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Biological and Chemical Characterizations of Allelopathic Potential of Diverse Accessions of the Cover Crop Sunn Hemp

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Abstract. **There has been increasing interest in recent years in sunn hemp (*Crotalaria juncea*), as a leguminous cover crop and green manure, for weed and pest management and improving soil health. Aqueous extracts and ground shoot tissue have previously been demonstrated to be phytotoxic. To further explore its allelopathic potential, bioassays and chemical characterization of water-soluble eluates of sunn hemp were undertaken. Lettuce (*Lactuca sativa*) radicle growth inhibition was conducted with aqueous eluates from thinly sliced sunn hemp leaves, stems, and seeds, and all three tissues exhibited the inhibitory potential. Fourteen accessions originating from the United States, India, Brazil, South Africa, Pakistan, and Nigeria had water-soluble allelochemicals in leaves, suggesting that allelopathic potential is widely distributed in this species. The highest level of inhibitory potential was found in accession IN-86. Further characterization of IN-86 leaf eluates indicated that the inhibitory compound(s) was/were not soluble in chloroform, but was/were stable when boiled for 15 minutes and resistant to 1 N HCl. Binding and elution from AG-1(OH$^-$) ion-exchange resin also were observed. An analysis of leaf eluates of IN-86 using high-performance liquid chromatography (HPLC) followed by mass spectrometry (MS) showed the presence of a compound with a mass-to-charge ratio of 148, consistent with the spectrum for hydroxynorleucine, a phytotoxic nonprotein amino acid previously reported in seeds of *C. juncea*. However, its low concentration (<1 μg·mL^{-1}) suggested that other components of the eluate were responsible for the observed allelopathic effect. The results indicate the feasibility for development of weed control strategies using allelochemicals derived from sunn hemp biomass of select genotypes IN-86, NG-71, and BR-20 from India, Nigeria, and Brazil, respectively.**

Sunn hemp, a multipurpose species used for fiber, fodder, and biomass (Cook and White, 1996), is widely grown in tropical and subtropical agricultural systems for its usefulness as a cover crop and green manure. A cover crop of sunn hemp can provide sufficient dry matter to protect the soil from erosion and add substantial amounts of nitrogen in its residues (up to ≈126 kg·ha^{-1}) for use by the subsequent crop (Mansoer et al., 1997). In addition to these benefits, sunn hemp competes effectively with weeds (Collins et al., 2007, 2008), making it especially useful for weed management in organic and sustainable production of row crops. A nonprotein amino acid delta-hydroxynorleucine was identified in sunn hemp seeds (Pant and Fales, 1974; Pilbeam and Bell, 1979) that was demonstrated to be phytotoxic, inhibiting hypocotyl and radicle growth during germination (Wilson and Bell, 1979). Nonprotein amino acids from other legumes also have been shown to be phytotoxic. These include L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA) from velvet bean [*Mucuna pruriens* var. *utilis* (Nishihara et al., 2005)], mimosine and albizziine from *Leucaena leucocephala* and *Albizia* species, respectively (Williams and Hoagland, 2007), and m-tyrosine from *Festuca* species (Bertin et al., 2009). Cole (1991) found that extracts of sunn hemp seeds reduced the growth of several weeds. Aqueous extracts of leaves also showed inhibitory effects on the roots of wheat seedlings [*Triticum aestivum* (Ohdan et al., 1995)] and extracts from macerated sunn hemp leaves and leachate from whole sunn hemp leaves inhibited maize (*Zea mays*) germination at 15% and 30% w/v (Cruz-Silva et al., 2015).

Because of the potential to use sunn hemp's allelopathic properties for weed suppression in horticultural crop production systems, we examined the phytotoxicity of its aqueous foliar extracts and ground, dried residues in an earlier study (Adler and Chase, 2007). Both sunn hemp extracts and ground, dried residues inhibited the germination and growth of livid amaranth (*Amaranthus lividus*), goosegrass (*Eleusine indica*), bell pepper (*Capsicum annuum*), and tomato (*Solanum lycopersicum*)

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germination with greater negative effects on livid amaranth than in other species (Adler and Chase, 2007). Skinner et al. (2012) reported that ground, dried sunn hemp residues inhibited germination of lettuce and smooth pigweed (*Amaranthus hybridus*), and aqueous leaf extracts reduced germination and seedling growth of various crops. Despite this progress, several aspects of allelopathy by sunn hemp are not well understood. Sunn hemp has considerable genetic diversity (Wang et al., 2006), but possible differences in allelopathic potential of different accessions and the nature and properties of the allelochemicals are not known. The objectives of the current study were to test the relative allelopathic potential of leaves, stems, and seeds of a commercially available sunn hemp cultivar and to evaluate the allelopathic potential of aqueous leaf eluates from 14 accessions from eight countries. In addition, we examined whether the phytotoxic compound—delta-hydroxynorleucine—was the major allelochemical in sunn hemp aqueous leaf eluate.

Materials and Methods

Plant material used. Leaves and stems from a commercially available sunn hemp cultivar (Kauffman Seeds, Haven, KS) were used immediately after harvest from 3-month-old plants grown in a field in Gainesville, FL, with overhead irrigation during Fall 2011. Since the provenance of this seed source was unknown, a voucher specimen of the plant Rathinasabapathi 1 (FLAS) was submitted to the Florida Museum of Natural History in Gainesville, FL. In the experiment in which 14 different sunn hemp accessions [U.S. Department of Agriculture, Agricultural Research Service, Griffin, GA (Table 1)] were screened, plants were grown one per 11.4-L container in potting medium (Fafard no. 2; Sun Gro Horticulture, Agawam, MA) in a greenhouse with mean maximum and minimum temperatures of 27 and 20 °C, respectively, during Fall 2011. The plants were fertilized with a commercial fertilizer (20N–8.7P–16.6K), irrigated manually once per week, and the leaves were harvested before flowering.

Table 1. Aqueous eluates from stem, leaf, and root sections of 14 sunn hemp accessions were evaluated for phytotoxicity in bioassays with the lettuce cultivar Green Ice.

