Phytotoxins identified from Jerusalem artichoke (*Helianthus tuberosus* L.) residues and their potential inhibitory activity in the field and laboratory

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Abstract

Weed management systems often seek biological solutions to minimize the environmental impacts related to the use of herbicides in agricultural systems. The suppressive effects of allelochemicals can sometimes be used effectively to provide biological pest and weed control. Jerusalem artichoke has been reported to be a highly invasive weed in European cropping systems, but this plant is also gaining interest as a cultivated crop in Italy. The aim of this study was to investigate the impact of Jerusalem artichoke and its extracts upon the germination and growth of seedling weeds and crops. HPLC coupled to MS was used for identification of the compounds associated with the observed growth inhibition. The weed suppressive activity of local biotypes of Jerusalem artichoke on weed growth and establishment was also evaluated in a field experiment. Shoot extracts of the cultivar Fuseau were consistently most inhibitory to germination and seedling growth of lettuce, particularly the diethyl ether extract. Solvent partitioning of the aqueous extracts of dried artichoke shoots resulted in greater inhibition in comparison to the aqueous extract itself. The diethyl ether extract provided 2 fold greater growth inhibition, in comparison to the aqueous extract, at concentrations of less than 0.5 mg extract per Petri dish. HPLC MS evaluation of the diethyl ether extract led to the identification of salicylic acid (o-hydroxybenzoic acid), and the closely related compound p-hydroxybenzaldehyde, as well as minor quantities of o-coumarinic acid and coumarin in the active fraction inhibiting lettuce seedling growth. Field observations provided further evidence for the allelopathic potential of H. tuberosus residues, as significant weed growth inhibition was observed in artichoke-infested plots with soil-incorporated residues in comparison to non-infested field sites, both in terms of weed seedling emergence and growth.

KEYWORDS: Helianthus tuberosus; phytotoxicity; residue degradation; phenolics; plant invasion
1. Introduction

Weeds are one of the major constraints to crop yield and quality. In the EU, herbicides are typically used in most agricultural production systems, due to the increasing cost of labour for weed removal and the lack of inexpensive and effective weed control alternatives to herbicides. If the trend of growing reliance on herbicides for weed management is to be checked, more effective alternative weed management strategies are needed. Recently researchers are attempting to find biological solutions to minimize environmental impacts related to the use of pesticides in agricultural systems, including herbicide resistance in competing weeds (Xuan et al., 2005). In coming years, EU countries will have to introduce a directive (2009/128/EC) which is aimed at encouraging the introduction of cost affective alternative approaches for weed management and greater use of integrated crop management (Zaghi, 2009).

In recent years, allelopathy and allelochemicals have been investigated as environmentally sustainable methods for potential pest and weed control (Weston, 1996). Researchers have documented the potential of allelopathic plants to reduce soil pathogens as well as weed emergence and growth (Weston, 2005). The mode of action of allelochemicals has been evaluated in some detail (Duke et al., 2000) and most commonly several phytoinhibitors are active in plants as secondary metabolites, but this is not always the case (Petchey, 2003; Reigosa et al., 1999). As demonstrated in many field studies, allelopathy can play an important role in plant interactions and ecosystem patterning (Batish et al., 2001). Additional possibilities for utilization of allelopathy exist through crop selection to increase the competitiveness of crop cultivars, but this requires considerable time and monetary investment in plant breeding systems (Kong et al., 2002; Weston and Duke, 2003). In addition, weed suppressive green manures or residues can be introduced into the crop rotation, or allelopathic plants can be utilized as living mulches (Bertin et al., 2009).

*Helianthus tuberosus* L. (Jerusalem artichoke), is a member of the *Asteraceae* and was introduced into Europe in the late 1500s from North America. Over the centuries, Jerusalem artichoke gained
wide popularity as a cultivated crop across Europe, both for human consumption and as feed for livestock (Swanton et al., 1992). However, the economic importance of this species has declined remarkably in recent years, mainly due to the increased popularity of alternative tuber crops such as potato. Unfortunately, the easy vegetative propagation of Jerusalem artichoke by means of tubers and stolons has resulted in this species becoming invasive in varied environments and locations across Europe and it is now a significant weed of field crops (Török et al., 2003). This plant is also widespread in natural settings, especially along riverbanks (Schittenhelm, 1996). The formation of tall dense stands of *H. tuberosus* has been associated with the reduction of native taxa in Europe, resulting in the development of altered plant ecosystems in Austria, for example (Wadsworth et al., 2000).

When Jerusalem artichoke is cultivated, the emergence of volunteer shoots, despite their burial at a depth of 30 cm or more, may result in significant losses in crop yield and quality. In addition, these shoots and tubers can be widely spread by later cultivation. For example, 31 to 71% reductions in soybean yield were reported with only a moderate infestation of *H. tuberosus* tubers (Wyse et al., 1986). In field corn, a density of approximately four tubers m\(^{-2}\) caused a yield reduction of 16 to 25% (Wyse and Young, 1980). Surveys in Italian fields have shown that this noxious weed was present in field row crops across most of northern Italy, particularly in fallow crop fields or in areas where *H. tuberosus* was cultivated for production in previous years (Tesio et al., 2008). Given its extensive underground root system, herbicides for control of Jerusalem artichoke must effectively translocate a considerable distance through the plant to its fibrous roots and tubers (Wall et al., 1986).

