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Allelochemicals of *Helianthus annus* L. and their effect on Germination Parameters of *Phaseolus aureus*

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Abstract
Several phytotoxic substances causing germination and growth inhibitions have been isolated from the plant *Helianthus annus* L. These substances, collectively known as allelochemicals, are usually secondary plant products or waste products of main metabolic pathways of plants, which exhibit toxicity towards other plants. Many allelochemicals have been characterized from the H. annuus, which inhibit the seed germination and seedling growth of several crops including ***Phaseolus aureus*.** In the current investigation, the allelochemicals were extracted from *Helianthus annus* L. using different organic solvent systems and their bioefficacy was assessed against chosen germination parameters of *Phaseolus aureus* viz a viz seed germination, seed vigour and mean seedling length. Inhibition of germination was observed at a higher concentration, and a reduction in speed of germination, seedling growth of *Phaseolus aureus* in lower concentration of aglycosidic and organic components.
Key words: Alleopathy, allelochemicals, bioefficacy.

Introduction

Helianthus annus L., commonly called as the sunflower is well known for its allelopathic activity. Leachates from the plants affect the germination, growth and development of other plants grown in the same soil (**Leather, 1987; Narwal, et al., 1999, Batish, et al., 2002 and Macias, et al., 1999**).
Apart from the various macromolecules of primary requirement, green plants also produce innumerable biomolecules generally referred to as secondary products involved in secondary metabolism. These secondary products get released from the plants of their synthesis into the environment. Their interaction with the plants leads to the effect on the growth and establishment of the latter (**Cheng, F., Cheng, Z., 2015**). Allelopathic effect therefore depends on the compound (phytotoxin or allelochemical) being added to the environment. Allelochemics are synthesized in the plants and stored in their cells as tannins, lignin and water-soluble glycosides. They get released into the environment due to the action of plant enzymes and/or environmental stress through any of the following ways:
1. Exudation through roots.
2. Release by volatilization of oils into environment.
3. Release upon microbial decomposition of litter.
4. Leaching aided by agencies like rain, mist, snow, fog and dew from aerial parts.

The water-soluble forms exist as glycosides of the organic components. The glycoside is made up of a large molecule attached to a secondary compound. The translocation of secondary plant product in or out of the plant is in glycosidic form itself (**Goss, 1973**).

It seems that the harmful effects of sunflower in crop rotations are due to release and accumulation of root exudates during crop growth in soil. Soil incorporation of its fresh (green manure) or dry biomass in soil is inhibitory to both crops and weed species (**Azania, A., et al, 2003**).
The current work was carried out to find out the nature of allelochemicals of *Helianthus annus* L. The allelochemicals were extracted with different organic solvent systems and their bio-efficacy was assessed in terms of a few germination parameters of *Phaseolus aureus* viz a viz seed germination, seed vigour and mean seedling length.

Materials and Methods
The leaves and other plant parts of *Helianthus annus* L. were collected from plants grown in the experimental plots of Botanical Garden, Panjab University, Chandigarh (30° N; 77° E; 280 mean sea level). The healthy, uniform and viable seeds of plant *Phaseolus aureus* Roxb. Var. ML-267 was used (viability ensured through 2, 3, 5-triphenyl tetrazolium chloride test). The plants were procured from Plant Breeding Department of Agricultural University, Ludhiana. Plants of *Phaseolus aureus* Roxb. were raised from seeds sown in earthenware pots (8 inches diameter) filled with sand and clay (1:1 v/v).

Extraction of Allelochemicals
Aqueous leachates
Freshly collected, surface cleaned, healthy leaves of *Helianthus annus* were chopped into pieces, weighed and suspended in pure water in the ratio of 1:1 w/v for 24 hours. The suspension was filtered using bilayer muslin cloth. The filtrate was used as aqueous leachate. The concentration used was selected on the basis of preliminary trial involving wide range of concentrations (0.1 to 1 g fresh weight/mL of water) of leachates on cell survival values of a few representative plant leaves (**Muhammad, Zahir, and Abdul Majeed. 2014**).

