Available online at www.sciencedirect.com
ScienceDirect
Thermochimica Acta 458 (2007) 84–87
thermochimica acta
www.elsevier.com/locate/tca

A calorimetric study of the allelopathic effect of cnicin isolated from *Centaurea diffusa* Lam. on the germination of soybean (*Glicine max*) and radish (*Raphanus sativus)*

Fanny I. Schabes, E. Elizabeth Sigstad*

Instituto de Química Orgánica, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, CP T 4000 INI, Tucumán, Argentina

Abstract

This work concerns the application of isothermal calorimetry to measure the effect of 'cnicin' on the germination of the soybean (*Glycine max* (L.) Merr., cv. A7636 RG) and the radish (*Raphanus sativus* L., cv. Sparkler). The sesquiterpenelactone, cnicin was isolated from a highly invasive plant, the diffuse knapweed *Centaurea diffusa* Lam. Calorimetric experiments were performed with seeds on wetted filter paper disks or in agar, both containing varying concentrations of cnicin. Results indicate that this substance blocks the water uptake by roots inhibiting subsequent seedling growth but has no effect during germination.

Keywords: Isothermal calorimetry; Allelopathy; *Centaurea diffusa*; Cnicin; *Glycine max*; *Raphanus sativus*

1. Introduction

Many chemical substances derived from plants seem to be potentially selective for weed control in certain conventional cultivation systems or of minimum tillage. They can be used as models for a type of totally new biorational synthetic herbicides. Allelochemicals – a class of biologically synthesized chemicals used by terrestrial plants in their fight against insect herbivores – are also important in plant–plant interactions (allelopathy). Thus there is an increasing interest in using this type of substances to control pests and weeds because they are considered environmentally more benign and secure; due to their mode of action, they are more specific in general [1–4].

Centaurea diffusa Lam., the diffuse knapweed, is a weed belonging to the Asteraceae family, recently introduced in Argentina and much earlier in North America from its origin in East Europe/Asia Minor. It contains a high concentration of a sesquiterpenelactone, called cnicin. This allelopathic compound acts more on the roots of other plants than on their stems or leaves and suppresses the growth of nearby native species, apparently in a competition for water and nutrients. Knapweed caused great economical damage in the US during the 1980's due to its allelopathic effects on cultivated species [5,6]. It is well known nowadays that non-native plants constitute a significant threat to native species and natural communities [7]. Therefore, is of great interest to elucidate the mechanisms of action that these species use to invade a field.

Preliminary studies with solutions of 500 and 1000 mg dm^{-3} of a chloroform extract of *C. diffusa* indicated such an effect on germinating quinoa seeds (*Chenopodium quinoa* Willd.). The seedling length and mitotic index were found to be reduced compared with the control at 24 h [8]. Moreover, the honeyweed *Leonurus sibiricus* experienced reduced germination (40%) and seedling length (93%) when exposed to a 1000 mg dm^{-3} solution [9]. As the *exo*-methylene-γ-lactone cnicin, isolated from the $CHCl_3$ extract of the aerial parts of *C. diffusa* [10], is the major metabolite, it was interesting to see if this sesquiterpenelactone had any effect on the germination of other species, as well as to elucidate its mechanism of action. In this sense, the germination of soybean (*Glycine max* (L.) Merr., cv. A7636 RG) and radish (*Raphanus sativus* L., cv. Sparkler), both dicotyledonous species, was studied using isothermal calorimetry.

---

* Corresponding author at: Paraguay 668, CP T 4107 DDN, Yerba Buena, Tucumán, Argentina. Tel.: +54 381 4247752x7070; fax: +54 381 4248169.
E-mail addresses: esigstad@arnet.com.ar, lizzie@fbqf.unt.edu.ar (E.E. Sigstad).

