination, while low concentrations (0.5 and 1.0%) were stimulatory. The 10.0% aqueous extract significantly inhibited the germination of dicotyledonous species. In monocotyledonous species, the inhibitory effects were observed during the early stages, but not in the final stages of test period. At 10.0% aqueous extract, both dicotyledonous species produced significantly shorter roots. GC-MS (gas chromatography mass-spectrometry) analysis of the extracts resulted in > 21 peaks. The major constituents were D-myo-Inositol and 2,3-Butanediol, whose relative content was 80.2%. The 2,3-Butanediol, L (+)-lactic acid and DL-homoserine can be considered as the allelochemicals for *Medicago sativa* Linn.

Keywords: Allelopathy, aqueous extract, bioassay, *Caragana intermedia*, dicots, *Elymus dahuricus*, GC-MS analysis, *Melilotus officinalis*, monocots, root, seed germination, seedling growth, *Sophora alopecuroides*, *Sorghum sudanense*.

INTRODUCTION

Desert steppes are characterized by drought, windy conditions and in northwestern China, the human activities are contributing to desertification (19), Vegetation restoration is effective way to combat and control the land desertification (34). Shrubs play an important role in ecological and evolutionary dynamics of desert steppe ecosystems (20,23). The shrub *Caragana intermedia* dominates in the desert steppe in north-western China, however its influence on adjacent crop species is unknown due to incomplete understanding of its allelopathy. *Caragana spp* are perennial, temperate, deciduous shrubs with high degree of drought tolerance (10,51). These have been planted widely in desert steppe ecosystems (33,47) to control the soil erosion and nutrients loss (49). *C. intermedia* is preferred by the local population, as it not only curbs the desertification, improves soil quality, but also used as livestock fodder (22,51).

Allelopathy is the process of chemicals released by plants into the environment that suppress the growth and establishment of other plants in their vicinity (13,15,28,30). In this process, an organic chemical (allelochemical) released from one plant influences the growth and development of other plants (32,37). play important role in structuring of plant communities (17) and also influences the plant competition (43). The plant organs contain

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different allelochemicals (31), which can be introduced into the environment through leaching, volatilization, root exudates and biomass decomposition. These play important role in explaining growth inhibition in interspecies interactions and in structuring the plant community (27). The root derived compounds, or rhizodeposits, have the ability to affect the growth of neighbouring plants species (6). Allelopathy is a composite phenomenon which has spacious potentials of application in agriculture. Understanding of interactions among plants, particularly cultivated crops, may be helpful in modifying crop cultivation pattern with consequent yields increments (26).

In harsh environment of desert steppe, belts of longlived *Caragana* shrub with multiple thorny stems and compound leaves. It grows over 2 m tall and its crown diameter is >10 m (18). It is widely distributed in desert steppe in Ningxia, China, where it is feed for domestic animals. The *Caragana* shrub was selected for long-term afforestation to stabilize the degraded land in this region. Its plantations, accounted for 33.37% of the whole sandy grassland (21). It has been planted vertical to the predominant northwest wind directions. Row to Row and plant to plant spacing is 1.0 to 1.5 m. Some plant species grow between the shrub belts. Very few studies have evaluated the interspecies interactions from an allelopathy perspective (3).

S. alopecuroides is wild perennial herb, used in traditional Chinese medicine and widely distributed in northwest China (9). It is used as livestock feed (11). It is rich in quinolizidine alkaloids and exhibit anti-tumor, anti-inflammatory, antibacterial and antiviral activities (8,16,38). *M. officinalis* is herbaceous plant with odorous smell due to the presence of coumarins. It is traditionally used to treat insect bite, circulatory disturbance in minor veins, liver disorders, hypertension, arthritis, hemorrhoids and bronchitis (25). It is legume, hence fix atmospheric nitrogen in soil (42). It is used as forage crop, for soil stabilization and nectar source for honey bees (24). *E. dahuricus*, is important gene pool for genetic improvement of cereal crops (7). It is large polyploid genus in tribe Triticeae and a native tufted perennial grass of semi-arid grassland in China. It is widely distributed in desert steppe and used for reseeding for soil erosion control (45,48). *S. sudanense*, is an essential staple food in Africa and India (41), with rapid growth, large biomass, high resistance to adverse conditions and ease of cultivation. It is fast-growing macrophytes capable of growing in polluted water (50) and regarded as promising bioenergy crop and may be an alternative to maize for biogas use in future. In US, it is used for ethanol production from its grain or sugar. In Europe, recently it has drawn attention as novel bioenergy crop (41).