Organic Component of Aqueous Leachate (Aglycones)
Pre- chilled 1 N HCl was added to the aqueous leachates (1:1 v/v). The precipitates were recovered using centrifugation and were washed 5-6 times with pure water. Every time the recovery was made through centrifugation. Finally, the recovered precipitates were air-dried. The requisite amount of precipitates was dissolved in a drop of ethyl alcohol and the desired concentration was built with pure water.

Organic Extract Fractions
Freshly cut, surface cleaned, healthy leaves of *Helianthus annus* L. were shade dried and crushed to form powder. The latter was suspended in petroleum ether (60° - 80°) for 24 hours. Filtration was done and the residue was rinsed again with petroleum ether to ensure that no other petroleum ether fraction was left in the residue. The filtrates were then pooled. The solvent was recovered using water bath. For further experiment, the requisite amount of fraction was weighed and dissolved in 2-3 drops of xylene and a drop of tween-20 (surfactant) was added. Desired concentration of the solution was obtained by making the final volume with pure water.
The residue from petroleum ether suspension (**marc**) was air dried to evaporate petroleum ether completely and then suspended in methanol for 24 hours and then filtered. The residue was discarded. The filtrate was divided into two parts:

i) From the first part, **methanol fraction** was recovered by distilling off the solvent on water bath. The requisite amount of fraction was weighed and the final concentration was achieved by making up the volume with pure water.
ii) From the second part, methanol was removed by distillation and residue was partitioned between chloroform and water (1:1 v/v). The two layers were separated using a separating funnel. The solvent from **chloroform fraction** was distilled off; the residue (chloroform fraction) was weighed and dissolved in a few drops of methanol and the final volume made up with pure water.

Germination Trial

The seeds of *Phaseolus aureus* Roxb. var. ML-267 were subjected to germination trials. International Rules for Seed Testing (ISTA rules) were followed (**Agarwal, 1980 & Tian, Y., Guan, B., et al 2014**). Uniform, healthy viable seeds of plants under investigation were used. For each treatment 120 seeds of each type were taken. These were presoaked in aqueous leachates, organic components of aqueous leachates and organic extracts for 24 hours. Seeds sown in water served as control. The soaked seeds of each treatment were placed in 6 inches diameter petri dishes. The petri dishes were lined with a wad of thin absorbent cotton and overlined with Whatman number 40 filter paper. These were moistened with treatment solution and 30 seeds were arranged in each petridish in concentric rings maintaining a proper inter-seed distance.) 4 petri dishes were set for each treatment and the set up was maintained in seed germinator for definite temperature (**table1**), relative humidity 74 $\pm$ 1 % and continuous light of 4000 lux for 24 hours daily.

Table1 Conditions of temperature, substratum and pretreatment to the seeds of various groups under observation for germination

BOTANICAL NAME	TEMPERATURE/ SUBSTRATUM	PRE-TREATMENT
Phaseolus aureus Roxb. Var. ML-*267*	25 $\pm$ 3 (BP)	-
Phaseolus mungo L.. var. Mash 1-1	25 $\pm$ 2 (BP)	Diffuse light
Cajanus cajan DC. Var. AL-15	25 $\pm$ 2 (BP)	-
Vigna umbellate Thunb. Var. RBL-	25 $\pm$ 2 (BP)	-
Lens esculentum L. var. LL-56	25 $\pm$ 2 (BP)	Pre-chill treatment
Triticum aestivum L.,	20 (BP)	Diffuse light, pre-chill KNO_3
Zea mays L.	25 $\pm$ 3 (BP)	-
Oryza sativa	25 $\pm$ 3 (BP)	-
Avena sativa	20 (BP)	Diffuse light, pre-chill KNO_3
Brassica camperstris L.	20 $\pm$ 3 (BP)	Light, pre-chill KNO_3
Linum usitatissimum L.	20 (BP)	Light, pre-chill, pre-dry
Hibiscus esculentum	25 $\pm$ 2 (BP)	Light
Lycopersicum esculentum (L.)	25 $\pm$ 2 (BP)	Light, KNO_3
Solanum melongena L.	25 $\pm$ 2 (TP)	Light, KNO_3

BP – between the folds of filter paper
TP- top of filter paper

Protrusion of radicle from seed coat signified initiation of germination. Daily observations with hand lens were made in one representative out of the four petri dishes. Observations were made for seven days till no more seeds germinated. The data was subjected to Duncan Multiple Range Test (**Duncan, 1955**).