Despite these invasive tendencies and its widespread occurrence as a weedy plant, Jerusalem artichoke is once again gaining interest in Europe as a food source for human consumption, as a raw material for different industrial uses including inulin production, a natural sweetener, or for ethanol for biofuel production. It can also be profitably grown as silage for livestock (Seiler, 1993; Seiler et
Plant breeders are also interested in the exploitation of its resistance genes to *Sclerotinia sclerotiorum* for the potential incorporation of this trait in commercial cultivars of sunflower (*H. annuus*) (Cassells and Walsh 1995).

Recent greenhouse and laboratory experiments showed the strong potential allelopathic activity of *H. tuberosus*, which was associated with its aqueous shoot extracts (Tesio et al., 2008), dried residues (Tesio et al., 2010; Vidotto et al., 2008) or root exudates (Follis et al., 2010). In particular, its impact on several weed and crop species was described under laboratory and greenhouse conditions. However, some variation in allelopathic interference was observed, which was dependant upon the cultivar of Jerusalem artichoke evaluated (Tesio et al., 2010).

We present the results of studies performed under laboratory conditions to assess the impact of Jerusalem artichoke extracts upon the germination and growth of lettuce, a fast germinating species often utilized in laboratory bioassays to assess growth inhibition. High pressure liquid chromatography coupled to mass spectrometry was used for isolation and identification of allelochemics responsible for the observed growth inhibition in laboratory assays. We also evaluated the activity of local Italian biotypes of Jerusalem artichoke under field conditions to assess impacts of artichoke establishment and soil incorporation of residues upon weed growth and establishment, directly after incorporation to determine if measured field responses also support the hypothesis that Jerusalem artichoke residues are indeed allelopathic in typical field settings.

### 2. Material and Methods

#### 2.1. Plant material

Multiplication of all vegetative material evaluated in laboratory experiments occurred in a series of collaborative studies performed at Cornell University’s glasshouses located in Ithaca NY USA in 2005 through 2006. Tubers of wild *Helianthus tuberosus* L. (hereafter indicated as “Italian” population) were collected during August 2005 in heavily infested corn fields in Northwestern Italy,
outside of Torino Italy. In addition, tubers of cultivated varieties “Fuseau” and ”Stampede” were purchased from Ronninger Potato Farm (Austin, CO 81410) in the USA. After receiving all tuber genotypes, including the Italian population, tubers were transplanted into plastic pots (30 cm diameter) filled with commercial potting media (Metromix 360). Pots were placed in a greenhouse maintained at daily temperatures of 23 to 30°C, with no supplemental lighting. Plants were watered overhead as needed and fertilized daily with soluble fertilizer (NPK 21-5-20). *H. tuberosus* shoots were harvested periodically (on a monthly basis) by cutting stalks 10 cm above the soil surface, and selecting healthy individuals. The leaves were immediately separated from the stalks and dried in open trays in the laboratory drying oven at 40°C. Dried material was stored in tightly closed plastic containers to maintain dryness until further extraction.

2.2. Extractions of plant material

Extractions were performed in both the USA and Italy with dried leaf tissues, previously powdered using a small electric coffee grinder. Approximately 45g of dried leaf tissue was extracted in a flask containing 800 mL of distilled water on an orbital shaker, for 24 hours, in a 4°C cold room, in order to avoid fermentation or microbial growth. The extract was subsequently filtered through four layers of cheesecloth and centrifuged for 20 minutes at 10,000 rpm to remove particulate material. A subsequent three step vacuum filtration was performed through Whatman No. 4, 1 and 42 filter paper, respectively. Part of the filtered aqueous extract was stored in a refrigerator at 4°C until further bioassay, while the remaining portion was used for further solvent extraction. The clear aqueous extract was subsequently partitioned with less polar solvents including hexane, ethyl ether, and ethyl acetate. The aqueous extract was partitioned with 1250 mL of each solvent (five times with 250 mL). Solvent was subsequently removed from each fraction by rotary evaporation at 30°C and the dried material was weighed and. Dried fractions were stored at -20°C in darkened containers to avoid light and temperature degradation.
2.3. Bioassay
The evaluation of potential growth inhibition was carried out by performing a bioassay with the solvent extracts. The water extract and the three organic extracts were redissolved with their own solvent used for the extraction, to obtain a dilution of 1 μg dry residue / mL solvent. The bioassay was carried out utilizing glass Petri dishes (60 x 150 mm) lined with No. 1 Whatman filter paper, to evaluate phytotoxicity of all extracts on germinating lettuce seeds. The extracts were formulated at 1, 0.5, 0.25 and 0.125 μg/mL, in order to obtain a corresponding quantity of 1, 0.5, 0.25 and 0.125 μg dried residue per Petri dish. Controls consisted of Petri dishes containing only water, an additional control containing 1 mL of each solvent used for resuspension of the dried extract. Solvents were allowed to evaporate from each Petri dish treatment, and 1.5 mL of distilled water was then added to each dish. Ten seeds of lettuce (cv. Meraviglia d’inverno) were placed in each Petri dish. Seeds were surface sterilized by placing them in a 50% (v/v) solution of ethanol / distilled water for 1.5 min. Treatments were replicated 4 times and arranged in a completely randomized design in a closed seed germination box maintained in the dark at room temperature. After seeding, dishes were sealed with Parafilm and maintained for 6 days at ambient temperature (25°C). Total germination, as well as shoot and root elongation were measured at this time.