M. sativa is cheap source of protein not only for animal fodder but also for nourishment in poorest and developing countries. It is most cultivated forage legume in world, rich in essential amino acids such as (Valine, leucine, threonine and lysine), hence, called the “the queen of forages” (5,29) and used in folk medicine (29). It gives highest yield of crude protein: 2000-3000 kg/ha, i.e. 3 times more than soyabean and 4 times than wheat.

In this study, 30-year-old *C. intermedia* plantation in desert steppe was selected to evaluate the allelopathic effects of *C. intermedia*. Understanding allelopathy in natural ecosystems is important to use it (31,39), hence 4-crops (*S. alopecuroides*, *M. officinalis*, *E. dahuricus* and *S. sudanense*) used as test plants to study the allelopathic effect of *C. intermedia*. The extract was also characterized by GC-MS. GC-MS to identify the chemicals present in this plant extracts (36). The main chemical compounds that may cause the allelopathic effect were also indicated.

MATERIALS AND METHODS

Plant material

In July to August, 2014, we collected the *C. intermedia* roots upto of 0-1 m depth at late fruiting stage from farmer's fields in Wanjigou Yanchi county, Ningxia Province, China (37lage in N, 107°29.846′ E, height: 1424 m, mean annual precipitation : 290 mm, 70% precipitation occurs between June and September, mean annual temperature : 8.1 ℃, with lowest and highest monthly mean temperatures of 8.7 ℃ in January and 22.4 ℃ in July.).

Preparation of aqueous extracts

Fresh roots were chopped into 0.5 to 1.0 cm lengths. One hundred g plant material was soaked in 1000 mL sterile distilled water for 48 h at 20±2℃. The extracts were then filtered twice through 3 layers of gauzes to remove all debris and centrifuged at 2500 rpm for 2 h. The supernatant was filtered again through 0.45 μm filter membrane and designated as 10% extract. The resulting extracts were further diluted with sterile distilled water to obtain concentrations of 1/2 (5% extract), 1/5 (2.0% extract), 1/10 (1.0% extract) and 1/20 (0.5% extract). The extracts were kept in a fridge at 4℃ until use in August, 2014.

Germination bioassay

Seeds of *S. alopecuroides*, *M. officinalis*, *E. dahuricus* and *S. sudanense* were chosen as test plants for bioassay because of their common cultivation in this area (Table 1). In September, 2014, before sowing, the germination of all test spp. was 98% except 95% in *S. alopecuroides*. Thirty seeds of test plants were placed per petri plate (9 cm dia) on double layer of filter paper containing 20 ml extracts (10%, 5%, 2.0%, 1.0% and 0.5% extract, respectively). Sterile distilled water was used as control (0% extract). Treatments were replicated 6 times in randomized block design. When moisture of the filter paper declined, 10 to 20 mL distilled water was added per dish. Germination was counted every 24 h for 8-days thereafter, germination (%), germination rate and length of longest root were determined.

Table 1. Crop species used to test allelopathic effects

Species	Monocot/dicot	Family	Life cycle
S. alopecuroides	Dicot	Leguminosae	Perennial
M. officinalis	Dicot	Leguminosae	Perennial
E. dahuricus	Monocot	Poaceae	Annual
S. sudanense	Monocot	Poaceae	Annual
M. sativa	Dicot	Leguminosae	Perennial

GC-MS analysis

The aqueous extract of *C. intermedia* roots was analyzed by GC-MS in July 12th, 2014. An aliquot of the stock solution (10 mL) was evaporated under a rotary evaporator (RE-52AA, Shanghai yuhua Company, China) and diluted to 5 mL using methyl alcohol.