Results

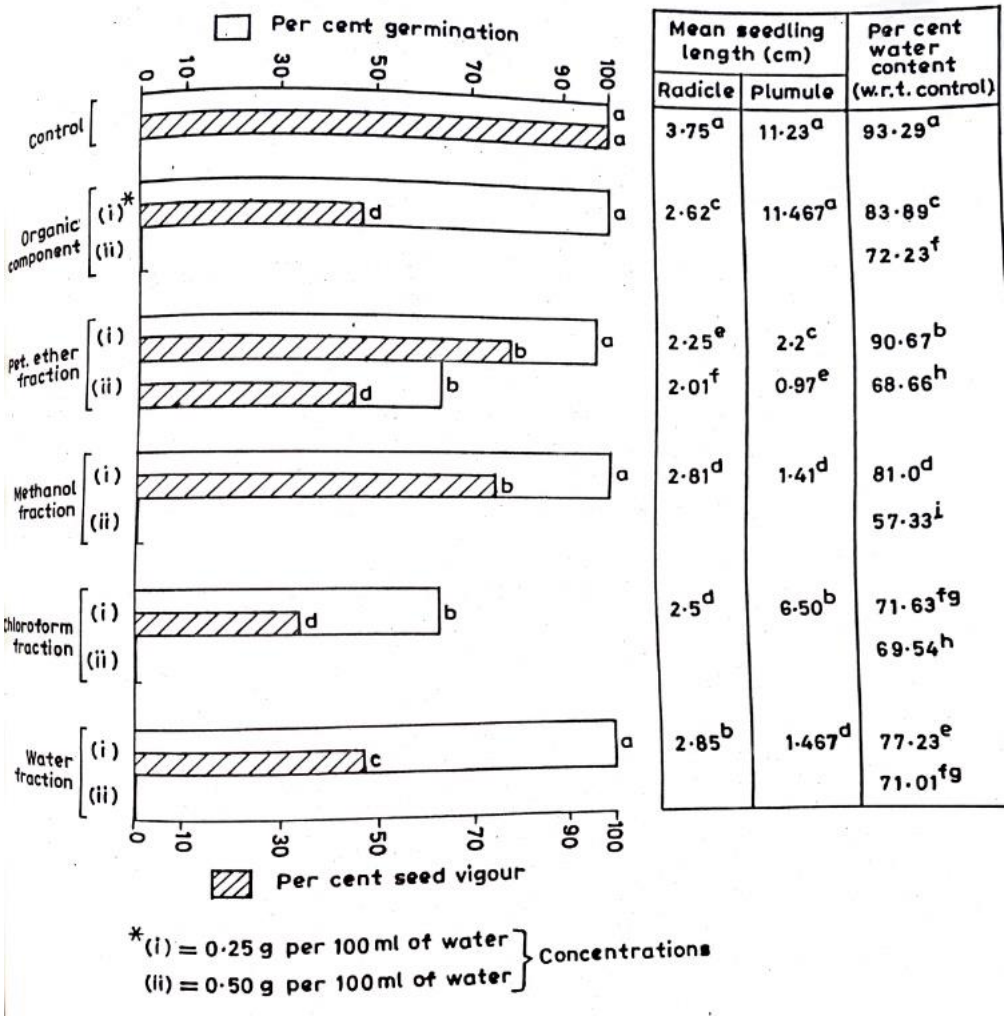
Compared to cent percent germination in control, none of the seeds treated with 0.5g/100mL of water of extracts made in methanol or chloroform or water fractions derived from marc or the aglycones from aqueous leachates derived from the fresh leaves showed cent per cent germination. Seeds treated with lower concentrations of any of the extract fractions other than chloroform fraction did not show any significant statistical change in percent germination over that of the control. In those treated with chloroform fraction, 63% of the seeds as compared to control germinated. Almost a similar number of seeds treated with 0.5g/100mL water of petroleum ether showed germination (**fig1**).

