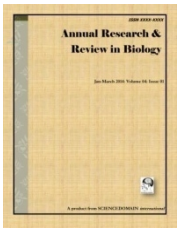

*Annual Research & Review in Biology*
*4(24): 3993-4003, 2014*

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# Persistent and Transient Inhibition of Seed Germination by Fractions of Methanol-Water Extract of Wheat Straw

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***Author's contribution***

*This whole work was carried out by author LSD.*

***Original Research Article***

***Received 13th June 2014***
***Accepted 8th July 2014***
***Published 19th July 2014***

## ABSTRACT

The objectives of this work were to investigate the effects of wheat straw constituents on total seed germination and on time needed for seed germination to start and to assess the usefulness of evaluating the recovery of germination to help guide the screening for a sequential search of bioactive natural phytochemicals. Dose-response bioassays of lettuce seed germination to increasing concentrations of six fractions of methanol-water extract were conducted and whenever inhibition of germination exceeded 90% non-germinated seeds were transferred to distilled water and recovery of germination investigated. Only the fractions essentially composed by neutral polar compounds and by acids failed to inhibit total germination. Fractions composed by strong acids, basic compounds and fatty acids plus steroids completely inhibited total germination at 5mg $mL^{-1}$, the former also inhibiting germination by more than 90% at 1mg $mL^{-1}$. In these three fractions the concentration necessary for 100% inhibition was estimated to range between 4.1 mg $mL^{-1}$ and 4.4mg $mL^{-1}$. Time needed for germination to start was always significantly increased even by fractions that failed to affect total germination. Basic compounds were the most effective in delaying the start of germination increasing it more than 4-fold at 2mg $mL^{-1}$ from 1.0 to 4.3 days. When non-germinated seeds were transferred to distilled water persistence of complete inhibition was only found with strong acids, almost full persistence with basic compounds. Thus the consideration of recovery of germination makes possible a more informed choice of the fraction to select for further bioassay guided search for phytochemical with herbicide activity. The fraction is the one composed by strong acids,

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mainly phenolic acids, all of them identified, provided that persistent seed inhibition is desired and long term weed control through depletion of weed seed banks in soil is aimed.

*Keywords: Allelopathy; fractions; germination; persistent effects; transient effects; wheat straw.*

## 1. INTRODUCTION

A high number of natural compounds from living or dead plant material are active on a large number of plant processes at various levels of organization [1,2]. Such chemicals may have a high potential to provide alternative and more natural ways of controlling and managing weed populations [3] and in the framework of allelopathic investigation the search for chemicals with herbicide activity has grown over the years. Microbes [4], fungi [5,6] and a variety of higher plants [7-9] have been investigated with that purpose.

Wheat and especially wheat straw has been intensively searched [10-12] generally by testing complete extracts. Water extracts of wheat straw have been found to be effective against a number of species sometimes with stimulatory activity [13] or more frequently inhibiting seed germination and seedling growth [13-15].

However bioassay-guided fractionation and structure optimization of selected compounds has been proposed and has received increasing attention [16-18]. In parallel, research has also been carried to establish more adequate and more rigorous bioassays and methods to investigate seed germination responses to allelopathins, namely in relation to the dependency of functional availability of phytochemicals on seed density [19,20], biologically meaningful parameterization of seed germination or growth over time [21,22] and the relevance of seed morphology including seed volume on seeds responses to allelopathic compounds [23].

Finding natural compounds that completely or almost completely inhibit germination of potential competitors like weed species has long been a goal of allelopathic research. However, if inhibition of germination is transient and disappears after the disappearance of the compounds responsible for it, the result will be the maintenance of a viable seed bank in soil requiring control of seed germination to be done year after year. Conversely, persistence of effects on germination after the inhibitory agents disappear would provide a much more efficient weed control because of the resulting progressive depletion of weeds seed banks.

Therefore this study was conducted 1) To investigate the effects of a series of concentrations of six different fractions of the methanol-water extract of wheat straw on total seed germination and on time needed for seed germination to start and 2) To assess the usefulness of evaluating the recovery of germination to help guide the screening for sequential search of bioactive natural phytochemicals.