0040-6031/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.tca.2007.01.017

2. Experimental

2.1. Plant material

Seeds of radish (*R. sativus,* L. cv. Sparkler) were obtained from the local market and seeds of soybean (*Glycine max* (L.) Merr, cv. A7636 RG) were obtained from the Agro-Industrial Experimental Station 'Obispo Colombres', in Tucumán. The mean weights per seed were 19.3 ± 3.8 mg or 144.8 ± 37.8 mg for radish (two seeds) or soybean (one seed), respectively; their water content was 5.7 ± 0.1% and 11.0 ± 0.2%, respectively, as determined by drying at 95 °C until constant weight. The sesquiterpenelactone cnicin was obtained from the Natural Products group of the Organic Chemistry Institute of the Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucuman, Argentina. The isolation and structure of cnicin have been reported elsewhere [10].

2.2. Preparation of cnicin stock solution

Twenty-five milligrams of cnicin were dissolved in 2.5 cm^3 DMSO and diluted to 25 cm^3 with deionised water. Aliquots were taken to produce 10, 50 and 100 mg dm^{-3} solutions of cnicin containing 1% DMSO and used in the germination experiments on filter paper disks. For experiments with agar, 25 mg cnicin were dissolved in 2.5 cm^3 DMSO from which aliquots were taken to produce agars containing 10 and 100 mg dm^{-3} cnicin and 1% DMSO.

2.3. Calorimetric determinations

A twin heat conduction calorimeter (a non-commercial isothermal-type instrument built at Lund University, Sweden, 25 °C, 8 cm^3 vessel, 7 cm^3 headspace, at least 10 experiments per treatment) was applied to these studies as described elsewhere [11–13]. One (1) seed of soybean or two (2) seeds of radish were used for each calorimetric curve. In a previous work we reported that the best conditions to germinate seeds in a calorimeter were with a solid medium of 1% agar for imbibition [12]. It was also stated that this setting was convenient to control the concentration of a metabolic effector. To confirm that conclusion, soybean seeds were germinated by either using filter paper disks (treatment 1, T1) or 1 cm^3 of 1% agar (T2) in this work. For T1, the seed was placed on a filter paper disk wetted with 0.2 cm^3 of 10, 50 or 100 mg dm^{-3} solution of cnicin and a further addition of 0.2 cm^3 after 9–10 h of imbibition. For T2, the seed was inserted into 1 cm^3 of 1% agar containing 100 mg dm^{-3} of cnicin. Radish seeds with their flat shape were placed over the agar layer containing 10 and 100 mg dm^{-3} cnicin. Control experiments were performed with either water or agar containing 1% DMSO. When agar was used, the ampoule was removed from the calorimeter after 9–10 h, opened, covered with polyethylene, left for 5 min to exchange gases with the surrounding atmosphere and returned to the calorimeter. After thermal equilibration for 30 min, the power (p)–time (t) curves of seed germination were recorded and further processed as reported elsewhere [12].

Table 1
Mean (±S.D.) values of specific enthalpy of imbibition, $\Delta_i h$, time of germination, Δt_g, and specific enthalpy of germination, $\Delta_g h$, as determined for soybean (S) and radish (R) seeds under different concentrations of cnicin

Seed/cnicin (mg dm^{-3})	$-\Delta_i h$ (J g^{-1})	Δt_g (h)	$-\Delta_g h$ (J g^{-1})
Soybean seeds			
S/0	29.2 ± 4.6	18.8 ± 3.7	81.9 ± 21.2
S/10	31.7 ± 4.7	22.9 ± 3.3	93.4 ± 20.1
S/50	31.0 ± 6.3	22.9 ± 3.7	117.4 ± 24.2[a]
S/100	29.9 ± 4.3	28.3 ± 3.5[a]	116.4 ± 10.9[a]
S/Agar/0	27.62 ± 3.3	20.1 ± 2.6	80.6 ± 12.5
S/Agar/100	23.3 ± 7.1	21.1 ± 7.0	65.2 ± 8.1[a]
Radish seeds			
R/Agar/0	57.3 ± 6.8	30.6 ± 6.2	252.1 ± 111.6
R/Agar/10	54.4 ± 14.6	29.9 ± 5.5	178.8 ± 88.0
R/Agar/100	64.3 ± 8.7	29.6 ± 5.1	146.1 ± 22.5[a]

[a] Significant differences against the control ($p < 0.05$).

