J Therm Anal Calorim (2011) 104:187–192
DOI 10.1007/s10973-010-1225-7

# Impact of sunflower and mustard leave extracts on the growth and dark respiration of mustard seedlings

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ISBCXVI Special Issue


**Abstract** The subject of the study was investigation of impact of extracts from sunflower and mustard leaves on growth of mustard seedlings. Seeds of mustard were germinated on water and then grew on aqueous extracts from sunflower or mustard leaves. The specific thermal power during seedlings growth was measured by isothermal calorimetry. Changes in the chemical composition stimulated by extracts were measured by FT-Raman spectroscopy and analyzed with the support of the cluster analysis. The heat production rate during growth of seedlings was related to the type of extracts. Crude sunflower and mustard extracts strongly inhibited the growth of seedlings when compared to non-treated control. FT-Raman spectroscopy confirms that allelopathic compounds have the greatest influence on the metabolism of fatty acids of mustard cotyledons. The obtained results indicate that sunflower and mustard extracts have varied impact on growth and heat production rate of mustard seedlings.

**Keywords** Allelopathy · Isothermal calorimetry · Mustard · Non-destructive Raman spectroscopy · Sunflower

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## Introduction

Allelopathy is a direct or indirect influence of chemical compounds (allelochemicals) released from one living plant on the growth and development of another plant [1]. Many allelochemicals can be toxic to higher plants and for this reason they can be used as a natural herbicide. Nowadays, a new method of weed control is searched for and allelochemicals are considered to be environmentally friendly herbicides [2].

*Helianthus annus* L. is a well-known plant which contains a lot of allelopathic components. Chemical studies of sunflowers have shown that in this plant several bioactive compounds are present: flavonoids [3], terpenoids [4], norsesquiterpenoids [5], sesquiterpene lactone [6, 7], annuionones [8, 9], and two new families of sesquiterpenoids, heliespiranes [10], and heliannanes [11–13]. Therefore, it is possible to use this plant as a natural herbicide.

The influence of sunflower extracts on the germination of mustard seeds has already been studied by using isothermal calorimetry. Preliminary studies indicate that the extract from sunflower leaves inhibits the germination of mustard seeds [14]. Also our previous studies indicate impact of mustard and sunflower leaves extracts on the mustard seedlings growth [15]. Another study shows the importance of calorimetry as a tool to indicate that cnicin has an allelopathic impact on the germination of soybean and radish seeds [16].

This article shows the potential of the calorimetric method for investigating the allelopathic interaction between plants. On the other hand, the development of Raman spectroscopy with Fourier transformation (FT-Raman) makes this technique useful for characterizing and identifying chemical compositions in living tissue. It allows you to study analytes in situ in their natural

environment which is not possible when using previously applied analytical techniques. Raman spectroscopy is a fast, non-invasive, and non-destructive method, which means that an analysis can be performed without any mechanical, chemical, photochemical, or thermal decomposition [17]. In addition, samples require no special preparation before measurement. Several articles show the usefulness of applying FT-Raman spectroscopy to investigate polyacetylenes and polysaccharides [18], carotenoids in various types of carrot roots [19], and flavonoids in green rooibos [20].

In this article the allelopathic effect of extracts from sunflower (*Helianthus annus* L.) and the autoallelopathic effect of extracts from mustard (*Sinapis alba* L.) leaves on the growth of mustard seedlings were studied both with the use of isothermal calorimetry and FT-Raman spectroscopy.

## Experimental

### Plant material

Mustard seeds, cultivars 'Nakielska', were received from Plant Breeding and Acclimatization Institute Oil seed Department in Poznań (Poland). 50 seeds were germinated in darkness for 24 h in a plastic Petri dish on the filter paper moistened with distilled water at 20 °C. Next 24-h-old seedlings were used to the future experiments (as described below).

### Preparation of extracts

The leaves of sunflower, cultivars 'Ogrodowy', and mustard, cultivars 'Nakielska', were received from Agriculture University in Kraków. The leaves were collected after flowering season. Then they were air-dried, next ground to powder, and crude extracts were prepared by mixing 5 g of powdered plant tissue with 95 g of water for 24 h in darkness at room temperature.

### Calorimetric measurements

The specific thermal power (STP) was measured (in mW) in an isothermal calorimeter (BioActivity Monitor 2277, Thermometric, Järfälla, Sweden). For the experiment, the ampoules (volume 20 mL) with tops enabling air exchange were used. Five seedlings were put into an ampoule on filter paper with 300 μL of sunflower or mustard extract, allowed to equilibrate for 15 min in high position and placed into the measurement chamber, then the next 15 min of equilibrating. In the reference ampoule there was only the filter paper moistened with extract. Seedlings on the filter paper with 300 μL of water were taken as absolute control. Measurements were recorded continuously for 24 h at 20 °C, and five replicates were performed. The STP was expressed per gram of dry mass. The enthalpy values were determined as the area under the calorimetric curve.