## 2. MATERIALS AND METHODS

### 2.1 Wheat Straw Extraction and Fractioning

Extraction and fractioning of wheat straw followed the procedures described elsewhere [24]. Dried baled wheat straw was finely chopped, washed with water, extracted with MeOH:$H_2$,

the liquid filtered and dried. The dry residue was re-dissolved in water, mixed with $KHCO_3$ to pH 8 and extracted with diethyl ether. The aqueous layer was acidified to pH 1 with HCl and extracted with diethyl ether. The resulting aqueous layer will be referred to as fraction *A*, and the organic layer as fraction *B*. The ether fraction was extracted with $KHCO_3$, the resulting aqueous layer was acidified to pH 1 with HCl, extracted with diethyl ether, washed with water, yielding the fraction *C*, and the ethereal layer was re-extracted with NaOH. The resulting aqueous layer was acidified to pH 1 with HCl, extracted with diethyl ether and washed with water (fraction *D*) and the organic layer was extracted again with HCl. The resulting aqueous layer was made alkaline with NaOH, the solvent evaporated and dried over $P_2O_5$ giving the fraction *E*. Fraction *F* was obtained by washing the organic layer with water and evaporating the solvent.

## 2.2 Germination Bioassays of Fractions

Because of the small amounts of dried fractions (minimum of 59.3 mg in fraction *E*) lettuce (*Lactuca sativa* L.) was selected as target species because of the small size of its seeds and its adequacy for experiments of this kind [25]. An exploratory experiment in which four lettuce cultivars were compared showed a higher performance of cv. 'Iceberg' (data not shown) prompting for its use to evaluate the effects of fractions on seed germination. Solutions 5, 2, 1 and 0.5mg·mL$^{-1}$ were prepared by dilution with distilled water of a stock solution of dried aliquots of fractions *A*−*F* made of 1mL of methanol plus distilled water and bioassayed on germination of lettuce seeds cv. 'Iceberg'. In each treatment four 60-mm glass Petri dishes were fitted with Whatman® No. 1 paper, sown with 10 seeds of lettuce, and wetted with 1mL of the appropriate solution. Controls were prepared likewise with 1 mL of methanol and distilled water at the same rates as in fraction treatments. Seeds were incubated under constant 20ºC and 8h photoperiod provided by seven fluorescent tubes (Philips TLD 18W/84). Seeds were considered germinated if the radicle was at least as long as seed length. Germinated seeds were regularly counted and discarded during 8 days. Non germinated seeds of treatments with inhibition of total germination larger than 90% were washed with distilled water and transferred to newly prepared glass Petri dishes wetted with 1mL of distilled water, incubated as described above and germinated seeds were regularly counted and discarded during 8 more days to evaluate the persistence of inhibitory effects.

## 2.3 Statistical Analyses

Total germination ($G_T$) before seed transferal to distilled water to evaluate recovery of germination was investigated by exact or approximate two-tailed Student's *t* tests after checking for homocedasticity using the two-tailed *F* distribution.

Time-course of germination was modelled separately for each treatment using the three-term Weibull function [26], a highly flexible and useful equation to describe germination in phytotoxic and allelopathic studies [22], and can be expressed as:

$$G = 1 - \exp - \{ [ ( T - l ) / k ]^c \} \qquad \text{Eq. (1)}$$

Where G is the cumulative germination at time T as proportion of total germination $G_T$, *l* is a location parameter that for all practical purposes estimates the time at which the first seed germinates (lag of germination), *k* is a scale parameter with *l*+*k* estimating the time at which approximately 63% of cumulative germination occurred and *c* is a shape parameter.

To investigate the relationship between total germination ($G_T$) and concentration $G_T$ was normed so that the control was 100%. An exploratory visual examination of the responses of germination to the concentration of fractions strongly suggested that a sigmoidal model expressed as a modified Weibull equation (Equation 2) could adequately describe the relationship between total germination and fractions concentration and that a polynomial model could adequately describe the relationship between the lag of germination (*l*) and fractions concentration:

$$G_C = \exp - (C / k)^c \qquad \text{Eq.(2)}$$

Where $G_C$ is the total germination in percentage of control at concentration C, *k* is a scale parameter estimating the concentration at which $G_C$ is approximately 37% and *c* is a shape parameter.