Then, the diluted solution was injected directly. A Hewlett Packard (HP) 6890A gas chromatograph equipped with Agilent DB-5ms capillary column (30 m×0.25 mm; 0.25 μm film thickness) was used. The carrier gas was helium, with a flow rate of 1.0 ml/min. The oven temperature was held at 60℃ for 5 min then programmed at rate of 15℃/min to 270℃ and then held at this temperature for 8 min. Mass spectra were taken at 70 eV. Mass range was from m/z 30 to 400 amu. Injector port temperature is 220℃, detector 270℃, injected volume 0.1 μL, split ratio 1:50. Relative amounts of individual components were calculated based on GC peak areas without any correction. Identification of the constituents was made by comparison of their mass spectra and retention indices with the National Institute of Standards Technology (NIST 2011) spectral library and those given in Wiley Online Library (WOL).

Exogenous compounds

Myo-Inositol (MW= 180.16, >99% purity), 2,3-Butanediol (MW=90.121, >99% purity), L(+)-lactic acid (MW= 90.08, >99% purity), DL-homoserine (MW= 119.12, >98% purity) were purchased from Wei-Min biology Ltd., Yinchuan, China. Four 50 μM of the stock solutions of technical grade were prepared in distilled water and the final volume was made by distilled water. The same volume of distilled water was used as control. All compounds solutions were buffered with phosphate buffer and adjusted to pH 6.5 to eliminate the effect of low pH (1).

Bioassay

M. sativa was used as test spp. in bioassay because it is commonly found near *C. intermedia* (46). The effects of Myo-Inositol, 2,3-Butanediol, L (+)-lactic acid and DL-homoserine (50 μM) were studied under laboratory conditions in a Petri dish bioassay on early growth of *M. sativa*. Its seeds were imbibed in respective solution of four compounds or distilled water (as control) for 12 h, Thirty seeds of test plants were placed per petri plate (9 cm dia) on double layer of filter paper seeds were placed equidistantly on two qualitative filter papers moistened with 10 mL of respective treatment solution. An artificial climate chamber was used to study the effects of compounds on *M. sativa*. The petri dishes were kept in chamber [25^{o}C temperature and 12h/12h light/dark photoperiod, 75% relative humidity]. Germination was recorded once daily. The bioassay was terminated when no increase in number of germinating seeds was observed after 6 d. Then, germination % (GP), root length (RL), seedling length (SL) (from root tip to shoot tip) and seedling diameter (SD) were determined. Treatments were replicated 6 times in randomized block design.

Statistical analysis: Means and standard deviations for all experimental parameters were calculated. One-way analysis of variance (ANOVA) and the Tukey's test (IBM SPSS version 21.0) were done to determine the significant differences between the treatments (P<0.05).

RESULTS AND DISCUSSION

Germination

The aqueous extracts from *C. intermedia* roots influenced the germination of all test species (Fig. 1). On the 8th and last day of experiment, the strongest response was observed with 10.0% concentration for dicotyledonous species (*S. alopecuroides* and *M. officinalis*). The aqueous extracts inhibited the germination of *S. alopecuroides* and *M. officinalis* to

30.16 and 24.79% of control, respectively. At 5.0% treatment, the germination was delayed. When the concentrations of aqueous extract from *C. intermedia* roots increased the germination of dicotyledonous species decreased by 14% to 25% compared to control. While in monocotyledonous species (*E. dahuricus* and *S. sudanense*) the decrease was 3% to 6%.

At low concentrations, monocotyledonous species germination was stimulated (Fig. 1). However, only 1.0% aqueous extracts from *C. intermedia* roots showed significant differences ($p < 0.05$), the extracts stimulated the germination of *E. dahuricus* by 13.46% than control. A similar phenomenon was observed in previous research examining the allelopathic effects of *Cannabis sativa* L. and *Solidago canadensis* L. (27,35). In these cases, the extracts of low concentrations also stimulated the seed germination but higher concentrations reduced the germination.