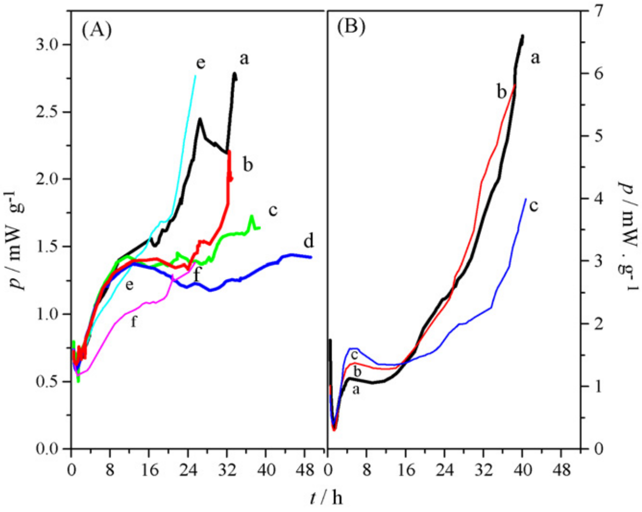

Fig. 1. The average specific thermal power (p)–time (t) curves of germination for (A) soybean seeds on filter paper disks wetted with 0.2 cm^3 and a further addition of 0.2 cm^3 after 9–10 h of: (a) distilled water; (b) 10, (c) 50 and (d) 100 mg dm^{-3} cnicin or inserted in 1 ml of 1% agar containing (e) distilled water or (f) 100 mg dm^{-3} cnicin and (B) radish seeds in 1 ml of 1% agar containing (a) distilled water, (b) 10 and (c) 100 mg dm^{-3} cnicin.

The enthalpy values shown in Table 1 were determined from the initial parts of the p–t curves in Fig. 1 as the area under the calorimetric curve for the indicated time period.

2.4. Germination in closed Petri dishes

Soybean seeds (10 × 3) were placed in Petri dishes (90 mm diameter) at 25 °C to germinate on filter paper disks wetted with 5 cm^3 water containing 1% DMSO (control) or 10, 50 and 100 mg dm^{-3} cnicin solutions with 1% DMSO. The percentage of germinated seeds, G (determined from the 30 seeds used for each treatment), root length, Lr, and seedling water content, WC, were determined at 72 h.

3. Results and discussion

The calorimetric experiments on germination had 10 replicates for each treatment and each concentration. Representative

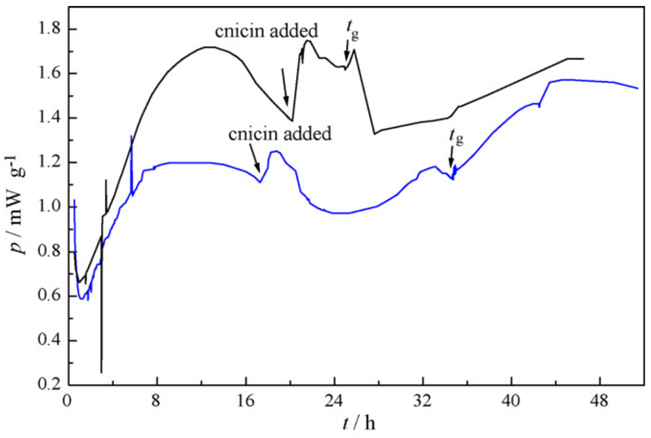

Fig. 2. Specific thermal power–time curves of soybean seed germination on filter paper disks wetted with 0.2 cm^3 of a 100 mg dm^{-3} cnicin solution and further addition of 0.2 cm^3 solution after 12 h.

curves are depicted in Figs. 1 and 2. The former shows the average specific thermal power–time curves of seed germination (A) for soybean seeds imbibed either by treatment T1 (curves a–d) or T2 (curves e and f) and (B) for radish seed imbibed on agar. A deep endothermic trough is seen – more or less distinctly expressed – in all curves. It is connected with the physico-chemical processes which occurred during wetting. After about 12 h the values of p for seeds under T1 (Fig. 1A, curves b-d) are lower than the control (Fig. 1A, curve a). The small differences observed for seeds under T2 in 100 mg dm^{-3} (Fig. 1A, curve f) with the control (Fig. 1A, curve e) during the first 4–6 h of the experiments become more pronounced after about 9 h. It is interesting to observe the differences in the p–t curves obtained with 100 mg dm^{-3} cnicin for the treatments T1 or T2 (Fig. 1A, curves d and f, respectively). In T2, the seed was inserted into the agar layer in such a way that three sides of the seed were exposed to imbibition.