### Calorespirometric measurements

Calorespirometric measurements were performed with the use of an isothermal calorimeter (BioActivity Monitor 2277, Thermometric, Järfälla, Sweden). Mustard seeds were germinated on water (as described in "Plant material"), then the seedlings grew for 12 h on the extract (mustard or sunflower). Next, five seedlings were put into a measurement ampoule with 300 μL of water (control), mustard, or sunflower extract. The same extract was entered into the reference ampoule. The thermal equilibration time of ampoules was 15 min. Heat rate measurements (q) were carried out at the temperature of 20 °C for 30 min, then 100 μL of 0.4 M NaOH was injected into the vessel in the ampoule to absorb $CO_2$ produced by seedlings during their growth. The second measurement (with NaOH and without $CO_2$–$q_{CO_2}$) was conducted for 15 min. All measurements were performed in four replicates. Results were converted to dry mass (per gram).

### Fourier transform Raman spectroscopy measurements

For FT-Raman measurements five cotyledons of mustard seedlings were taken, which grew on each kind of the investigated extracts. FT-Raman spectra were recorded using a spectrometer Nicolet NXR 9600 equipped with a Nd:YAG laser, emitting at 1064 nm, and a germanium detector cooled with liquid nitrogen. The instrument was provided with an *xy* stage, a mirror objective, and a prism slide for re-directing the laser beam.

All spectra were accumulated from 64 scans, measured with a laser power of 150 mW using an unfocused laser beam with the diameter of approx. 100 μm. Measurements were performed with a spectral resolution of 4 $cm^{-1}$ in the range from 100 to 4000 $cm^{-1}$. Raman spectra were registered by the Omnic/Thermo Scientific software program. For each sample five spectra were collected.

### Chemometrics

Similarities between FT-Raman spectra were studied using Hierarchical Cluster Analysis (program Opus/Bruker package 5.1). The spectra were not baseline-corrected. Spectral distances were calculated with the factorization algorithm (3 factors), Ward's algorithm, followed by vector normalization. The cluster analysis was carried out for the wavenumber range from 1320 to 1680 $cm^{-1}$.

## Results and discussion

### Seedlings heat production

The source of heat production from seeds rests in various biochemical reactions in plants, e.g., respiration. Calorimetric measurements allowed us to obtain information about the response of plant tissue to stress conditions, such as allelopathic compounds in extracts. The value of STP provides information about the viability of germinated seeds or seedlings growth. The STP of control seedlings was increasing continuously during 24 h of growth on water, Fig. 1. Differences in the STP between seedlings growing on sunflower and mustard extracts were already evident in the first hour of the measurement. The seedlings growing on mustard extract had higher values of metabolic activity, and the peak of heat production for these seedlings was over 400% higher than the peak related to seedlings growing on sunflower extract. The STP for the seedlings treated with mustard extract reached the maximum in 11.9 h of measurement, whereas, for seedlings growing on sunflower extract in 12.1 h. The highest value for seedlings growing on mustard extract was 20.22 mW $g_{DW}^{-1}$ and the maximum value for the seedlings treated with sunflower extract was 4.51 mW $g_{DW}^{-1}$.

Calorimetric results indicate the difference in STP between seedlings growing on various extracts. Sunflower extract is more toxic than mustard extract. For the control seedlings, STP is considerably lower than for the latter. This shows that sunflower extract is a stronger stress factor due to the concentration of toxic compounds.

Figure 2 shows the values of specific enthalpy determined for mustard seedlings growing on mustard and sunflower extracts and water as a control. They were calculated from each individual specific thermal power curve obtained during their growth. The value of specific enthalpy for seedlings growing on mustard extract is significantly much higher than the control values. In contrast, enthalpy value for seedlings growing on sunflower extract is lower than the control, but the differences between these values are not statistic significantly.

### Calorespirometric ratio

The calorespirometric ratio ($q/R_{CO_2}$) provides information on energy efficiency [21]. The ratio $q/R_{CO_2}$ is estimated as a quotient rate of heat production ($q$) to the rate of carbon dioxide production ($R_{CO_2}$). $R_{CO_2}$ was calculated according to [21]. The data for calorespirometric ratio are gathered in Table 1. The highest value of the ratio was obtained for the seedlings which were grown on sunflower extract. For control seedlings the value of this ratio was the lowest. The effect of toxins from sunflower extract is stronger than the effect of toxins from mustard extract. With increased concentration of toxins and stress, the calorespirometric ratio is also growing.