Equations (1) and (2) were fitted by least squares nonlinear regression with replication with the Marquardt method [27]. Lack of fit was tested for *P*=.05, parameters and estimates were checked against original data, and coefficients of determination ($R^2$) are expressed as proportion of the maximum $R^2$ possible [28]. Polynomials were fitted by least squares stepwise linear regression without replication and an experiment-wise error rate for regression coefficients of .05 estimated by Dunn-Šidak method [29]. Coefficients of determination are presented after being adjusted to degrees of freedom ($R^2_{adj}$).

Recovery of germination was calculated in percentage as $100(G_R/G_F)$ with $G_R$ being the number of seeds that germinated after being transferred from treatments to distilled water and $G_F$ the number of non-germinated seeds in treatments before that transferal to distilled water [30]. Total germination and recovery of germination after seed transferal to distilled water was investigated by exact or approximate one-tailed Student's *t* tests after checking for homocedasticity using the two-tailed *F* distribution.

A significance level of *P*=.05 was used throughout and data is presented as mean ± standard error. Linear and nonlinear regression analyses and ANOVAs were done with Statgraphics 4.2 (STSC, Inc., Rockville, MD, USA), all other statistics with Excel®2010 (Microsoft Corporation).

## 3. RESULTS AND DISCUSSION

### 3.1 Total Germination

Data of total germination before transferal of non-germinated seeds to distilled water are summarized in Table 1. Total germination ($G_T$) was 97.5±1.2% in controls and significant inhibition of $G_T$ was found in all fractions except when seeds were treated with fractions *A* or *C* which are essentially composed by neutral polar compounds like sugars and amino acids or by acids respectively.

Conversely fraction *B* significantly inhibited seed germination at all concentrations tested while the remaining fractions (fractions *D*−*F*) significantly inhibited seed germination but only at the highest concentration tested. In addition complete inhibition of $G_T$ was found in seeds treated with fractions *B*, *E* and *F* but only at 5mg $mL^{-1}$.

**Table 1. Total germination of lettuce seeds treated with fractions of methanol-water extract of wheat straw**

| Concentration ($mg \cdot mL^{-1}$) | Fraction | | | | | |
|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** |
| 0 | 97.5±1.2 | 97.5±1.2 | 97.5±1.2 | 97.5±1.2 | 97.5±1.2 | 97.5±1.2 |
| 0.5 | 97.5±2.5 (≈1) | 85.0±6.5 ($.35\times10^{-2}$) | 100±0 (.38) | 100±0 (.38) | 97.5±2.5 (≈1) | 97.7±2.3 (.94) |
| 1 | 100±0 (.38) | 5.0±2.9 ($.17\times10^{-18}$) | 100±0 (.38) | 97.5±2.5 (≈1) | 90.0±7.1 (.07) | 100±0 (.38) |
| 2 | 92.5±4.8 (.16) | 2.5±2.5 ($.64\times10^{-19}$) | 97.5±2.5 (≈1) | 97.5±2.5 (≈1) | 42.5±7.5 (.05) | 92.5±2.5 (.11) |
| 5 | 92.5±2.5 (.11) | 0±0 ($.98\times10^{-20}$) | 65.0±10.4 (.05) | 25.0±5.0 ($.48\times10^{-15}$) | 0±0 ($.20\times10^{-24}$) | 0±0 ($.98\times10^{-20}$) |

*Values are means ± standard errors (in percentage). Between brackets are significance levels (P) of exact or approximate two-tailed Student's t comparisons with control. Sample size of controls n=20, otherwise n=4*

Equation 2 could always be fitted to the relationship between total germination normed so that control is 100% and concentration except for bioassays involving fraction *A* (Fig. 1). The coefficient of determination $R^2$ of equations ranged between 0.940 (fraction *B*) and more than 0.999 (fraction *E*) with a mean value (± standard error) of 0.985±0.011. Lack of fit was never significant (P≥.57) except for fraction *B* ($F_{2,31}$=51.41, $P$=$.14\times10^{-9}$).