Accession	USDA PI no.[z]	Other names[y]	Origin
US-56	PI 468956	Tropic Sun	United States
IN-87	PI 250487	K681	India
BR-77	PI 322377	IRI 2473	Brazil
FS-39	PI 314239	No 524	former Soviet Union
BR-80	PI 337080	n.a.	Brazil
US-39	PI 652939	374	Texas, United States
SA-67	PI 391567	T'ai-yang-ma	South Africa
PK-26	PI 426626	Sanni, K-98	Pakistan
IN-86	PI 250486	K680	India
IN-85	PI 250485	K679	India
NG-71	PI 234771	n.a.	Nigeria
BR-20	PI 561720	IAC-1	Brazil
SR-57	PI 207657	n.a.	Sri Lanka
IN-97	PI 346297	n.a.	Delhi, India

[z]United States Department of Agriculture's Plant Introduction Number.
[y]Local names, cultivar name, or other designation associated with accessions; n.a. indicates that information is not available.

Bioassay for allelopathic potential. A lettuce bioassay for allelopathy using surface-sterilized seeds of cultivar Green Ice (Park Seed, Greenwood, SC) was conducted as described in Ferguson et al. (2004). Briefly, 5 g fresh weight of plant tissue of each of the various accessions cut into 1-cm strips were incubated in 50 mL of water for 24 h, the aqueous fraction filtered with four layers of cheesecloth, centrifuged at 300 g_n for 10 min, filtered using a 0.2-µm filter, and stored at 6 °C until use. It is to be noted that this relatively gentle method of elution preferentially elutes water-soluble phytochemicals only and elutes about 10% of the phytochemicals in the tissue compared with a typical phytochemical extraction where the tissue is macerated in a solvent (Ferguson et al., 2004; Rathinasabapathi et al., 2005). No visual signs of microbial growth could be observed in these eluates after the 24-h incubation. Lettuce seeds were surface sterilized by washing in 10% (v/v) commercial bleach and rinsed five times in sterile water. At least 20 surface-sterilized lettuce seeds were used in each bioassay. Three milliliters of eluates per test were used on a Whatman no. 2 filter paper (GE Healthcare BioSciences, Pittsburgh, PA) placed in a petri dish. Seed germination was assessed and radicle lengths after were measured following after 24- and 72-h incubation, respectively, under a light bench [16/8 h (light/dark), 150 µmol·m^{-2}·s^{-1}].

Tests on stability and chemical nature of phytochemicals. Leaf eluates of sunn hemp accession IN-86 were used. For testing stability to heat, the eluate was boiled at 100 °C for 15 min. Acid stability was tested by hydrolyzing the

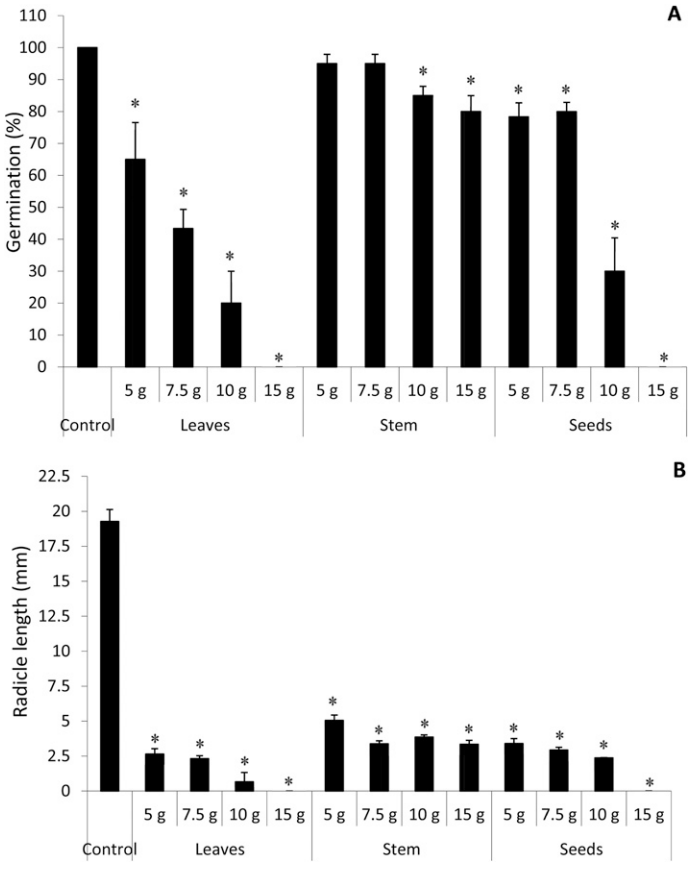

Fig. 1. Inhibition of (**A**) lettuce seed germination and (**B**) lettuce radicle elongation by aqueous eluates from a commercially available sunn hemp cultivar. Bars show means and SE for observations on at least 15 lettuce seedlings. Bars marked by an asterisk indicate significant difference at $P \leq 0.05$ compared with the control determined using Fisher's protected least significant difference test.

eluate in 1 N HCl for 15 min at 50 °C. Then HCl was evaporated in a nitrogen evaporator and the residue was redissolved in water and pH was adjusted to 7 as needed before testing. A negative control was included that contained HCl, but no eluate, which provided results similar to a water control (data not shown). For a solubility test, aqueous eluate (3 mL) was extracted with an equal volume of chloroform and the aqueous and organic phases were separated and evaporated and residues were redissolved in water. To test the ionic nature of the compounds, an aliquot of leaf eluate (7 mL) was passed through 1.5-mL columns of AG-1(OH–) and AG-50(H+) ion-exchange resins, placed in tandem, the anion exchange column above the cation exchange column (Bio-Rad, Hercules, CA). The unbound fractions were collected and the columns were eluted with 15 mL of 0.1 N HCl followed by 15 mL of 2.5 N HCl. The liquid was evaporated using a stream of air and residues were redissolved in 5 mL water. All treated (temperature, acid, and ion exchange) fractions were tested for bioactivity using the lettuce bioassay described above.