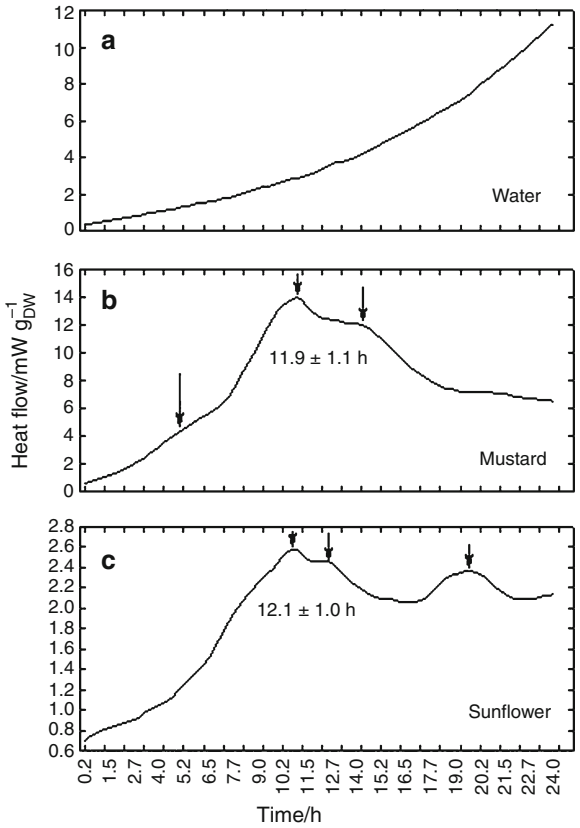


**Fig. 1** Heat flow of mustard seedlings during growth on extracts from mustard and sunflower leaves and water (control)

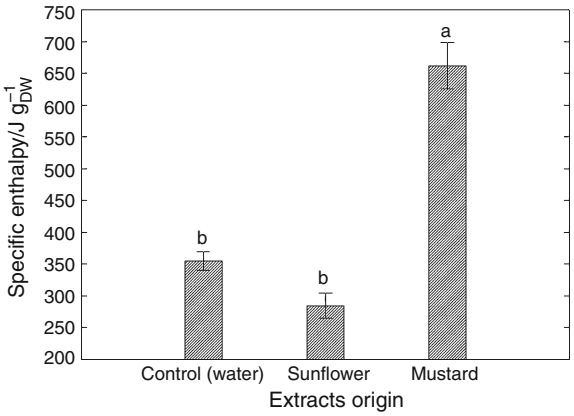


**Fig. 2** Mean (± SD) values of specific enthalpy of mustard seedlings during growth on extracts from mustard and sunflower leaves and water (control)

**Table 1** Calorespirometric ratio for mustard seedlings growing on water, aqueous extract from mustard and sunflower leaves

| Extract | Calorespirometric ratio $(q/R_{CO_2})$/kJ $mol^{-1}_{CO_2}$ |
|---|---|
| Control | 24.6 |
| Mustard | 163.4 |
| Sunflower | 278.0 |

## FT-Raman spectroscopy

Calorimetric measurements provide information only about general metabolic activity and the level of respiration of mustard seedlings. More details about the metabolic pathway are described with the Raman spectroscopy method.

Raman spectroscopy is used as an analytical technique applied in agricultural, food, and industrial laboratories. Its potential for the detection of vegetable oils is also well known. Raman measurements allow you to monitor simultaneously the degree of unsaturation and isomer formation during hydrogenation processes of edible oils [22].

FT-Raman spectra obtained from mustard cotyledons germinated on extracts from sunflower as well as from mustard leaves are presented in Fig. 3. In addition, the spectrum of the cotyledons germinated on the filter paper soaked with pure water was taken as control. The most characteristic wavenumber range with several distinct bands can be pointed out between 1700 and 1200 $cm^{-1}$. The signals occurring there are broadened since they consist of several overlapping bands and can be only tentatively assigned to individual plant components. However, the prominent band at about 1440 $cm^{-1}$ together with signals near 1655, 1300, and 1266 $cm^{-1}$ can be associated with fatty acids, but also lignin can be correlated with

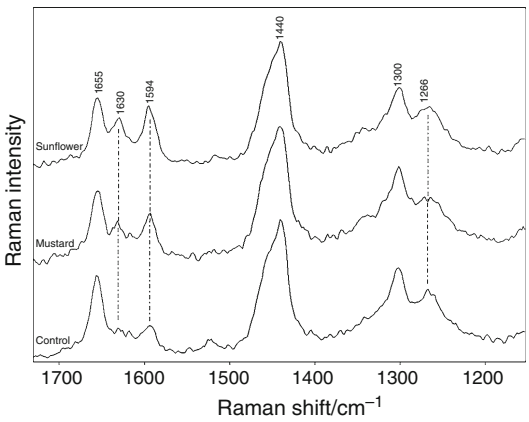


**Fig. 3** FT-Raman spectra of mustard cotyledons germinated on extracts from sunflower (top) and mustard (middle) leaves, and control (bottom)

signals near 1600–1650 $cm^{-1}$. In addition, a characteristic flavonoid band near 1590 $cm^{-1}$ can be detected.