2.4. Purification of extracts
Leaf tissue extracts of Jerusalem artichoke cultivar Fuseau were selected for further fractionation based on greatest assessed inhibitory activity to lettuce seed germination and growth. Extracts were fractionated using liquid-liquid partitioning and column chromatography for further purification. Column chromatography was performed on silica gel (BDH 60-120 mesh) using various eluting solvents with increasing polarity ranging from: chloroform, 19:1, 9:1 and 5:1 chloroform-methanol (v/v), followed by methanol. Pressure for the column was provided by a laboratory compressed air pump that was adjusted to provide a solvent flow rate of 4 ml / min. Each active fraction was loaded on the column separately and eluting fractions of approximately 20 ml each were collected. Fractions collected were further evaluated by thin layer chromatography (TLC - silica gel
developed with 7:1 chloroform-methanol (v/v), and similar fractions were combined on the basis of their TLC profile. Each fraction was bioassayed for inhibitory activity at 250 μg and 500 μg dried material / Petri dish, as described above, using the lettuce seed germination and growth bioassay.

2.5. Identification of allelochemicals

The most toxic fractions were combined and chromatographed again by silica gel TLC (Whatman LK5F plates) using a eluent system of 7:1 chloroform-methanol (v/v). Visualization under UV (254 nm) light revealed 8 distinct bands. Eight bands were scraped off developed thin layer plates (3 in total) and corresponding separate bands of all plates were combined across plates, in order to have sufficient material for the following analyses of each of the 8 fractions. Silica was separated from the extracts through a fine fritted glass filter.

The chemical nature of the phytotoxic and potentially allelopathic compounds were examined using high-pressure liquid chromatography (HPLC) and liquid coupled to mass-spectrometry (LC-MS/MS).

Analysis of the extracted bands was conducted using a Hewlett Packard 1050 system, equipped with a reverse phase C18 column (Novapack, 3.9 x 1.5 mm). Each band was redissolved in 65% acetonitrile / 35% water acidified with 2.5% acetic acid (v/v), and then filtered as above. The isocratic mobile phase was 60% water acidified with 2.5% acetic acid / 40% acetonitrile (v/v), with an injection volume of 50 μl. The UV detector wavelength was set at 254 nm. Standards for comparative analysis included the following phenolic compounds: p-hydroxybenzoic acid, p-hydroxybenzaldehyde, salicylic acid, coumarin, o-coumarinic acid, and p-coumaric acid by comparing the retention times of the standards with those obtained from separation of active extracts by HPLC.

An LC System (Agilent 6410 QQQ), equipped with a Phenomenex Synergy 4μ Fusion RP-80° 150x4.60 mm was used for LC-MS/MS analysis to confirm identification of the compounds detected with the HPLC, and to quantify their presence in each of the 8 distinct fractions collected.
from preparative TLC. The parameters associated with the chemical standards are reported in table 1. The mobile phase utilized was 25% water and ammonium acetate (1 nM), and 75% methanol. The injection volume was 1 μl, run time was 40 min, with a post run wash of 0.5 min. All chemical standards utilized were purchased from SigmaAldrich as high purity standards and all solvents were HPLC spectral grade. The entire analysis using HPLC-MS was repeated twice.

The concentration of the compounds detected with the HPLC-mass spectrometry was determined by comparison of peak area obtained with those of the standards:

\[ y = \frac{A \times V}{W} \]

where \( y \) is the concentration of the compound in the band, expressed in mg / kg; \( A \) is the peak area formed by the standard and; \( V \) is the final volume of the standard (mL); \( W \) is the weight of the band analyzed.

2.6. Observations on allelopathic potential of *Helianthus tuberosus* residues under field conditions

Experimental observations were carried out from March 2009 to June 2010 in a field located in Valle Sauglio (TO, northwestern Italy), to weed suppressive or allelopathic effects of Jerusalem artichoke residues at a field scale. Soil type was a sandy-loam textured alluvium soil (Typic Udifluvents). The soil was previously ploughed and cultivated with a rotary harrow, and 0.5 t ha\(^{-1}\) of mineral fertilizer N-P-K 5-10-25 was broadcast before Jerusalem artichoke tuber transplanting. In the previous year, potatoes were produced at this site. The experiment consisted of two main treatments, tubers present and tubers absent. Locally collected *H. tuberosus* tubers were transplanted during March 2009 in plots consisting of two parallel rows of 45 m length with 0.8 m between rows to provide a final density of approximately 3 tubers / m\(^2\). The control treatment consisted of plots of similar size in which no tuber transplanting was performed. The *H. tuberosus* plants developed uniformly over time during the summer months, and invading weeds were
controlled with several applications of rotary harrowing between rows of the planted Jerusalem artichokes. Tuber harvest occurred during October 2009 by digging the tubers with a hoe, and the plant and root residues were ploughed into the soil at a depth of about 20 cm. In the following growing season, tuber transplanting occurred on April 10\textsuperscript{th} 2010 in the same plot areas planted the previous year. Weed biomass was assessed two separate times in the second year (2010) in terms of weed density and percentage of soil cover on May 15\textsuperscript{th} and June 12\textsuperscript{th}. After each survey a rotary harrowing between rows was conducted to remove above ground plant material, including that of *H. tuberosus* itself. An assessment of weed seedling establishment was performed at each rating in treated and untreated plots (with and without presence of Jerusalem artichokes). Any remaining above ground artichoke biomass was periodically removed from the field in order to try to eliminate any further competitive effects of artichoke regrowth and establishment upon successive weed establishment, before the second weed survey. Data was collected for 5 replications per treatment at both weed sampling dates.