Analyses of leaf eluates for phytochemicals. Leaf eluates (with or without ion-exchange purification) were analyzed using HPLC (Agilent Technologies, Santa Clara, CA) equipped with a 1100 series binary pump, fitted with a 2 × 150-mm column (Synergi 4µ Hydro-RP80A, serial no. 106273-5; Phenomenex, Torrance, CA). The mobile phase consisted of an isocratic gradient of solvents A (0.2% acetic acid in water) and B (0.2% acetic acid in methanol) at 0.15 mL·min^{-1}. The peaks were detected using an ultraviolet/visible (ultraviolet/Vis) detector (1100 G1314A; Agilent Technologies) at 230 nm. Mass spectral analyses were performed using a mass spectrometer (Thermo-Finnigan; Thermo Fisher Scientific, Waltham, MA) with electrospray ionization [ESI (sheath gas nitrogen)] at a heated capillary temperature of 250 °C. The (+)ESI spray voltage was 3.3 kV at heated capillary voltage of 12.5 V and (–)ESI spray voltage was 3.2 kV at a heated capillary voltage of 10 V.

Estimation of delta-hydroxynorleucine. A standard of L-6-hydroxynorleucine (formula weight 147.17; ChemSamp Co., Dallas, TX) was used to quantify delta-hydroxynorleucine in leaf eluates of accession IN-86 using HPLC-MS-MS equipment and the method described above.

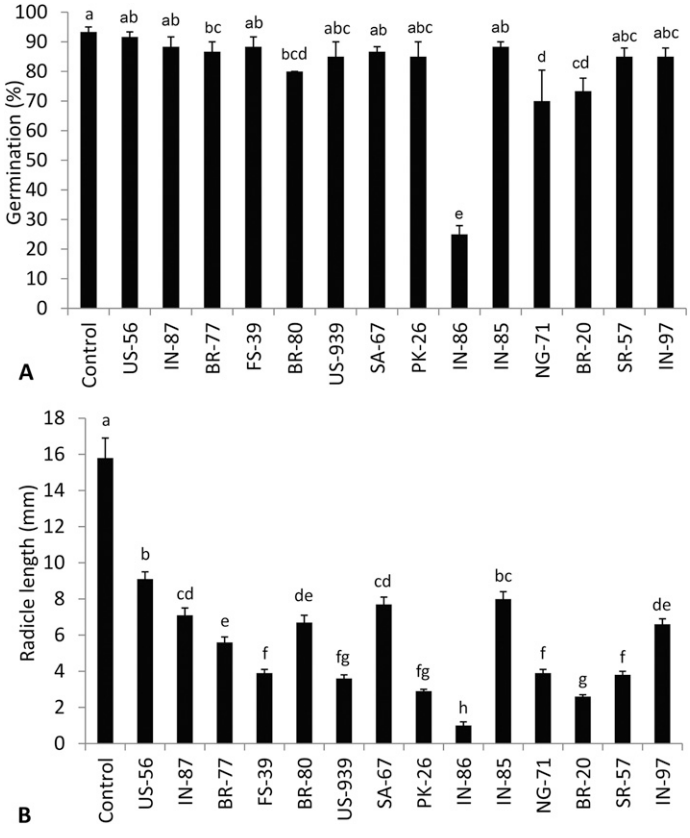

Fig. 2. Aqueous eluates from the leaves of 14 sunn hemp accessions showed allelopathic potential when (**A**) seed germination and (**B**) radicle elongation were assessed in bioassays with 'Green Ice' lettuce. Bars illustrate means and SE for observations on at least 15 lettuce seedlings. Bars marked by different letters indicate significant difference at $P \leq 0.05$ determined using Fisher's protected least significant difference test.

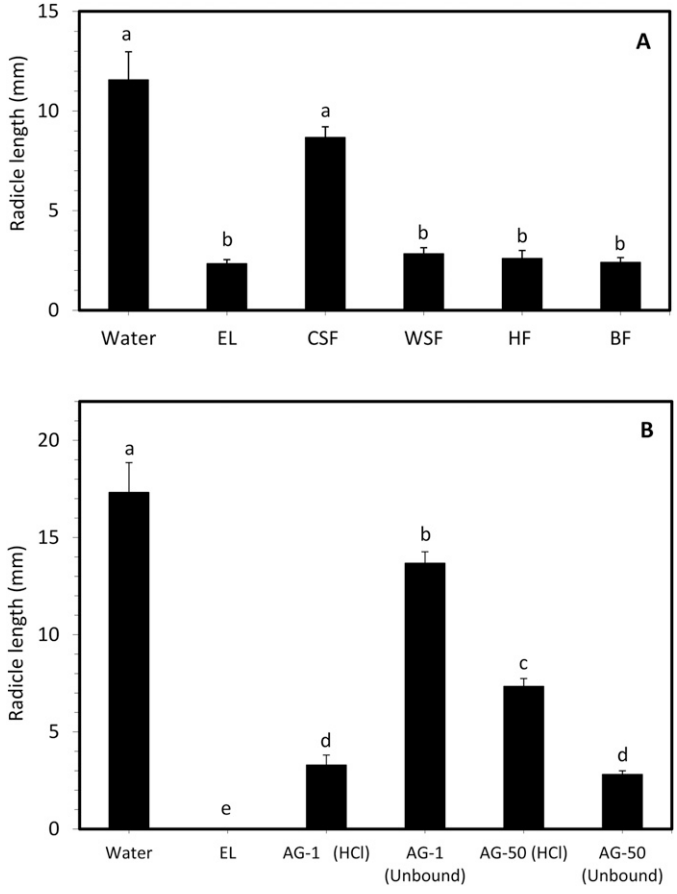

Fig. 3. Assessment of solubility and stability characteristics of putative allelochemicals in leaf eluates from sunn hemp accession IN-86. Bars represent means and SE for observations on at least 15 seedlings each. Bars marked by different letters indicate significant difference at $P \leq 0.05$ determined using Fisher's protected least significant difference test. (**A**) water (control), EL (eluate), CSF (chloroform-soluble fraction, chloroform evaporated, redissolved in water), WSF (water-soluble fraction left after removal of chloroform phase), HF (hydrolyzed fraction, eluate treated with 1 N HCl, 50 °C for 15 min, HCl evaporated, redissolved in water), BF (eluate boiled at 100 °C for 15 min). (**B**) Ion-exchange purified fractions: water (control); EL (eluate); AG-1 (HCl) [eluate loaded on 1.5 mL AG-1(OH$^-$) resin, unbound washed with water, column eluated in 2.5 N HCl, HCl evaporated and redissolved in water]; AG-1 (Unbound) [eluate from AG-1(OH$^-$) column as unbound]; AG-50 [eluate loaded on 1 mL AG-50(H$^+$) resin, unbound washed with water, column eluted in 2.5 N HCl, HCl evaporated and redissolved in water]; AG-50(Unbound) [fraction that did not bind to either AG-1(OH$^-$) or AG-50(H$^+$) resin].