According to the response of lettuce germination, fractions *B*, *E* and *F* are highly promising sources of compounds able to completely inhibit germination at concentrations lesser than 5 mg $mL^{-1}$. In the re-parameterized Weibull equation (Equation 2) the scale parameter *k* estimates the concentration at which total germination is approximately 37% which equates to the effective dose to reduce total germination by 63% ($IC_{63}$). However, despite that $IC_{63}$ or even the more common and less stringent $IC_{50}$ [18,31] might be a good result to temporarily reduce the density of weeds and thus their competition with crop species, it is hardly a desirable target if a more permanent control is desired because it does not prevent the new production of seeds and thus the partial or complete replenishment of weed seed banks.

Therefore $IC_{100}$ was deduced from fitted equations and concentrations for complete inhibition of germination of seeds by fractions were estimated by extrapolation to be 8.7mg $mL^{-1}$ in fraction *C* and 5.9mg $mL^{-1}$ in fraction *D*, and by interpolation to be 4.4mg $mL^{-1}$ in fraction *E*, slightly less (4.3mg $mL^{-1}$) in fraction *F* and 4.1mg $mL^{-1}$ in fraction *B*. Thus $IC_{100}$ values for fractions *B*, *E* and *F* lie within a very narrow range of concentrations. All considered, inhibition of total germination and $IC_{100}$ fraction *B* emerges as the most promising source for bioherbicides, followed by fraction *E* while fraction *F* ranks third.

Fraction *B* is composed by strong acids including phenolic acids, fraction *E* by basic compounds and fraction *F* by neutral compounds. According to published work [32] fraction *B* was in all likelihood essentially composed by phenolic acids which were very likely to include benzoic acid, *trans*-*p*-coumaric acid, *cis*- and *trans*-ferulic acids, fumaric acid, *p*-hydroxibenzoic acid, protocatechuic acid and vanillic acid. Similarly fraction *F* was in all likelihood composed by fatty acid methyl esters and steroids, which might include azelate, palmitate, oleate, linoleate, campesterol, cholesterol, ergosterol and several ergosterol-

derivatives, spinasterol and also a number of new or unusual ketosteroids [32,33]. So far no compounds have been identified in fraction *E*.

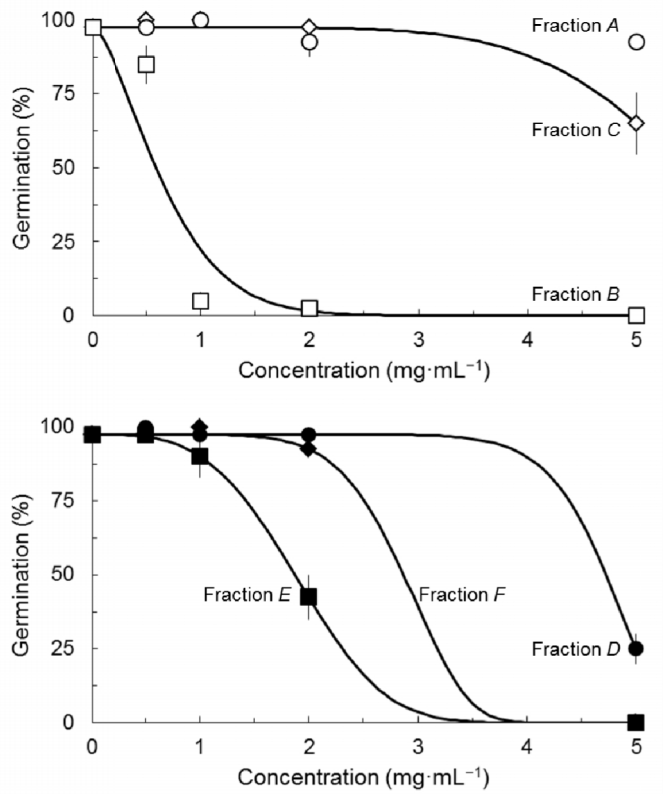


**Fig. 1. Expected (mean ± standard error) and observed effects of fractions of methanol-water extract of wheat straw on total germination of lettuce in percentage of control**