2.7. Statistical analysis

The data obtained from the bioassay of solvent extracts were analyzed using ANOVA analysis (SPSS version 16) to compare the activity of the solvent extracts with the activity of the water controls.

All combinations of cultivar and solvent were subjected to ANOVA analysis using the free software R (agricolae package) to evaluate the effect of extract rates upon lettuce seedling growth. After ANOVA, the combinations that were statistically significant (*H. tuberosus* extracts with P >= 0.05), were subjected to regression analysis using R software (drc package) (Cedergreen et al., 2005; Ritz and Streibig, 2005). A logistic 3 parameters model was adopted for the regression analysis:

\[
y = \frac{1}{1 + \left(\frac{x}{EC50}\right)^b}
\]
where \( y \) is the value, \( b \) is the slope of the curve, \( EC50 \) is the extract rate at the point of inflection halfway between the upper and the lower (equal to 0) asymptotes, and \( x \) is the extract rate. With the curves obtained, \( GR_{50} \), \( GR_{80} \) and \( GR_{90} \) values were calculated for each combination of cultivar and solvent extract. These values represent the extract treatment or allelochemical rates required to obtain a germination or growth reduction of 50%, 80% and 90%, respectively.

In the second bioassay, after additional purification of the bioactive extract, any potential negative effects of purified fractions on lettuce germination and growth were tested, and a t-test was performed to evaluate the effects of the extract rate of each sample obtained from TLC fractionation in comparison to controls.

In the field experiment, differences in weed density, soil cover and weed composition were detected between treatments using a t-test, using the statistical software SPSS (version 16).

3. Results

3.1. Bioassay of solvent extractions

A comparison of the results obtained with the untreated control (water only) and the solvent controls (where solvent only was evaporated from each Petri dish before addition of water) demonstrates the absence of any significant negative effect due to the presence of any solvent residues remaining after hexane, ethyl ether and ethyl acetate evaporation (Figure 1). Therefore, preparation of extracts in solvents and removal of solvent by evaporation before seeding should not present any confounding effects in the lettuce assay utilized in these studies.

In a comparison of the tested populations or cultivars of Jerusalem artichoke, Fuseau extracts were most effective in reducing lettuce germination and growth. With increasing rates, all cultivar extracts showed increasing growth inhibition. Extracts of the local Italian population were also generally active on the indicator species; however, the ethyl acetate extracts had limited activity when compared to hexane and ethyl ether extracts. In regards to the Stampede cultivar, the original
aqueous extract was not inhibitory, while ethyl ether extract obtained from partitioning the aqueous extract affected all measured parameters. Lettuce shoot length was inhibited by both hexane and ethyl acetate extracts.

When considering all populations, the ethyl ether extracts generally showed the greatest inhibitory activity on lettuce seedling growth and germination, in comparison to other solvent extracts. Seedling radical elongation was generally more inhibited by the presence of extracts in comparison to shoot elongation.

When the crude aqueous extract of the Italian population was evaluated, the calculated GR$_{50}$, GR$_{80}$ and GR$_{90}$ (Table 2) for lettuce germination and shoot length were always higher than the tested rates of solvent extracts of the Italian aqueous extracts (at concentrations ranging from 0.125 to 1 mg), indicating that inhibition was limited for solvent extracts of Italian Jerusalem artichoke at these concentrations. However, the crude aqueous extract remained active and showed an 80% reduction in radical elongation at a concentration of 1 mg / dish.

In comparison to the Italian population, the aqueous extract of cultivar Fuseau inhibited both radical and shoot length by 50% and 80% with 0.27 and 0.64 mg of extract, respectively (Table 2). Reductions of 90% were obtained with both hexane and ethyl ether fractions on all parameters measured. Surprisingly, low concentrations of less than 0.08 mg per dish resulted in up to 50% inhibition of lettuce seedling growth. A quantity of 1 mg / dish ethyl acetate extract resulted in up to 80% germination reduction, and significant reduction of radical and shoot length.

The hexane and ethyl ether extracts of cultivar Fuseau showed considerable phytotoxic activity on lettuce germination and growth in comparison with the water extract. On average, lettuce shoot length was the most significantly affected parameter by $H. tuberosus$ extracts obtained from the population Fuseau, although radical elongation and seed germination were also impacted.

The extracts obtained from the cultivar Stampede did not show strong phytotoxicity to lettuce seedling growth, as observed previously in laboratory assays (Tesio et al., 2010). Among the tested
fractions, only the ethyl acetate extracts inhibited lettuce germination, with GR values lower than 0.2 mg / dish (Table 2).

However, the ethyl ether fraction was highly inhibitory to radical elongation, with 90% reduction observed with 0.80 mg of extract. Calculation of GR80 and GR90 values for shoot length reduction with the hexane fraction was not possible because an accurate regression could not be calculated as estimated response did not result in 80% or greater reduction of lettuce seedling growth.

3.2. Fractionation of extracts

Evaluation of the solvent mixture controls versus the water controls utilized in the bioassay (Table 3), indicated that neither phytotoxic or stimulatory effects of the purified fractions were caused by the solvent evaporation from the bioassay dishes.