Statistics. Analysis of variance of quantitative data were performed using the general linear models procedure of SAS (version 9.2; SAS Institute, Cary, NC) and mean comparisons were performed using Fisher's least significant difference test at $\alpha = 0.05$.

Results

Allelopathic potential. When aqueous eluates of leaves, stem, and seeds of the commercial sunn hemp cultivar were evaluated, it was found that lettuce seed germination and radicle growth were negatively affected in a concentration-dependent manner (Fig. 1A and B). As 10 g tissue in 50 mL water was sufficient to inhibit germination and root growth, additional experiments were performed using this ratio of tissue to water. Leaf eluates from 14 sunn hemp accessions grown under uniform conditions were compared using the lettuce bioassay for allelopathic potential. Leaf eluates of accessions BR-77, BR-80, IN-86, NG-71, and BR-20 significantly ($P \leq 0.05$) inhibited lettuce seed germination compared with the water control (Fig. 2A). Eluates from all 14 accessions ($P \leq 0.05$) inhibited radicle growth and the eluate from IN-86 resulted in the greatest inhibition (Fig. 2B).

Stability and chemical nature of allelochemicals. To evaluate sunn hemp allelochemicals for amenability to procedures that may be needed for development of a bioherbicide, leaf eluates of IN-86 were subjected to chloroform partitioning, acid hydrolysis, boiling, and ion-exchange chromatography. Figure 3A shows that allelopathic compounds are not present in the chloroform fraction (CSF) following phase separation with an aqueous eluate. Sunn hemp allelochemicals also were stable to limited acid hydrolysis and boiling. In a test using ion-exchange resins, the inhibitory compounds were effectively bound to AG-1 (OH–) but were only partially bound by AG-50 (H+), suggesting that the majority of allelochemicals were either negatively charged or had no charge (zwitterionic) (Fig. 3B).

Analyses of leaf eluates for putative allelochemicals. Ion-exchange resin-purified fractions had at least 12 ultraviolet-positive peaks each eluting within the first 35 min of the gradient (Fig. 4). More than 70% of the peaks were eluted within the first 8 min of the run. HPLC/ultraviolet/(+)ESI-MS^n analysis identified the presence of protonated compounds with (m/z) 178.6, 132.0, 153.2, 166.0, 260.8, 422.9, 534.0, 758.6, 205.0, 158.1, 406.5, 422.0, 209.0, 190.8, 236.8, 287.3, 285.2, 291.3, 293.1, 293.2, 353.3, 295.2, 277.3, 268.9, 268.9, 376.3, 618.2, 465.1, 449.1, 595.2, 625.1, 463.2, 617.1, 433.0, 529.5, 695.3, 699.3, 663.3, 514.8, 432.8, 514.8, 959.2, 874.4, 858.3, 773.0, 757.0, 826.3, 840.2, 933.0, 1211.1, 1179.0, 997.0, 695.3,