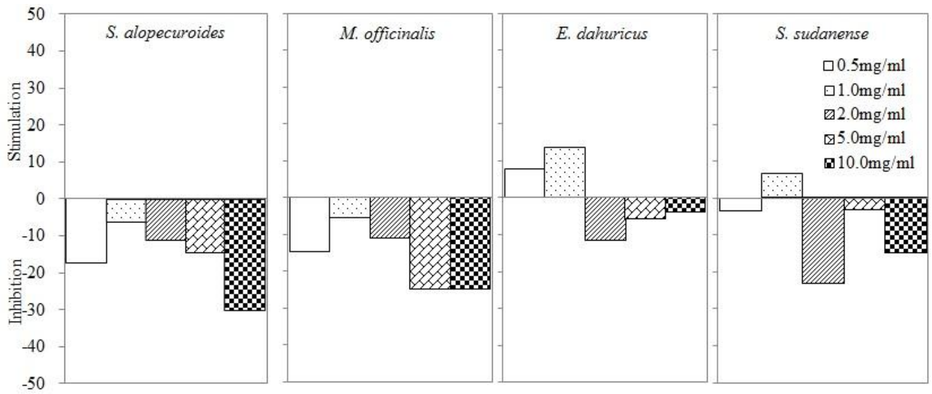

Figure 1. Effects of *C. intermedia* roots on the germination of test plants at 8 days after sowing

Residues of these compounds affects the crop seedlings during the early stages of development (27). Analysis of germination dynamics confirmed the allelopathic effects of 10.0% aqueous extract from *C. intermedia* roots on dicotyledonous species (**Fig. 2**). The inhibitory effects on germination (%) of dicotyledonous species were significant, but rotting of young seedlings was not observed. In monocotyledonous species, an inhibitory effect occurred on germination during the early stages of their development, but it decreased over time and did not suppress germination on the final sample date.

Seedling growth

The radicle is the first organ to come directly into contact with phytotoxic compounds in the soil (27). The 1.0% extract stimulated the root lengths (39.6%) of *S. alopecuroides* over the control treated with distilled water. However the 10.0% extract decreased the root length up to 51.2%. An increase in root length under mid to low aqueous extract concentrations (≤ 2.0%) up to 27.02 mm was recorded. The reduction in root length with increasing concentrations (≥ 5.0%) was statistically significant ($p < 0.05$). The extracts of 5.0 and 10.0% concentrations inhibited the root length of *S. alopecuroides* to 14.09 and 51.20% of control, respectively. This was similar to *M. officinalis*. The two dicotyledonous species produced significantly shorter ($p < 0.05$) roots at 10.0% aqueous extracts than other concentrations (Table 2, Fig. 3). Thus the allelochemicals from *C. intermedia* roots at low concentrations had stimulatory effects on *S. alopecuroides* and *M. officinalis*, while higher (10.0%) concentrations were highly phytotoxic to the two dicotyledonous species.

Table 2. Effect of *C. intermedia* extract on the length (mm) of the longest root of the tested species

Species	Concentration (%)					
	0.0	0.5	1.0	2.0	5.0	10.0
S. alopecuroides	34.55b	47.74a	57.26a	51.22a	29.69b	16.86c
M. officinalis	17.34b	27.02a	22.02b	26.66a	19.48b	11.95c
E. dahuricus	4.54c	9.13a	9.93a	5.92b	8.78a	10.79a
S. sudanense	34.92a	42.81a	44.15a	33.46a	42.49a	46.71a

Note: different small case letters among treatment means indicate significant differences according to Fisher's LSD test (p<0.05)

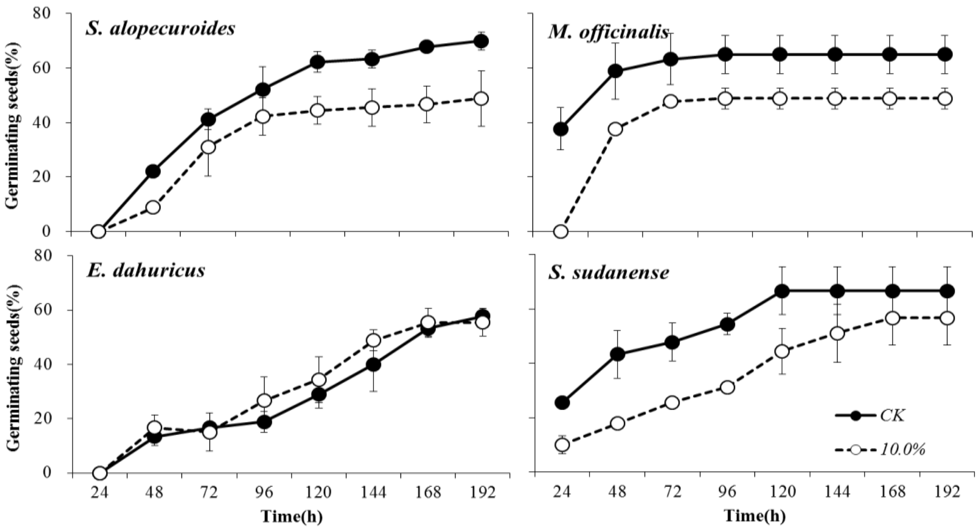

Figure 2. Effects of aqueous extract from *C. intermedia* roots on the germination patterns in control and 10.0% concentration