The purification of the extract with greatest overall inhibitory activity, cultivar Fuseau, resulted in 5 distinct samples on the basis of the TLC profile. Sample 1 showed a highly significant stimulation of root growth at both tested rates (Figure 3); however, no effects were observed on lettuce germination (Figure 2) and shoot length (Figure 4). Sample 2 inhibited shoot length, with a reduction of about 15% at both concentrations evaluated. Greatest germination inhibition was observed with sample 3 (about 70%), and 0.5 mg rate resulted in greatest inhibition of radical elongation. Samples 4 and 5 were not generally inhibitory to radical elongation and were less inhibitory to lettuce germination.

Germination was generally the parameter most affected by further sample fractionation (Figure 2). Conversely, radical elongation seemed to be influenced mainly by fractions 1, 3 and 5 only (Figure 4).

Overall greatest inhibition was observed with samples 2 and 3. These two samples were combined and used for the further evaluation with HPLC coupled to MS.
3.3. Identification of allelochemicals

Analysis of the collected bioactive fractions by HPLC resulted in separation and identification of \( p \)-hydroxybenzaldehyde; salicylic acid, cinnamic acid, \( o \)-coumarinic acid; \( p \)-coumarin acid; and coumarin by comparing retention times with chemical standards, as well as co-elution with the same standards. Using LCMS, salicylic acid was found to be most prevalent in all fractions, and ranged in quantity from 2.57 mg / kg to 22.46 mg / kg dried plant material (Table 4). The second most prevalent phenolic was \( p \)-hydroxybenzaldehyde, with a minimum amount of 1.21 mg / kg in band 5, and a maximum of 10.59 mg / kg in band 3. \( O \)-coumarinic acid was found in four bands out of eight (2, 3, 5, and 6), while only a minor amount of \( p \)-coumaric acid was found in bands 4 and 7. In band number 8 a small quantity of cinnamic acid was found (0.5 mg / kg), and bands 1, 2, 3, 4, 5, and 7 contained traces of coumarin. This result might be due to the fact that it occurs at concentrations below detector thresholds. Out of the six acids identified, the presence of three compounds were identified in five bands (2, 3, 4 5 and 7).

3.4. Observations on allelopathic potential of Helianthus tuberosus residues under field conditions

The weed composition observed in the field was characterized by the typical weedy flora recorded in the cropped horticultural systems of northern Italy. Among the dicot weeds, the most commonly observed species were Galinsoga ciliata (Raf.) Blake, Chenopodium album L. and Portulaca oleracea L., while Digitaria sanguinalis (L) Scop. was the most common grass weed (Table 5).

Total weed seedling density was inhibited by approximately 60% and 70% during the first and second survey, respectively (Figure 5) in the plots in which \( H. \) tuberosus residues were planted. During the first survey (May 15\textsuperscript{th}) total biomass cover was inhibited by 65% (Figure 6). On June 12\textsuperscript{th}, total biomass coverage from weeds was not significantly influenced by the presence of residues, but was lower than that encountered in control plots. When evaluating individual species composition in the HELTU plots, \( D. \) sanguinalis and \( G. \) ciliata were strongly affected by previous
establishment of *H. tuberosus* with a reduction of 95% and 68%, respectively (Table 5). Differences observed in other weed species such as *A. retroflexus, C. album, C. arvensis, P. oleracea,* and *S. media,* even if not individually significant likely contributed to the significant difference in total weed reduction.

4. Discussion

Powdered dried leaves of Italian, Fuseau and Stampede cultivars were evaluated for their phytotoxicity by performing sequential solvent extractions. Extracts were tested for their phytotoxicity on germinating lettuce seeds in the laboratory. Fuseau extracts were consistently the most phytotoxic to germination and seedling growth of lettuce. The GR\textsubscript{50}, GR\textsubscript{80} and GR\textsubscript{90} values calculated for all cultivars from regression analysis showed that the highest suppression was obtained with the ethyl ether fraction, and the cultivar Fuseau in particular. The logistic model adopted properly fitted the data of all solvent fractions of this cultivar, and showed increased inhibition directly correlated with increasing rate of *H. tuberosus* extract. The toxic effects of the extracts were enhanced in the solvent extraction experiment, as only 0.28 mg / dish of Fuseau ethyl ether extract were needed to obtain 90% inhibition of germination, and 0.27 and 0.22 mg for 90% root and shoot length reduction, respectively. The separation and purification of the extracts, performed sequentially through the use a silica column for preparatory chromatography, was followed by thin layer chromatography of most active fractions. Separation using silica gel column and thin layer chromatography lead to the procurement of a highly active set of fractions containing numerous biologically active phenolic compounds. The GR values calculated for purified solvent fractions also showed more potent values than the GR values obtained for initial aqueous extracts of each Jerusalem artichoke cultivar or population.

High pressure liquid column chromatography coupled to mass spectrometry was utilized to further separate and evaluate chemical constituents in the phytotoxic Fuseau fractions under evaluation for allelopathic activity. HPLC separation coupled to UV detection lead to the identification of salicilic
acid (o-hydroxybenzoic acid), and the closely related compound p-hydroxybenzaldehyde, as the key potential allelochemicals in Jerusalem artichoke shoot extracts. In addition, significant amounts of o-coumarinic acid were also detected, along with trace levels of coumarin. These phenolic compounds have all been associated with allelopathic activity of other plant residues or extracts, and have been shown to be present in a number of plant species associated with allelopathic or plant growth inhibition (Inderjit, 1996) coumarin in lavender (Haig et al., 200; Weston et al., 1989).