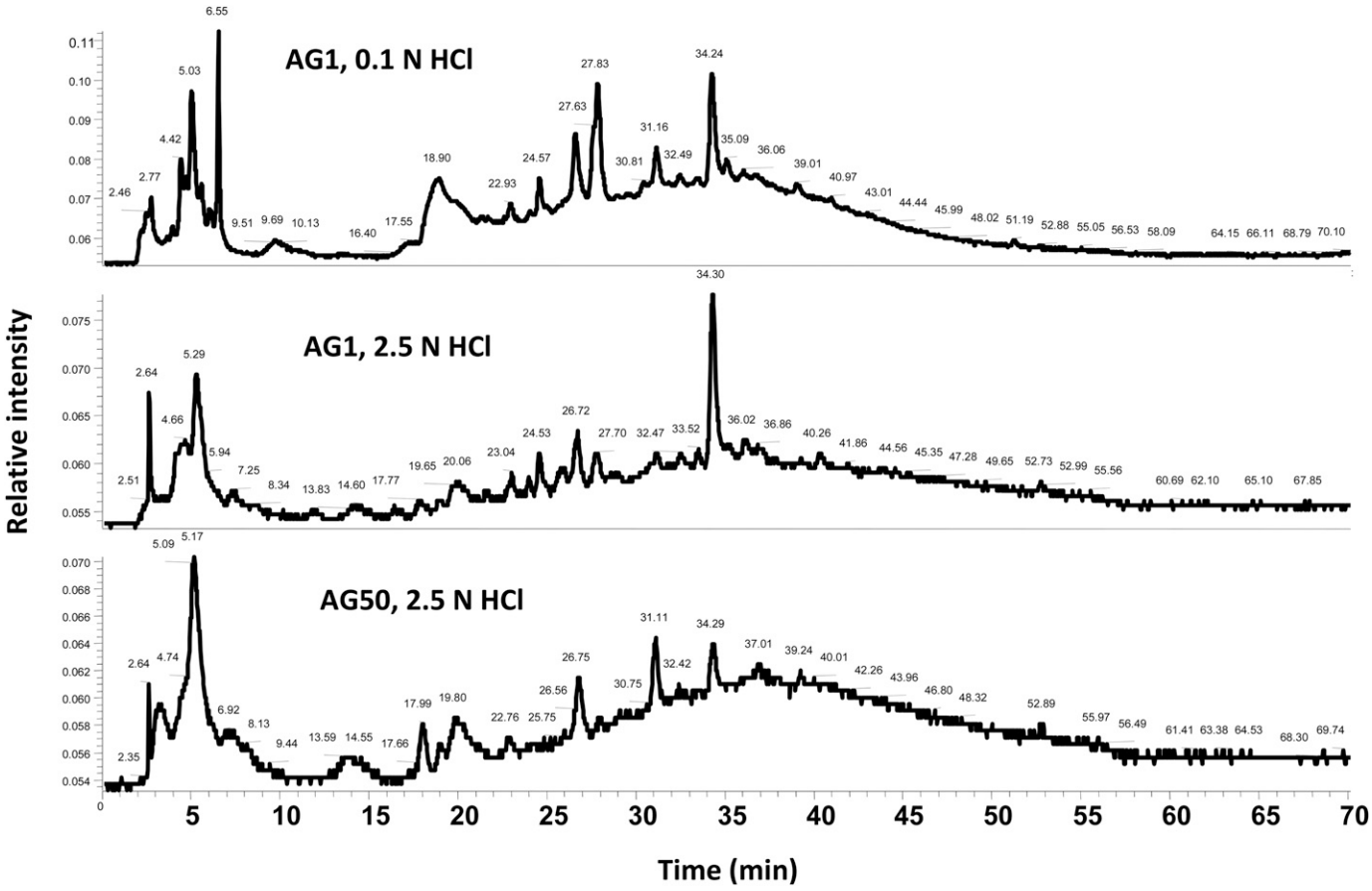

Fig. 4. Distribution of ultraviolet-positive phytochemicals in sunn hemp leaf eluate. Leaf eluates of sunn hemp accession IN-86 were fractionated using ion-exchange resins and eluted using different strengths of HCl. After evaporation of HCl, the compounds were dissolved in water and separated using high-performance liquid chromatography. The ion-exchange fractions were from AG-1(OH–) with 0.1 N HCl (AG-1, 0.1 N HCl), AG-1(OH–) eluted with 2.5 N HCl (AG-1, 2.5 N HCl) and AG-50(H+) resin eluted with 2.5 N HCl (AG-50, 2.5 N HCl). Peaks detected at 230 nm in a gradient high-performance chromatograph are shown with retention time marked on the peaks.

and 695.4 in the leaf eluate (Fig. 5). HPLC/ultraviolet/(–)ESI-MS^n analysis identified the presence of compounds with (m/z) 191.1, 167.3, 164.3, 343.1, 421.1, 443.0, 763.4, 327.4, 593.2, 577.2, 677.4, 771.3, and 755.3 in the leaf eluate (data not shown). A comparative analysis of mass spectral data from both (–)ESI and (+)ESI modes confirmed molecular weights for 65 compounds (Table 2).

Because of the unavailability of standard delta-hydroxynorleucine, 6-hydroxynorleucine was used for quantifying delta-hydroxynorleucine in a leaf eluate of IN-86. With (+) ESI-MS, 6-hydroxynorleucine produced m/z 148 [M+H]+ ions, which dissociated to form (m/z) 130 and 102 product ions (Fig. 6). The standard showed acceptable linearity between 50 and 5000 pg·mL^{-1}, when estimated at (m/z) 148, 130, or 102 (data not shown). Because of the absence of interferences, the product ion m/z 102 was used for quantitative estimation of hydroxynorleucine. On the basis of four analyses, the leaf eluate had 892 ng·mL^{-1}, with 15% relative standard deviation.

Discussion

The allelopathic potential of sunn hemp tissues of multiple sunn hemp genotypes from various countries was consistent with the results for individual genotypes tested in previous studies (Adler and Chase, 2007; Ohdan et al., 1995; Skinner et al., 2012). The current study further expanded upon previous work to show that water-soluble allelochemicals are present in seeds, leaves, and stems of sunn hemp, and that stems had the lowest allelopathic potential of the three organs (Figs. 1 and 2). During the first 3 weeks of growth, sunn hemp produces higher leaf than stem dry matter; but by 6 weeks after planting, stem dry matter is reported to be greater than that contributed by the leaves (Mansoer et al., 1997). However, the contribution of the leaf to the biomass can be manipulated by pruning the main stem (Abdul-Baki et al., 2001). Hence, these results showing allelopathic qualities for all tissues tested suggest that all biomass from this species could potentially be used for weed-suppression purposes, but leaf tissue could be the most useful for using the allelochemical potential.

All 14 accessions of sunn hemp had strong allelopathic potential in our tests, although some accessions had greater inhibitory effects than others (Fig. 2). Together these results suggest evolution of allelochemicals in this species was an ancient one, perhaps predating speciation. The results are useful for choosing accessions with higher or lower allelopathic potentials, a critical decision in sustainable crop management. In addition, the information will be useful to breed new cultivars with enhanced or reduced allelochemical content.

Water-soluble allelochemicals were stable for a boiling treatment and acid hydrolysis and could bind and elute from ion-exchange resins (Fig. 3). Such stability is important for using the allelopathic potential of this species for weed suppression in the field. The results also suggest methods to purify and store the active principles for such applications.