In T1, a second addition of cnicin was necessary to maintain the moisture high enough until the end of experiments. If this addition were done after 12 h of imbibition – a time at which the values of p had either reached a steady state or were decreasing (Fig. 1A, curves b–d) – and the experiment was started again, a burst in heat production, p, was observed which decreased again after 1 or 2 h (Fig. 2). This thermal effect was not observed when water was added instead of the cnicin solution or when cnicin was added between 9 and 10 h of imbibition that means prior to soybean seed coat hydrolysis (12–13 h) [12]. Probably, the lactone interacts with proteins at the root membrane level; therefore, a further addition is seen in the curve as the heat involved in the interaction once roots enlarged to protrude from the seed. When the addition was done before 10 h of imbibition, the p–t curves with cnicin become lower than the control soon after it. In total, the p–t curves of the agar treatment T2 look smoother and with less fluctuation than those with filter disks T1 underlining the fact that it is the treatment of choice, easier in performance because there is no need for a second addition of the liquid.

The p–t curves for the germination of the radish seeds (Fig. 1B) show an initial decrease in heat production followed by an increase approximately returning to the initial level before reaching a steady state. After 16 h, the values of p for the seeds in the control and of 10 mg dm^{-3} cnicin (Fig. 1B, curves a and b, respectively) are similar and higher than those for seeds exposed to 100 mg dm^{-3} cnicin (Fig. 1B, curve c), indicating that their behaviour was similar to that of the soybean seeds.

Table 1 shows the values for the specific enthalpy changes of imbibition, $\Delta_i h$, determined from zero up to 9 and 14 h for soybean and radish, respectively, and the specific enthalpies of germination, $\Delta_g h$, determined from zero to the time of germination, Δt_g. They were calculated from each individual p–t curve of germination. We reported previously [12] that the water content, WC, of soybean seeds of the cultivar A7636 RG had a linear relationship with the corresponding specific enthalpy change, $\Delta_m h$, determined from the p–t curves between 3 and 9 h of imbibition. After 9 h, the roots of this cultivar were about to emerge and the specific enthalpy of imbibition, $\Delta_i h$, calculated up to this time accounted for the value calculated from imbibition p–t curves determined in the presence of the respiratory poison, KCN, after 19 h (the average time of root protrusion). Radish seeds have a similar relationship until 14 h of imbibition (results not shown) and therefore, the $\Delta_i h$ value for radish was calculated up to this time. Table 1 also indicates the differences between the results obtained for soybean seeds under protocols T1 and T2. Seeds germinated under protocol T1 have germination enthalpy values, $\Delta_g h$, for 50 and 100 mg dm^{-3} cnicin that are significantly higher than the control values. In contrast, the corresponding enthalpy value for 100 mg dm^{-3} under T2 was significantly lower than the control. The time of germination (Δt_g) was also significantly prolonged for seeds under T1 in 100 mg dm^{-3} cnicin. The higher values of $\Delta_g h$ calculated for seeds under T1 in 50 and 100 mg dm^{-3} cnicin than for T2 were probably due to the interaction of an increased amount of cnicin provided by the second addition of the lactone. It is reasoned that more channels were blocked and thus there was a delay in germination time. On the other hand, the lowered values of $\Delta_g h$ for the soybean seeds under T2 or those of the radish are due the lower p values observed with respect to the control after 9 and 16 h of imbibition, respectively. These investigations clearly indicate that the effect of cnicin on soybean seeds might involve a chemical interaction at the root level: probably the blockage of water channels by the reaction between the –SH group of the terminal cysteine residue constituting aquaporines [13] with the exomethylene group of the γ-lactone as previously reported for other exomethylene γ-lactones [14].

