

Impact of temperature stress on secondary metabolite profile and phytotoxicity of *Amaranthus cruentus* L. leaf extracts

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ABSTRACT

In this study *Amaranthus cruentus* plants were grown under controlled optimal conditions (28/21 °C) for three months and then subjected to cold (14/7 °C) and hot (33/40 °C) temperatures. We investigated the influence of these temperature regimes on the metabolite profile of the leaves through analyses of data by TLC, HPLC and GC-MS spectrometry. The phytotoxic potential of a methanol-water (MW) and dichloromethane (DCM) extract from the aerial parts were examined through in vitro screening of germination and growth of lettuce and pepper. The optimal extracts displayed the highest diversity of secondary metabolites, and the highest total phenolics and flavonoids content. Through TLC and HPLC analysis the significantly lower phenolic content in the hot temperature treated samples was confirmed. A wide range of metabolites were detected in the DCM extracts through GC-MS analyses. The phytotoxicity of both the MW and DCM extracts were demonstrated, as germination and growth of pepper and lettuce were significantly inhibited, indicating the presence of more than one allelochemical compound which may affect the allelopathic activity of *A. cruentus* during changes in environmental temperatures.

Key words: *Amaranthus cruentus*; temperature; stress; phytotoxicity; metabolites; phenolic compounds

IZVLEČEK

VPLIV TEMPERATURNEGA STRESA NA PROFIL IN FITOTOKSIČNOST SEKUNDARNIH METABOLITOV V LISTNEM IZVLEČKU ZRNATEGA ŠČIRA (*Amaranthus cruentus* L.).

Rastline zrnatega ščira so bile za namene te raziskave gojene v nadzorovanih optimalnih temperaturnih razmerah tri mesece (28/21 °C) in nato izpostavljene hladu (14/7 °C) in vročini (33/40 °C). Preučevan je bil vpliv temperaturnih režimov na profil metabolitov v listih ščira z metodami kot so TLC, HPLC in GC-MS spektroskopija. Fitotoksični potencial metanolvodnih (MW) in diklormetanskih (DCM) izvlečkov nadzemnih delov ščira je bil analizirana preko *in vitro* analize kalitve vrtno solate in paprike. Optimalni izvlečki so imeli največjo raznolikost sekundarnih metabolitov in največjo vsebnost celokupnih fenolov in flavonoidov. S TLC in HPLC analizo je bila potrjena značilno manjša vsebnost fenolov v vročinsko obdelanih vzorcih. Z GC-MS analizo je bil ugotovljen širok nabor metabolitov v diklormetanskih izvlečkih (DCM). Fitotoksičnost MW in DCM izvlečkov se je izrazila v značilno zmanjšani kalitvi in rasti solate in paprike, kar kaže na prisotnost več kot ene alelokemične spojine. To lahko posledično vpliva na alelopatsko aktivnost zrnatega ščira med spremembami temperature v okolju.

Ključne besede: *Amaranthus cruentus*; temperatura; stres; fitotoksičnost; metaboliti; fenolne spojine

1 INTRODUCTION

Several studies documented on the increase of secondary compounds or changes in chemical profile within specimens of the same plant species growing under different or environmental stress conditions

(Gobbo-Neto & Lopes, 2007; Ramakrishna & Ravishankar, 2011; Gouvea et al., 2012). The interaction between plants and their environment influence synthesis and accumulation of secondary

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metabolites and their roles as a response to the environment. (Rhoads et al., 2006). The exposure to various environmental stresses can strengthen the allelopathic potential of many plants (Einhellig, 1987, 1996; Gershenzon, 1984; Tang et al., 1995; Kobayashi, 2004) and can affect allelopathy in at least three ways: 1) the production of allelochemicals by the donor species, 2) their bioavailability and 3) modify the effect of an allelochemical on the target plant (Einhellig, 1996, Trezzi et al., 2016).