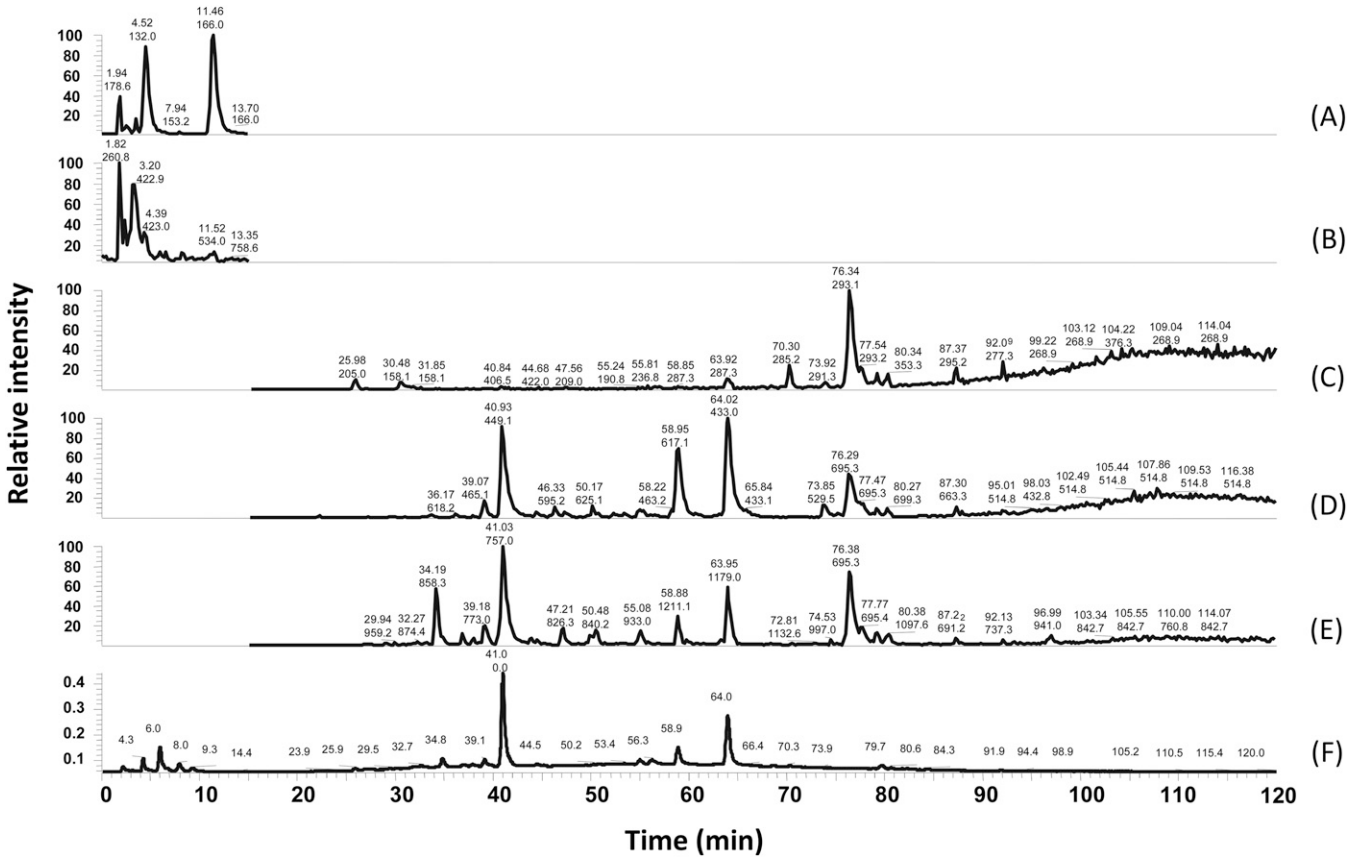

Fig. 5. Mass spectral analyses of leaf eluate from sunn hemp IN-86 under (+)ESI (electrospray ionization, positive ion mode). Mass spectral peaks in fractions eluting at different times are shown with their elution time in minutes and mass-to-charge ratio (m/z) values in m/z ranges of 125 to 200 (**A**), 190 to 800 (**B**), 125 to 430 (**C**), 420 to 700 (**D**), and 690 to 1300 (**E**). The ultraviolet-analog peak chromatogram from the high-performance liquid chromatography is shown in (**F**).

Table 2. Analysis of sunn hemp leaf eluate purified by anion exchange resin-purified fraction using electrospray ionization (ESI) mass spectrometry (MS). The molecular weight (MW) deduced, tentative identification, retention times (RT) in the positive and negative ion modes (+ and –ESI) and fragmentation products (MS^n) in each of the modes are listed.

MW	Tentative identification	RT-MS (+)ESI	RT-MS (–)ESI	MS^n (+)ESI	MS^n(–)ESI
121	Cysteine	2.22	ND	122, 104	
150		ND	2.65		131, 103(b), 87, 75, 59, 43
145		2.61	2.37	146=>128, 100, 82	
175			2.78		
398	Diglycoside of MW 89	2.68	ND		398=>324, 308, 293, 268, 252(b), 248; 398=>252=> 90(b), 72
192		2.8	2.86		
164		ND	3.42		
129		3.2	ND		
126		3.99	3.56		
90		3.56	ND		89=>43(bp; –46u)
360		ND	4.03		359=>341, 323(b), 315, 305, 297, 288, 280, 269, 261, 255, 239, 226, 213, 195; 359=>323=>305(b)
338		ND	4.22		337=>320, 277(b), 203, 175, 157, 139, 115; 337=>277=> 157
200		4.37	4.22	201 > 183(b), 165, 140, 123, 102, 84, 58; 201 > 183=>165(b), 148, 140, 123, 99, 84	
323	Cytosine monophosphate	4.8	4.96		322=>306, 294, 277, 264, 211(b); 322=>211=>97, 79(b)
131	Leu/Isoleucine	4.7	ND	132=>86	
151		4.96	ND	152=>134 (weak MS/MS)	
310		ND	5.34		
237		5.22	ND	238=>220, 179(b), 151; 238=>179=>177, 151(b), 133, 117	
258		5.61	5.94		257=>240, 213, 195(b;-62u), 183, 177; 257=>195=>177(b), 152
135		5.54	6.91		
146	Lysine	5.92	ND	147=>129, 101(b), 83, 69,59	
324	Uracil monophosphate	7.04	6.99		323=>250, 211(b), 182; 323=>211=>97, 79(b)
228		6.38	6.99	229=>211(b), 203, 193(b2), 185, 165, 130, 112, 86, 84; 229=>211=>193(b), 165, 112, 99	
165		6.5	ND		
173		6.5	6.54		
193		6.5	ND	194=>135(bp), 58; 194=>135=>107	
203		6.5	7.55		
187		6.69	9	188=>170, 146(b), 138, 82; 188=>146=>128, 118(b), 101, 100, 82, 74	246=>186(b), 171; 246=>186=>42(b), 124, 83
347	Adenosine monophosphate	9.81	12.66	348=>330, 136(b)	346 => 211(b) => 79
146		11.22	12.66		145=> 127, 101(b), 99, 83, 71, 57; 145=>101=>83, 71(b)

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Table 2. Continued.