The monocotyledonous species showed different responses to allelochemicals. With increasing extract concentration, a statistically significant (p < 0.05) increase in root length of *E. dahuricus* seedlings was observed. *S. sudanense* was different with little change in root length.

Although few studies demonstrated the probable allelopathy in nature, many were limited to *in-vitro* studies. This difficulty reflects the complexity of allelopathic interactions (14). The allelopathic effects of *C. intermedia* under field conditions, and whether the action concentration could impose allelopathic effects on seed germination and plant growth requires further study (4).

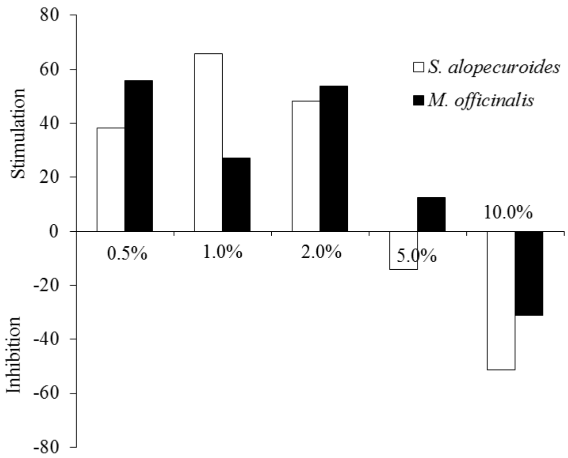

Figure 3. Influence of *C. intermedia* roots extracts on root elongation of *S. alopecuroides* and *M. officinalis* after 8 days.

GC-MS analysis

Recent improvements in the cost and capabilities of bench-top chromatography-mass spectrometry instruments has made this tools more powerful and more widely available to assist with molecular studies in the expanding field (36). The total ion current chromatogram of GC-MS of aqueous extract from *C. intermedia* roots is shown in Fig. 4. GC-MS analysis of the extractions resulted in more than 21 peaks. The main peak at 16.79min was D-myo-Inositol, which was dominant (50.6%). The second was 2,3-Butanediol, which accounted for 29.6%. The aqueous extract was composed of 21 compounds, including alcohols, terpenoid, esters, aldehydes, ketones, ethers, organic acids, haloalkanes and nitrogen-containing compounds. These compounds made up a total of 97.2% of the compounds present in the extract. Most of the identified compounds have been previously reported in plant species (30,44), but D-myo-Inositol and 2,3-Butanediol have been reported less often, especially as dominant components. The results in this study together with related previous finding suggest that the majority of compounds identified in the aqueous extract from *C. intermedia* roots are likely act as important allelochemicals (2,30,40). However, the compounds with greater allelopathic effects on the monocot and dicot plant species needs to be further characterized.

Research on the chemical basis for allelopathy has been hindered by the complexity of plant and soil matrices, making it difficult to track active compounds (36). Although compounds present in plant tissues may possess allelopathic potential (residue allelopathy), they may not be leached or exuded from the plant into the environment and cause seedling allelopathy under natural conditions (43). However, chemicals with allelopathic functions have other ecological roles, viz., plant defense, nutrients chelation and regulation of soil biota to the biomass affect decomposition and soil fertility (15).