The speed of germination in all the treated cases was seen to be significantly low compared to control, where all the seeds germinated on the first day. Among the seeds that germinated relatively slow seed vigor was calculated in case of seeds treated with 0.25g/100mL of water of aglycones, chloroform fraction, or the water fraction or the 0.5g/100 mL of petroleum ether fraction (**fig 1**)

The length of the plumule was almost three times to that of radicle in case of water treated control seeds. In each of the seedling from the seed that germinated, the length of radicle or plumule length was statistically almost the same to that of control. Among the two, the effect was relatively more pronounced in case of plumule than the radicle. Shortest plumule length was measured in case of those seeds treated with 0.5g/100mL of water of methanol or water fraction (**fig 1**).

The content of water in case of seedlings of any of the treated samples was measured to be low than that of control.

Minimum water content was seen in case of those treated with 0.5g/100mL water of pet ether extract followed by 0.25g/100mL of water of chloroform extract. Among those seeds, which, did not germinate at all, the minimum water content was seen in those, treated with 0.5g fresh weight/100mL water of methanol fraction. Inhibition of germination was observed at high concentration, and a reduction in speed of germination, seedling growth of Phaseolus aureus even in lower concentration of aglycosidic and organic components.



Mean seedling length (cm)		Per cent water content (w.r.t. control)
Radicle	Plumule	
$3·75^{a}$	$11·23^{a}$	$93·29^{a}$
$2·62^{c}$	$11·467^{a}$	$83·89^{c}$
		$72·23^{f}$
$2·25^{e}$	$2·2^{c}$	$90·67^{b}$
$2·01^{f}$	$0·97^{e}$	$68·66^{h}$
$2·81^{d}$	$1·41^{d}$	$81·0^{d}$
		$57·33^{i}$
$2·5^{d}$	$6·50^{b}$	$71·63^{fg}$
		$69·54^{h}$
$2·85^{b}$	$1·467^{d}$	$77·23^{e}$
		$71·01^{fg}$

Fig 1 Effect of organic component of aqueous leachates of *Helianthus annus* L. on germination factors of *Phaseolus aureus* Roxb seeds.

Discussion

In the current work, the aim was to assess allelopathic potential of the plant by extraction of phytotoxins through leachation in water using different solvent systems based on their differential solubility. The extraction procedure was so designed that the highly nonpolar components got extracted first in petroleum ether from slightly polar, nearly polar or highly polar compounds. In the residue, the compounds with polarity of varying degree were further subjected to fractionation on the basis of polarity. Therefore, the **highly polar fraction** was obtained in water and least polar in chloroform. Fractions made in chloroform, representing the less polar allelopathic compounds were seen to be relatively more effective than those of water fractions, i.e. chemicals that were less polar. The effect of **methanol fraction**, representing the polar allelochemics in contrast to non-polar ones, extracted in petroleum ether represented the chemicals extracted in chloroform or water because the latter two were derived from the former (i.e., methanol fraction). The **non-polar** chemical extracted in petroleum ether were seen to be relatively not as effective as those from marc in relatively polar solvent, methanol. 0.5g/100mL of the extract fractions made in petroleum ether showed 63.3% germination compared to no germination of the seeds of *Phaseolus aureus*. The dose-response relationship shown by the aqueous leachates reflected that at 0.25%

concentration of almost all the aglyconic and organic fractions (except chloroform, where germination was 63.3%), the germination was found to be cent percent. In 0.5% concentration, none of the seeds germinated (except petroleum ether fraction where it was 63.3%).

Conclusion

Based on the experimental results, it can be concluded that the allelochemics from *Helianthus annus*, which get released from the aerial part of the plant upon death and decay appear not to be a single component but a combination of more than three types. One of them was highly nonpolar, and the other one relatively less non-polar (extracted in petroleum ether) and third being highly polar (extracted in water). Inhibition of germination was observed at high concentration, and a reduction in speed of germination, seedling growth of *Phaseolus aureus* even in lower concentration of aglycosidic and organic components clearly demonstrates that secondary products in their organic or aglycosidic forms also show inhibition. The results also signify that the action of allelochemicals is because of the phytochemics rather than physical or mechanical means.

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