MW	Tentative identification	RT-MS (+)ESI	RT-MS (–)ESI	MS^n (+)ESI	MS^n(–)ESI
363	Guanine monophosphate	14.32	13.25		
244		15.61	16.6		243=>225, 213(b), 199, 195, 181, 169
574		ND	16.65		573=>513(b), 485, 452, 412
285		15.21	16.81		284=>266(b), 253, 198, 196
763		16.79	ND	764=>577(b; –187u), 559, 390, 372; 764=>559, 517, 473, 415, 397, 390(b; –187u), 372, 311, 228, 209	
412		ND	17.07		411 => 413, 374, 349, 309, 267(b), 249; 433 => 415, 389, 373, 371, 331, 289, 272, 267, 249
281		16.6	17.44	282 => 136(b); –146 u	
297		17	17.66		296=>268, 234, 192, 150(b)
347	AMP	18.35	18.95		346=>308, 284, 265, 211(b), 164
950		19.1	ND	951=>764(b,-187u), 746, 577; 951=>764=>746, 577(b), 559, 390	
286		18.73	19.16	287=>269, 158, 140(b), 128, 112; 287=>140=>112(b), 70	285=>267(b), 241, 236, 198, 156, 128
763		19.59	ND	764=>577(b), 559, 415, 390, 372, 327, 226; 764=>559, 390(b), 372	
594		22.34	22.79	298=>466, 385, 367, 357, 340, 289, 257, 256, 249, 239, 211(b), 193, 165, 140, 130, 112, 95; ; 595=>577(b), 550, 514, 385, 367, 257; 595=>577=>559, 433, 499, 367(b), 257, 221, 193	593.5=>575(b); 593=>575=> 557(b), 513, 365, 320, 254
264		22.92	ND	265=>247, 221, 205(b), 176, 144, 134, 129, 105, 84	
473		24.52	24.99	474=>456, 428, 359, 345(b), 327; 474=>345=>345(b; 327 + H2O), 327, 359(327 + 32); 301	472=>454, 429, 411, 404(b), 385, 369, 343, 312, 300, 284; 472=>404=>386, 360, 275(b), 248
622		25.59	25.81	312=>481, 466, 385(b), 367, 303, 263, 257, 239, 221, 140, 123, 95; 623=>605, 385(b), 367, 257, 239, 221; 623=>385=>367(b), 349, 324, 310, 257, 239, 221, 212, 194, 129	621=>603(b), 383, 366; 621=>603=>559, 541, 507, 366(b), 323
166		25.59	25.96		165=>121
426		26.35	ND	427=>345, 328, 312(b), 308, 284, 281, 213; 427=>312=>284, 213(b), 185	
1558		26.5	ND	1559=>1413, 1397, 1267, 1251, 1105, 1089, 968, 943(b), 925, 907, 871, 823, 805, 781. 751, 737, 703; 1105=>943(b), 925, 907, 823, 805, 763, 727, 703, 661	
772	Triglycoside: O-162–146, C-glycoside	26.81	27.23	773=>755, 653, 627, 611, 557, 525, 504, 465(b), 447, 429, 399, 369, 345, 315, 303, 235; 773=>465=>447(b), 429, 411, 399, 381, 369, 345, 341, 327, 315, 305, 263	771=>734, 717, 711, 705, 693, 681, 668, 651(b; NL120), 642, 627, 618; 771=>651=>633, 385, 343(b2), 342(b1), 314, 285, 268, 257, 221

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Table 2. Continued.

MW	Tentative identification	RT-MS (+)ESI	RT-MS (–)ESI	MS^n (+)ESI	MS^n(–)ESI
756		27.96	28.2	757=>611, 595, 559, 541, 491, 473, 449(b), 431, 413, 383, 353, 329, 311, 299,; 757 => 449 => 431(b), 413, 395, 383, 367, 353, 329, 311, 299, 287, 243; 779 (M+Na)+=>761, 743, 689(b), 659(b2), 633, 615, 563, 471, 331(m/z 309-H+Na)	755 =>701, 665, 635(b); 755 => 635 => 617, 607, 590, 399, 369, 341, 327(b), 326(b2), 298, 283, 269, 255, 241, 223, 211 813=>777, 755(B), 657; 813 => 755 => 665, 635(B), 313
1250		32.4	ND	1273=>1127, 965(b), 947(b2), 845; 1105=>943(b), 925, 907, 823, 805, 719, 703, 479	785=>695, 665(B), 785 > 665 > 637, 399, 372, 357(B), 356, 342, 328, 314, 285
464	C-glycoside	32.7	32.5	465=>447(b), 429, 411, 399, 381, 369, 345, 327, 315, 300, 259, 219; 465=>447=>439, 411, 399, 369(b), 347, 327, 315, 285	463=>433, 409, 403, 373, 343(b), 301; 463=>343 => 315, 300, 297, 281, 221(b), 205, 193, 165, 149, 121
448	C-glycoside	34.22	34.39	449=>431(b), 413, 395, 383, 368, 354, 329, 311, 299; 449 => 431 => 413, 395, 383, 371, 367, 353(b), 339, 329, 311, 299, 257	447=>357, 327(b); 447=>327=>299(b), 271, 219, 193
478	C-glycoside	35.9	35.98	479=>461(b), 443, 425, 413, 383, 359, 341,329; 479=>461=>443, 425, 413, 401, 383, 341(b), 329, 275, 234, 154	477=>387, 357(b), 477=>357=>342, 329(b), 314, 301, 286, 285, 254, 217
942		36.73	ND	943=>925, 907, 889, 871, 829, 823, 805(b' –138u), 793, 787, 769, 763, 751, 739, 733, 727, 703, 685, 661, 643	
594	Di-O-glycoside of 286 aglycone	36.66	36.76	617[M+Na]+ => 471 (449, Na); 617=>471=>449, 326, 309(287 + 22u), 185	593=>447(NL 146); 593=>447=>419, 353, 285(b2), 284(b1), 254, 243, 219, 203
924		39.03	ND	925=>907, 889, 871, 853, 841, 829, 811, 805(b, –120u), 787, 769, 763, 751, 721, 709, 705, 691, 685, 667, 643, 611	
578	Di-O-glycoside of MW 286 aglycone	39.1	39.13	433(579–146) => 287(b), 269; 433=>287=>287(b), 269, 259, 241(di-BP), 213, 165	577=> 447, 431(b), 285; 577=>431=> 357, 339, 327, 285(b), 255, 217, 153
446		41.1	ND	447=>411(-HCl) => 249, 231(b2), 209(b1), 203, 167, 151	
432	O-glycoside of MW 286	43.3	43.45	433=>287(b,-146u); 433=>287=>269, 241(b2), 165, 133, 121, 111; m/z 433 NaOAc cluster in MS/MS but m/z 287 is bp	431=>285(b,-146u), 151; 431=>285=>257, 241, 213, 183, 151(b), 107

ND indicates that the compound was not detected in the indicated mode.