*Values for k and c in Equation 2 are: 0.773 and 1.499 (fraction B); 5.7996 and 6.106 (fraction C); 4.880, 12.643 (fraction D); 2.112 and 3.405 (fraction E); 3.041 and 7.046 (fraction F)*

## 3.2 Time-course of Germination

Germination over time could almost always be fitted by the three-term Weibull equation (Equation 1) except for concentrations 1 and 2 mg $mL^{-1}$ of fraction *B* in which $G_T$ was too low and naturally for concentration 5 mg $mL^{-1}$ of fractions *B*, *E* and *F* (Table 1). $R^2$ of Weibull equations ranged between 0.598 and 1 with a mean value (± standard error) of 0.958±0.002. Lack of fit was never significant ($P \geq .17$). Therefore the relationship between the lag of germination and concentration was investigated in all fractions except in fraction *B*.

Polynomials could always be fitted and $R^2_{adj}$ ranged between 0.879 (fraction *A*) and 0.983 (fraction *D*) with a mean value (± standard error) of 0.939±0.020. Significance levels of individual coefficients and models were always less than .01. Fractions *A*, *C* and *D* were fitted by straight line equations while fractions *E* and *F* required exponential forms (Fig. 2).

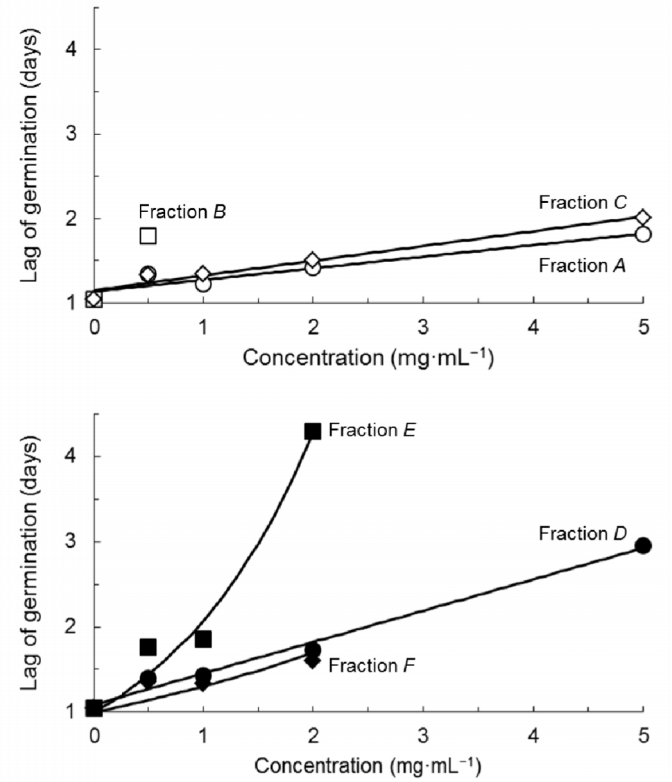


**Fig. 2. Expected and observed effects of fractions of methanol-water extract of wheat straw on lag of germination of lettuce seeds**

*Equations for fractions A, C and D are of the type Y= a+bX and values for a and b are: 1.137 and 0.137 (fraction A); 1.150 and 0.175 (fraction C); 1.087 and 0.368 (fraction D). Equations for fractions E and F are of the type Y=exp (aX) and values for a are: 0.727 (fraction E); 0.264 (fraction F)*

Lag of germination (*l*) increased with concentration in all fractions meaning that regardless of the sensitivity of total germination $G_T$ to fractions and concentrations, the time needed for the first seed to germinate increased always with concentration. This is especially noticeable in fractions *A* and *C* which failed to affect $G_T$ (Table 1) but at 5mg $mL^{-1}$ increased *l* two-fold from 1.0 to 1.8 days or 2.0 days respectively.

In relation to *l* fraction *E* was clearly the most effective significantly increasing it more than 4-fold at 2 mg mL−1 from 1.0 to 4.3 days with some tradeoff between inhibition and delay of germination. Again fraction *E* fares better than fraction *F*.