Additional field observations also provided further evidence for the allelopathic potential of H. tuberosus residues, as significant weed growth inhibition was shown in infested plots in comparison to non-infested field sites, both in terms of weed seedling emergence and growth. In Northern Italian fields where Jerusalem artichoke is commonly found as an invasive weed, D. sanguinalis and Galinsoga ciliata were most affected by the presence of decomposing residues (Tesio et al., 2010). In this field site, the tubers as well as the leaves, considered the most toxic part of the plant, were tilled into the soil prior to weed growth evaluation. The effects reported in this study, together with the observations reported from previous experiments (Tesio et al., 2010; Vidotto et al., 2008) of Jerusalem artichoke residues upon seedling germination and growth may help to explain the ecological dominance of this plant in natural and agricultural environments. The recent spread of H. tuberosus in cultivated fields across Europe and Italy in particular, may be associated with its ability to interfere with the growth of both crops and weeds, not only by allelopathic effects, but its ability to reproduce vegetatively by tubers, and produce large quantities of biomass in a short period of time (Schnitzler et al., 2007). In natural environments, H. tuberosus has been noted as a serious weed, and when cultivated in high density in agricultural settings, it is known for the production of great quantities of residue. Dense plantings of cultivated Jerusalem artichoke can also affect the establishment and growth of subsequent crops, especially by impacting the establishment of a crop that is highly sensitive to its decomposing residues (Vidotto et al., 2008).
Ben-Hammouda et al. (1995) evaluated the chemical basis for the allelopathic potential of *Sorghum* hybrids and reported that the total concentration of phenolic acids (*p*-hydroxybenzoic acid, vanillic, syringic, *p*-coumarin and ferulic acid) was positively correlated with the allelopathic potential. However, the relationship observed in sorghum hybrids was more qualitative than quantitative. Therefore, in this case it is important to investigate the activity of a mixture of potentially active phenolic compounds rather than just a single allelochemical, as well as activity in multiple cultivars.

Salicylic acid and *p*-hydroxybenzaldehyde have been widely reported as inhibitors of weed germination and growth (Inderjit, 1996; Jung et al. 2004; Patterson, 1981). Previous studies on the activity of phenolic compounds reported that the application of 56 kg ha\(^{-1}\) of salicylic and *p*-hydroxybenzoic acids and umbelliferone to the soil reduced shoot dry weight of several crop and weed species, while at about 11 kg ha\(^{-1}\), none of the chemicals inhibited soybean growth (Shettel and Nelson, 1983). Although the rates generally used in experimental settings are significantly higher in comparison to those recommended for most commercial herbicides, several phenolics have been extracted from different soils containing allelopathic residues at high concentrations in the soil solution (Whitehead, 1964). It has been shown that variable concentrations of phenolics exist in regions of the soil in which plant residue has accumulated. Allelochemicals, including simple phenolic acids, may be rendered less active in soil because of adsorption to soil colloids and organic matter (Blum et al., 1999). Previous experiments carried out by the authors also showed that soil incorporation of 2 t ha\(^{-1}\) of *H. tuberosus* dried residues inhibited germination and growth of seeded crop plants including rice, tomato, winter wheat, and zucchini crops, as well as *Digitaria sanguinalis*, *Echinochloa crus-galli* and *Solanum nigrum* weeds (Vidotto et al., 2008).

The present study has shown that *H. tuberosus* shoot extracts contain significant amounts of bioactive phenolic compounds that could be associated with plant growth inhibition in field situations. Our results are in general agreement with other studies investigating the allelopathic potential of other species in the Compositae family (Chon et al., 2003), or with the *Helianthus*
genus, in particular (Azania et al., 2003; Hall et al., 1982; Saggese et al., 1985). These results, together with those obtained with the previous studies on *H. tuberosus* dried residues, provide ecologically relevant evidence for the phytotoxicity of these residues and potential for allelopathic activity of this species.

The results of several recently conducted experiments showed differential toxicity of the compounds obtained from the allelopathic species. Activity was dependant upon the rate and method of chemical application. Compared to commercial herbicides, higher rates of allelochemicals or natural plant extracts are generally required to inhibit plant growth. Therefore, it is improbable that the chemicals identified in *H. tuberosus* residues would be useful as field-applied herbicides. However, it is possible that in certain agricultural settings, phenolic compounds could accumulate due to residue decomposition and allelochemicals released could selectively inhibit weed species. Selective inhibition of weed growth would depend upon concentration and distribution of these chemicals in the soil rhizosphere, as well as the crops following in the crop rotation. In any case, our studies also provide additional evidence for the role of Jerusalem artichoke as an increasingly important invasive weed of agricultural settings, and while its residues might be associated with weed suppression over time, its presence may also lead to subsequent interference with agronomic crops as well.

**References**


Figure captions

**Figure 1.** Effects of the different solvent controls on germination, root and shoot of lettuce. All values are expressed as percent of untreated (black line) (± SE, n = 9).

**Figure 2.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce germination. Bars are standard error (n = 4). * refers to significant differences from the control treatment with p <= 0.05 or ** with p <= 0.01 (independent sample t-test) All values are expressed as percent of control treatment (black line).