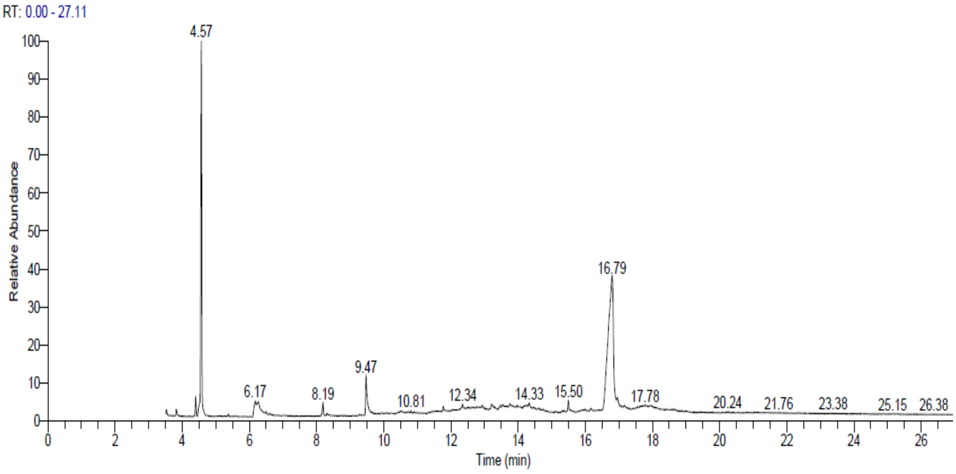

Figure 4. GC-MS analysis of aqueous extract from *C. intermedia* roots

The bioactivity of major compounds

Three compounds (2,3-Butanediol, L (+)-lactic acid and DL-homoserine) affected the early growth of *M. sativa* measured in terms of GP, RL, SL and SD, but Myo-Inositol had no significant effect (Fig. 5, Fig. 6). The GP was reduced by 10.3% and 50.0% of the L (+)-lactic acid and DL-homoserine than control treatment. A similar trend was also observed for the RL, SL and SD, which were significantly decreased. GP, RL, SL and SD increased significantly with the addition of 50 μM 2,3-Butanediol treatment compared to the control treatment.

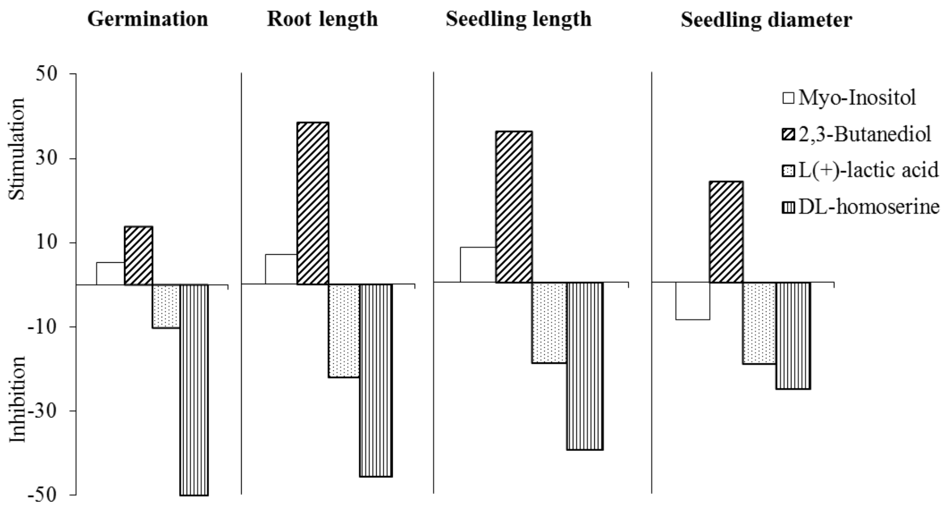

Figure 5. Effects of four compounds on the germination % (GP), root length (RL) and seedling length (SL) of *M. sativa* measured at 6 d after treatment