To confirm the blockage of water channels of roots in the presence of cnicin, soybean seeds were placed in Petri dishes for germination. As seen in Table 2, a non-significant decrease (17%) in the percentage of germinated seeds, G, was observed in 50 and 100 mg dm^{-3} cnicin with respect to the control. A plot of the root length values, Lr, against the final water content, WC, for the control, 50 and 100 mg dm^{-3} cnicin gave a straight line ($R^2 = 0.99$). The slope of this relationship indicated that Lr increased by 0.21 ± 0.02 mm mg^{-1} of water uptake. These results confirm that cnicin blocked the root water channels but had no effect during the previous germination processes. This was already found for the action of the whole extract of *C. diffusa* on quinoa seeds [8]. Moreover, results demonstrated the power

Table 2
Petri dish experiments of the percentage of germinated seeds, *G*, seed weight, SW, root length, Lr, and water content, WC (dW), of soybean seeds after 72 h from the onset of imbibition in a cnicin solution at 25 °C

Treatment	*G* (%)	SW (mg)	Lr (mm)	WC (mg)
Control	100	171.6 ± 14.0	68.2 ± 11.3	546 ± 114
10 mg dm^{-3}	100	170.4 ± 12.8	53.8 ± 18.2	537 ± 76
50 mg dm^{-3}	83.4	172.2 ± 17.8	33.4 ± 4.1[a]	414 ± 62[a]
100 mg dm^{-3}	83.4	164.0 ± 12.6	9.8 ± 2.4[a]	295 ± 37[a]

[a] Indicates a significant difference against the control ($p < 0.05$).

of calorimetry as a tool to elucidate the mechanism through which an allelochemical like cnicin interacts with seeds.

Acknowledgments

We thank Dr. César Catalán from the Organic Chemistry Institute, Faculty of Biochemistry, Chemistry and Pharmacy and Drs. Mario Fortuna and Elmira Riscala from Organic Chemistry, Faculty of Agronomy and Zootechnology, National University of Tucumán, Argentina for kindly providing us with cnicin. This work was financially supported by ANPCyT-UNT (PICTO 2004, No. 844-6) and CONICET.

References

[1] Inderjit, K.M.M. Dakshini, Ch.L. Foy (Eds.), Principle and Practices in Plant Ecology: Allelochemical Interactions, CRC Press, Boca Ratón, London, New York, Washington, DC, 1999.
[2] A.R. Putnam, J. Defrank, J.P. Barnes, J. Chem. Ecol. 9 (1983) 1001–1011.
[3] E.L. Rice, Allelopathy: an overview, in: G.R. Waller (Ed.), Allelochemicals: Role in Agriculture and Forestry (ACS Symposium series, no. 330), American Chemical Society, Washington, DC, 1987, pp. 8–22.
[4] A.R. Putnam, Chem. Eng. News 4 (1983) 34–45.
[5] Forest Service, USDA (United State Department of Agriculture), Botanical and Ecological Characteristics. Species*: Centaurea diffusa*, 2000.
[6] S. Hirsch, The impact of knapweed on Montana’s economy, Series Reports No. 355, North Dakota State University, Department of Agricultural Economics, 1996.
[7] T.J. Stohlgren, C. Crosier, G.W. Chong, D. Guenther, P. Evangelista, Plant Soil 277 (2005) 7–18.
[8] P. Fontana, E. Riscala, J. Trimarco, M. Krautmann, A. Fortuna, E.E. Sigstad XVIII Jornadas de la Sociedad de Biología de Tucumán, Book of Abstracts 2001.
[9] E. Riscala, Personal communication.
[10] A.M. Fortuna, E. Contrera de Riscala, C.A.N. Catalán, T. Gedris, W. Herz, Biochem. Syst. Ecol. 30 (2002) 805–808.
[11] E.E. Sigstad, F.E. Prado, Thermochim. Acta 326 (1999) 159–164.
[12] F.I. Schabes, E.E. Sigstad, Thermochim. Acta 450 (2006) 96–101.
[13] E.E. Sigstad, F.I. Schabes, Thermochim. Acta 349 (2000) 95–101.
[14] E. Rodríguez, G.H.N. Towers, J.C. Mitchell, Phytochemistry 15 (1976) 1573–1580.