## 3.3 Recovery of Germination

Five treatments resulted in more than 90% inhibition of $G_T$ and were investigated for the persistence of inhibitory effects. Cumulative results after transferring non-germinated seeds to distilled water are shown in Table 2.

Except fraction *B* at 1mg $mL^{-1}$ in which complete recovery was observed, all other treatments inhibited lettuce germination at various rates after seeds were transferred to distilled water. However only seeds treated by fraction *B* at 5mg $mL^{-1}$ were permanently

inhibited after seeds were transferred to distilled water. Partial or almost full recovery was observed in the remaining treatments with fraction *E* at 5mg $mL^{-1}$ closely resembling fraction *B* at the same concentration.

In addition, recovery data of fraction *B* strongly suggests that persistence of inhibitory effects might be concentration-dependent. Thus strong acids and phenolic acids that constitute this fraction may be able to permanently inhibit seed germination at concentrations between 2 and 5mg $mL^{-1}$ but further research is needed to ascertain whether or not, and how, such concentration-dependency could be used to allow a broader range of options for concentrations aimed at specified levels of permanent inhibition of seed germination.

**Table 2. Total germination and recovery of non-germinated lettuce seeds treated with fractions of methanol-water extract of wheat straw after transferal to distilled water**

| Fraction | Concentration ($mg \cdot mL^{-1}$) | Germination (%) | Recovery (%) |
|---|---|---|---|
| Control | 0 | 97.5±1.2 | 0±0 |
| *B* | 1 | 94.7±3.1 (.19) | 94.4±3.3 ($.45\times10^{-4}$) |
| *B* | 2 | 62.2±9.8 (.02) | 60.7±10.8 (.01) |
| *B* | 5 | 0±0 (≈0) | 0±0 (≈1) |
| *E* | 5 | 7.5±4.8 (≈0) | 7.5±4.8 (.11) |
| *F* | 5 | 67.5±10.3 (.03) | 67.5±10.3 ($.36\times10^{-2}$) |

*Values are mean ± standard error (in percentage). Between brackets are significance levels (P) of exact or approximate one-tailed student's t comparisons with control*

Results of recovery of germination agree with and strengthen the choice of fraction *B* for further studies aimed at finding highly effective natural compounds against germination of weed seeds. The fact that fraction *B* is essentially composed by phenolic acids adds to the interest of its choice.

According to our data permanent inhibition of germination by fraction *B* should occur at concentrations between 4.1mg $mL^{-1}$ (estimated $IC_{100}$) and 5 mg $mL^{-1}$. Because fraction *B* is a mixture of compounds this means that each of the twenty compounds already identified in it are present in lower or much lower concentrations. Fraction *B* is a mixture mostly composed of phenolic acids which essentially have the same sites of activity thus implying that their combined action is antagonistic or at most, additive [34]. Implications are that a search for the individual compounds responsible for the effects of fraction *B* might result in finding phenolic acids permanently inhibitory of seed germination at very low dosages.

Also phenolic acids are known to degrade rapidly [35,36] which means that in the case of its use as weedicide there is a good chance that after provoking permanent inhibition of weed seeds they will rapidly decompose and disappear from soil.

## 4. CONCLUSIONS

Three of the six fractions of the methanol-water extract of wheat straw (fractions *B*, *E*, *F*) completely inhibited lettuce seed germination at 5 mg $mL^{-1}$ and one of them, fraction *B* essentially composed by phenolic acids also inhibited seed germination by more than 90% at 1mg $mL^{-1}$.

All fractions significantly increase the time necessary for the first seed to germinate with the most effective fraction (fraction *E*) being composed by basic compounds still unidentified.

The consideration of recovery of germination eliminates the appeal for fractions *E* and *F* as choices for further investigation because only fraction *B* was able to completely and permanently inhibit germination.

## ACKNOWLEDGEMENTS

Thanks are due to Elvira Gaspar (Chemistry Department, New University of Lisboa) for extraction and fractioning of wheat straw, to Gertrudes Mariano and Isabel Robalo (Biology Department, University of Évora) for technical assistance in bioassays, and to one anonymous referee for comments on the manuscript.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

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