The intensities of Raman bands near 1660 $cm^{-1}$ and 1670 $cm^{-1}$ are related to the content of individual *cis* and *trans* isomers present in various edible oils [23]. The ratio of scattering intensity arising from the C=C stretching vibration (1600 $cm^{-1}$) to that obtained from the $CH_2$ scissoring mode (1444 $cm^{-1}$) could be used to predict reliably the iodine values of unconjugated vegetable oils. The total degree of unsaturation can be also determined by calculating the ratio of the intensity of a band at 1266 $cm^{-1}$ to that of a band at 1300 $cm^{-1}$ [23, 24].

Flavonols and anthocyanins have an intensive band near 1590 $cm^{-1}$, but this band is stronger for the former if the C(3) position of the molecule is glycosylated. The band can be assigned to the 8a and 8b modes of the phenyl rings A and B of the flavonol molecule [25, 26], and it is very characteristic for this group of compounds. Usually, signals do not occur in this wavenumber range due to other plant components, so the observed band near 1590 $cm^{-1}$ can be attributed to flavonoids.

## Cluster analysis

Some changes can be seen in the intensities of Raman bands originating from various plant components. Since there is a correlation between signal intensity and the concentration of analyte, the observed differences indicate various contents of the discussed components, i.e., fatty acids, carotenoids, and flavonoids. The spectral information about various plant components is usually spread in the broad range of the spectrum where signals assigned to these constituents are overlapping. To extract this information, some chemometrics methods should be used. In order to find meaningful and systematic differences among the measured spectra of mustard cotyledons germinated on extracts from sunflower and mustard leaves as well as control cotyledons, the cluster analysis was applied. Despite the application of the standard procedures of spectra pre-treatment and various mathematical algorithms, no discrimination could be achieved if the whole spectral range was considered. Analyses based on various narrow wavenumber ranges gave a variety of outputs, with randomly distributed plants from the experimental groups, except for one specific spectral range. When the analysis was limited to 1320–1680 $cm^{-1}$ (Fig. 4), a distinct discrimination between cotyledons germinated on extracts from sunflower and mustard leaves was achieved. These results indicate that changes of plant constituents caused by germination conditions are related to characteristic Raman signals in this range. The performed cluster analysis showed that the range between 1320 and 1680 $cm^{-1}$ could be used for discriminating between seeds germinated on

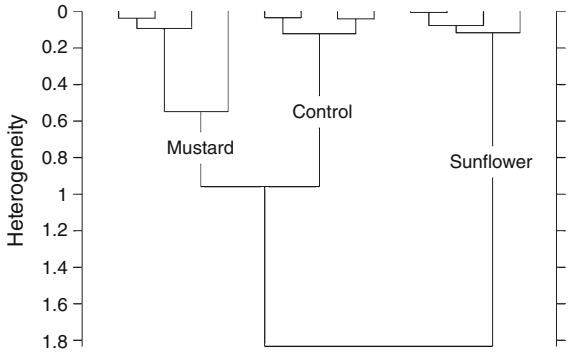


**Fig. 4** Dendrogram showing classification of mustard cotyledons treated with mustard and sunflower extracts and control cotyledons after cluster analysis of the FT-Raman spectra at the wavenumber range 1320–1680 $cm^{-1}$ using the vector normalization and factorization (3 factors) algorithm for calculating spectral distances and the Ward's algorithm. *Mustard* cotyledons germinating on mustard extract, *Control* cotyledons germinating on water, *Sunflower* cotyledons germinating on sunflower extract

extracts from sunflower and mustard leaves. As can be seen, it covers the most intense signals coming from the plant constituents mentioned above.

## Conclusions

This article has shown that isothermal calorimetry is a useful method for investigating allelopathy. It allows for monitoring the impact of one plant on another. The heat production rate–time curves obtained for mustard seedlings which grew on extracts from mustard and sunflower leaves can be correlated with different allelopathic compounds from extracts that influence the metabolism of seedlings. Seedlings growing on mustard extract have higher values of metabolic activity than those growing on sunflower extract.

In addition, FT-Raman spectroscopy with the hierarchical cluster analysis is a good tool to compare the seedlings treated with mustard and sunflower extracts for the purpose of investigating their chemical composition. The spectra of mustard cotyledons showed that extracts mainly influenced the degree of saturation of fatty acids, but also the metabolism of flavonoids.

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