**Figure 3.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce root growth. Bars are standard error (n = 4). * refers to significant differences from the control treatment with p <= 0.05 or ** with p <= 0.01 (independent sample t-test). All values are expressed as percent of control treatment (black line).

**Figure 4.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce shoot growth. Bars are standard error (n = 4). * refers to significant differences from the control treatment with p <= 0.05 or ** with p <= 0.01 (independent sample t-test). All values are expressed as percent of control treatment (black line).

**Figure 5.** Effect of *H. tuberosus* residues into the soil on total weed density. Bars represent the standard errors (n=4). Letters refer to significant differences (T-test P <= 0.05).

**Figure 6.** Effect of *H. tuberosus* residues into the soil on soil cover caused by all weed species. Bars represent the standard errors (n=4). Letters refer to significant differences (T-test P <= 0.05).
### Tables

**Table 1.** Phenolic compounds detected by LC MS and parameters utilized for detection.

<table>
<thead>
<tr>
<th>Name</th>
<th>Precursor Ion</th>
<th>Product Ion</th>
<th>Polarity</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-hydroxybenzaldehyde</td>
<td>142.16</td>
<td>93.0</td>
<td>negative</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>salicylic acid</td>
<td>134.12</td>
<td>92.9</td>
<td>negative</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>cinnamic acid</td>
<td>148.12</td>
<td>103.0</td>
<td>negative</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>$o$-coumarinic acid</td>
<td>164.16</td>
<td>118.9</td>
<td>negative</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>$p$-coumaric acid</td>
<td>164.16</td>
<td>119.1</td>
<td>negative</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>coumarin</td>
<td>146.14</td>
<td>100.5</td>
<td>negative</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2. Values of GR<sub>50</sub>, GR<sub>80</sub> and GR<sub>90</sub> expressed in mg, of the fractions extracted with water (W), hexane (H), ethyl ether (EE) and ethyl acetate (EA) from Italian, Fuseau and Stampede (± SE with n = 4), on the combinations of Cultivar, solvent and parameter resulted significant (P> 0.05).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Solvent</th>
<th>Parameter</th>
<th>Sig (F)</th>
<th>GR&lt;sub&gt;50&lt;/sub&gt;</th>
<th>GR&lt;sub&gt;80&lt;/sub&gt;</th>
<th>GR&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italian</td>
<td>W</td>
<td>Germination</td>
<td>0.0001</td>
<td>1.30 ± 0.070</td>
<td>1.58 ± 0.216</td>
<td>1.76 ± 0.336</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0005</td>
<td>0.28 ± 0.147</td>
<td>0.99 ± 0.517</td>
<td>2.09 ± 1.532</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0229</td>
<td>1.14 ± 0.265</td>
<td>1.83 ± 0.882</td>
<td>2.41 ± 1.653</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>Germination</td>
<td>0.0000</td>
<td>0.52 ± 0.092</td>
<td>1.28 ± 0.365</td>
<td>2.17 ± 0.850</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0000</td>
<td>0.59 ± 0.107</td>
<td>1.26 ± 0.882</td>
<td>1.97 ± 0.797</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0002</td>
<td>0.61 ± 0.133</td>
<td>1.30 ± 0.456</td>
<td>2.00 ± 0.930</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>Germination</td>
<td>0.0000</td>
<td>0.80 ± 0.156</td>
<td>2.21 ± 0.792</td>
<td>4.02 ± 1.949</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0361</td>
<td>0.58 ± 0.250</td>
<td>1.00 ± 0.619</td>
<td>1.37 ± 1.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0040</td>
<td>2.15 ± 1.775</td>
<td>9.40 ± 9.399</td>
<td>22.27 ± 22.270</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>Shoot length</td>
<td>0.0108</td>
<td>1.27 ± 0.504</td>
<td>2.85 ± 2.260</td>
<td>4.58 ± 4.839</td>
</tr>
<tr>
<td>Fuseau</td>
<td>W</td>
<td>Germination</td>
<td>0.0000</td>
<td>1.07 ± 0.043</td>
<td>1.14 ± 0.086</td>
<td>1.19 ± 0.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0000</td>
<td>0.41 ± 0.089</td>
<td>1.09 ± 0.323</td>
<td>1.95 ± 0.791</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0000</td>
<td>0.27 ± 0.049</td>
<td>0.64 ± 0.167</td>
<td>1.07 ± 0.386</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>Germination</td>
<td>0.0001</td>
<td>0.07 ± 0.009</td>
<td>0.18 ± 0.026</td>
<td>0.32 ± 0.069</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0003</td>
<td>0.06 ± 0.019</td>
<td>0.14 ± 0.049</td>
<td>0.25 ± 0.139</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0006</td>
<td>0.06 ± 0.012</td>
<td>0.16 ± 0.031</td>
<td>0.28 ± 0.081</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>Germination</td>
<td>0.0002</td>
<td>0.08 ± 0.010</td>
<td>0.18 ± 0.025</td>
<td>0.28 ± 0.057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0001</td>
<td>0.07 ± 0.017</td>
<td>0.16 ± 0.046</td>
<td>0.27 ± 0.113</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0002</td>
<td>0.05 ± 0.012</td>
<td>0.13 ± 0.029</td>
<td>0.22 ± 0.079</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>Germination</td>
<td>0.0001</td>
<td>0.23 ± 0.088</td>
<td>0.76 ± 0.411</td>
<td>1.53 ± 1.129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0002</td>
<td>0.13 ± 0.060</td>
<td>1.03 ± 0.780</td>
<td>3.45 ± 3.440</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0005</td>
<td>0.26 ± 0.679</td>
<td>1.13 ± 0.680</td>
<td>2.70 ± 2.348</td>
</tr>
<tr>
<td>Stampede</td>
<td>H</td>
<td>Shoot length</td>
<td>0.0000</td>
<td>1.18 ± 3.629</td>
<td>NA ± 0.000</td>
<td>NA ± 0.000</td>
</tr>
<tr>
<td></td>
<td>EE</td>
<td>Germination</td>
<td>0.0000</td>
<td>1.80 ± 0.641</td>
<td>10.93 ± 8.613</td>
<td>31.38 ± 31.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root length</td>
<td>0.0009</td>
<td>0.35 ± 0.121</td>
<td>0.59 ± 0.299</td>
<td>0.80 ± 0.501</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot length</td>
<td>0.0000</td>
<td>0.47 ± 0.058</td>
<td>1.33 ± 0.292</td>
<td>2.46 ± 0.765</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>Germination</td>
<td>0.0225</td>
<td>0.01 ± 0.014</td>
<td>0.01 ± 0.014</td>
<td>0.11 ± 0.110</td>
</tr>
</tbody>
</table>
Table 3. Effects of solvent control on germination, root and shoot length of lettuce.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>F value</th>
<th>P (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination</td>
<td>0.10</td>
<td>0.768</td>
</tr>
<tr>
<td>Root length</td>
<td>0.56</td>
<td>0.458</td>
</tr>
<tr>
<td>Shoot length</td>
<td>1.06</td>
<td>0.307</td>
</tr>
</tbody>
</table>