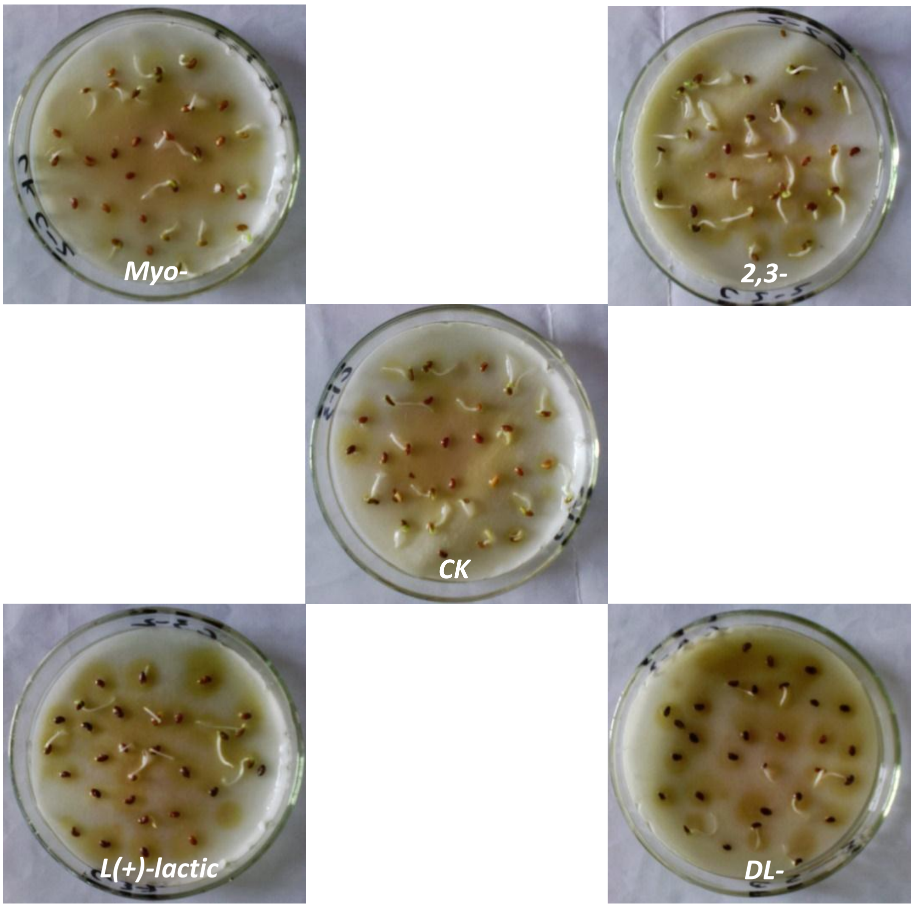

Figure 6. *M. sativa* seeds imbibed in respective solution of four test compounds treatments and distilled water (as control)

It is well-known that plants produce growth-regulating compounds. Some of these compounds are released from plants (donors) and absorbed by other plants (acceptors). The bioactive compounds transferred from the donor plant to the acceptor plants are termed allelochemicals (12). The inhibitory effects of the L (+)-lactic acid and DL-homoserine on early growth is not surprising, as the phenolic acids are potent germination and growth inhibitors (30). The 2,3-Butanediol, L (+)-lactic acid and DL-homoserine are allelochemicals for *M. sativa*, but the Myo-Inositol was not. A compound with high total activity could be a chemical communication tool among organisms in the natural ecosystem. Compounds with high total activity (biological activity per weight of compound contained in unit weight of the donor plant) should be addressed for the purpose of allelopathic research (12).

CONCLUSIONS

The aqueous extract from *C. intermedia* roots inhibited the germination of all test plants. The drastic inhibition was at 10.0% concentrations in dicotyledonous species (*S. alopecuroides* and *M. officinalis*). In monocotyledonous species (*E. dahuricus* and *S. sudanense*), high concentrations (2.0, 5.0 and 10.0%) of extracts reduced the germination but low concentrations (0.5 and 1.0%) were stimulatory. The root length of *S. alopecuroides* increased to 39.6% at 1.0% extracts and then rapidly decreased to 51.20% at 10.0% extract concentration. The same effect was observed in *M. officinalis*. The root length of dicotyledonous species significantly decreased ($p < 0.05$) at 10.0% aqueous extract than other concentrations. While the root length of *E. dahuricus* seedlings increased significantly ($p < 0.05$) with increasing extract concentration. In *S. sudanense*, increasing extract concentration did not influence the root length. The major constituents in extrant from *C. intermedia* roots by GC-MS analysis were : D-myo-Inositol and 2,3-Butanediol. The aqueous extract mainly contained 21 compounds (Alcohols, terpenoid, esters, aldehydes, ketones, ethers, organic acids, haloalkanes and nitrogen-containing compounds), which constituted 97.2% total of all compounds present in the extract. Three compounds (2,3-Butanediol, L (+)-lactic acid and DL-homoserine) are considered allelochemicals for *M. sativa*.

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