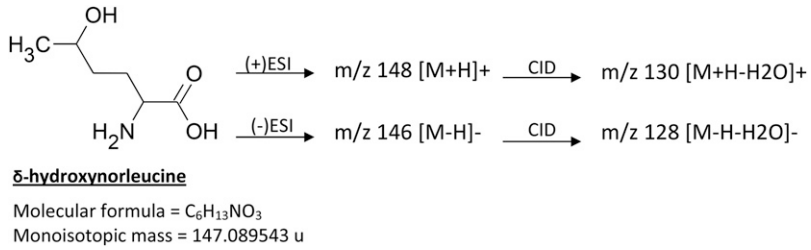

Fig. 6. Identification of δ-hydroxynorleucine using mass spectrometry. With (+)ESI-MS (electrospray ionization mass spectrometry, positive ion mode), δ-hydroxynorleucine readily forms a mass-to-charge ratio (m/z) of 148 [M+H]+, which upon collision-induced dissociation (CID) produced the m/z 130 [M+H-H2O]+ product ion. With (–)ESI-MS (electrospray ionization mass spectrometry, negative ion mode), δ-hydroxynorleucine forms an m/z 146 [M-H]– ion, which upon CID results in an m/z 128 product due to loss of H_2O. Loss of 46 u and 44 u from the [M+H]+ and [M-H]– ions, respectively, as shown in the figure are common for amino acids.

While chemical identification of the allelochemicals in sunn hemp leaf tissue was beyond the scope of this study, because elutions from the anion exchange resin and zwitterionic compounds had inhibitory effects in the lettuce bioassay, the inhibitory compounds could be amino acids and their derivatives including alkaloids, flavonoids, carotenoids, and phenolic compounds. The HPLC-MS-MS analysis of leaf eluates showed that the m/z value and its product ions consistent with the presence of delta-hydroxynorleucine could be identified in such fractions (Fig. 6). However, delta-hydroxynorleucine was identified to be only a minor component in the leaf eluate (i.e., 0.9 $\mu g \cdot mL^{-1}$) a comparatively low concentration for allelopathy. Since delta-hydroxynorleucine is highly soluble in water, the extraction method used should have been successful in eluting it. The eluates were kept in cold storage or analyzed immediately after preparation, reducing probability of degradation. Therefore, it is likely that the phytotoxicity observed in aqueous eluates of sunn hemp leaves is due to compounds other than the nonprotein amino acid delta-hydroxynorleucine, which was previously isolated from seeds of sunn hemp (Pant and Fales, 1974; Pilbeam and Bell, 1979) and shown to be inhibitory to lettuce (Wilson and Bell, 1979). The yield reported in the seeds after an isolation procedure was 1.47 $mg \cdot g^{-1}$ seeds (Pilbeam and Bell, 1979) and the concentration we found is equivalent to about 4.5 $\mu g \cdot g^{-1}$ leaves. Future studies should examine the contributions of differences in tissue distribution and extraction methods in estimating this nonprotein amino acid in sunn hemp. However, our results indicate that when sunn hemp biomass is incorporated into the soil, some delta-hydroxynorleucine is added. Nonprotein amino acids form a significant store of organic nitrogen in many ecosystems (Vranova et al., 2011) and the contribution of such organic nitrogen to the soil by cover crops and their ramifications for the soil ecosystem are currently not well understood. Nonprotein amino acids could favorably be used as sources of nitrogen by certain groups of microbial flora and fauna and likely affect the abundance of weed seeds and soil-borne arthropods, pathogens, and nematodes.

As expected, HPLC-MS-MS analysis of leaf eluates of sunn hemp indicated a complex profile (Fig. 4 and 5). A recent study on the leaf eluates of sunn hemp cultivar Tropic Sun indicated the presence of dehydropyrrolizidine alkaloids (Colegate et al., 2012). These alkaloids had M+H m/z values of 284 (hemijunceine and its isoforms), 370 (junceine and trichodesmine *N*-oxide), 386 (junceine N-oxide), 336 (senecionine), 326 (acetylisohemijunceine), 300 (isohemijunceine), and several partially identified compounds (Colegate et al., 2012). However, none of these m/z values matched the m/z values of ions that were observed in the analyses of this study done under ESI-MS-MS positive ion mode. Colegate et al. (2012) used methanol for extraction of different tissue samples, and enriched their fractions for alkaloids using cation-exchange chromatography before their analyses by MS while in this study the focus was only on water-soluble compounds. Differences in the germplasm used between the two studies and methodologies in the preparation of the samples could be reasons for the differences in the mass spectral profiles.

The use of plants with allelopathic properties or bioherbicides derived from such plants for weed suppression can be used to improve the sustainability of weed management in horticultural crops. Research on both the biological and chemical properties of phytochemicals in such plants is crucial for understanding their usefulness for weed management. Sunn hemp provides multiple agroecosystem services including as a cover crop with green manure, nematode deterrence, and has potential for use as a bioenergy feedstock. The present study provided evidence for allelopathic potential in 14 accessions of sunn hemp with the strongest inhibition of lettuce germination occurring with the IN-86, NG-71, and BR-20 accessions from India, Nigeria, and Brazil, respectively. Phytotoxic compounds were stable after chemical characterization procedures suggesting potential for use in developing formulations of natural herbicides that could be employed for weed management in organic and sustainable production systems.

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