Table 4. Quantity of allelochemicals (mg/kg) and total weight of each tested band.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>p-hydroxybenzaldehyde</td>
<td>10.22</td>
</tr>
<tr>
<td>salicylic acid</td>
<td>20.32</td>
</tr>
<tr>
<td>cinnamic acid</td>
<td>-</td>
</tr>
<tr>
<td>o-coumarinic acid</td>
<td>-</td>
</tr>
<tr>
<td>p-coumarin acid</td>
<td>-</td>
</tr>
<tr>
<td>coumarin</td>
<td>traces</td>
</tr>
<tr>
<td>Total weight (mg)</td>
<td>6.80</td>
</tr>
</tbody>
</table>

Table 5. Weed composition in plots with the presence (HELTU) and absence (Control) of *H. tuberosus* in the soil, combining the results of both surveys.

<table>
<thead>
<tr>
<th>Weed species</th>
<th>Control</th>
<th>HELTU</th>
<th>Sig. (t)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amaranthus retroflexus</em></td>
<td>28.0</td>
<td>8.0</td>
<td>0.159</td>
</tr>
<tr>
<td><em>Chenopodium album</em></td>
<td>44.0</td>
<td>26.0</td>
<td>0.190</td>
</tr>
<tr>
<td><em>Convolvulus arvensis</em></td>
<td>26.0</td>
<td>12.0</td>
<td>0.284</td>
</tr>
<tr>
<td><em>Digitaria sanguinalis</em></td>
<td>46.0</td>
<td>2.0</td>
<td>0.013</td>
</tr>
<tr>
<td><em>Galinsoga ciliata</em></td>
<td>62.0</td>
<td>20.0</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Portulaca oleracea</em></td>
<td>24.0</td>
<td>0.0</td>
<td>0.056</td>
</tr>
<tr>
<td><em>Stellaria media</em></td>
<td>10.0</td>
<td>4.0</td>
<td>0.512</td>
</tr>
<tr>
<td>Others</td>
<td>18.5</td>
<td>14.5</td>
<td>0.662</td>
</tr>
</tbody>
</table>
Figures

**Figure 1.** Effects of the different solvent controls on germination, root and shoot of lettuce. All values are expressed as percent of untreated (black line) (± SE, n = 9).

**Figure 2.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce germination. Bars are standard error (n = 4). * refers to significant differences from the control.
treatment with $p \leq 0.05$ or ** with $p \leq 0.01$ (independent sample t-test). All values are expressed as percent of control treatment (black line).

**Figure 3.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce root growth. Bars are standard error ($n = 4$). * refers to significant differences from the control treatment with $p \leq 0.05$ or ** with $p \leq 0.01$ (independent sample t-test). All values are expressed as percent of control treatment (black line).

**Figure 4.** Effects of different rates of fractions extracted from the cultivar Fuseau on lettuce shoot growth. Bars are standard error ($n = 4$). * refers to significant differences from the control treatment.
with \( p \leq 0.05 \) or ** with \( p \leq 0.01 \) (independent sample t-test). All values are expressed as percent of control treatment (black line).

**Figure 5.** Effect of *H. tuberosus* residues into the soil on total weed density. Bars represent the standard errors (\( n = 4 \)). Letters refer to significant differences (T-test \( P \leq 0.05 \)).

**Figure 6.** Effect of *H. tuberosus* residues into the soil on soil cover caused by all weed species. Bars represent the standard errors (\( n = 4 \)). Letters refer to significant differences (T-test \( P \leq 0.05 \)).