Amaranth is one of the few multi-purpose crops which can supply grain as well as tasty leafy vegetables of high nutritional quality (Mensah et al., 2008; Maiyo et al., 2010; Nana et al., 2012; Alemayehu et al., 2015). The chemical constituents and medicinal value of amaranth have been well described in the literature (Stintzing et al., 2004; Steffensen et al., 2011; Kraujalis et al., 2013). Most of the reported compounds include: carotenoids, steroids (Maiyo et al., 2010; Oboh et al.,

2008; Bishop & Yokoto, 2001), terpenoids (Connick et al., 1989), ascorbic acid, betacyanins (Cai et al., 1998), α -spinasterol, spinoside, amarantoside, amaracine (Shah, 2005), phenolic compounds (Kraujalis et al., 2013) and saponins (Vincken et al., 2007). Some of these compounds are considered to be allelochemicals which are able to affect surrounding plants once released into the environment (Rice, 1984; Waller, 1987). These phytotoxic compounds offer the opportunity to act as natural herbicides, since there is an increasing need for more cost-effective, safer, and more selective herbicides.

This study evaluated the influence of temperature on plant secondary metabolite production of *A. cruentus* L. and whether the chemical-mediated interaction is involved in *A. cruentus* allelopathy. Thus, phytotoxicity of *A. cruentus* was evaluated with extracts of the different temperature treatments.

2 MATERIALS AND METHODS

2.1 Plant material

Amaranthus cruentus 'Anna' seeds were planted in pots containing a soil-compost (80 : 22 v/v) mixture and grown at 28/21 °C; day/night temperatures in climate controlled chambers at The Department of Agriculture, University of the Free State as described by Allemann et al. (2017). Vegetable seeds used in this study were obtained from Starke Ayres: 'California Wonder' Sweet Pepper and 'Great Lakes' Lettuce.

2.2 Crude extracts

Methanol-water (70 : 30 v/v) and dichloromethane (DCM) were used as solvents. Ten grams of the powdered *A. cruentus* leaf material (oven dried at 40 °C) was extracted twice by shaking overnight in the different solvents (1 : 20 w/v). The pooled extracts were dried and kept at 4 °C until further analyses.

2.3 Allelopathy determination

A combination of the 'sandwich method' of Fujii et al. (2003) and Hill et al. (2007) was used to determine the *in vitro* phytotoxicity of the crude leaf extracts from the different temperature treatments of *A. cruentus* on the vegetable seeds. For this method 5 and 20 mg of each extract was dissolved in 1 ml of their own solvent, and 1 ml pipetted onto a filter paper. The filter papers were allowed to dry then placed on the bottom layer of agar resulting in 0.5 or 2 mg ml⁻¹ extract per well. Controls contain only the solvents on filter paper.

Lettuce (*Lactuca sativa* L.) and pepper (*Capsicum annuum* L.) seeds were surface sterilised as described by Allemann et al. (2017) and each of the experiments was done in triplicate and presented as the mean of the replicates.

2.4 Total phenolic and flavonoid content

Total phenolic content was evaluated in the methanolic extract, using the Folin-Ciocalteu method as reported by Singleton & Rossi (1965). The absorption was measured at 550 nm and the content in phenolics was expressed as mg gallic acid equivalents (GAE) of dry mass extract. Total flavonoid content was determined as reported by Zhishen et al. (1999). The absorption was measured at 510 nm and the content in flavonoids was expressed as mg quercetin equivalents (QE) of dry mass extract.

2.5 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out using silica gel 60 F₄₅₀-aluminium backed pre-coated plates. Extracts (50 mg ml⁻¹) were dissolved in their appropriate extraction solvents and 10 µl applied to the TLC. The mobile phase for development of the MW extracts was chloroform-methanol-water-acetic acid (65:35:5:1), while for the DCM extracts, plates were developed in toluene-ethyl acetate (93:7). Compounds resolved on the plate were visualized using ultraviolet light (UV) at 365 nm and 254 nm, ninhydrin (Piflung, 2006), *p*-anisaldehyde-sulphuric/acetic acid, 5 % ferric chloride and dragendorff reagents, prepared according to

the standard methods described by Wagner & Bladt (1996).

2.6 High Pressure Liquid Chromatography

The MW extracts (20 mg ml⁻¹) were separated and identified through high pressure liquid chromatography (HPLC) by comparing the retention times to standard phenolic compounds. Standards were prepared in methanol (3 mg ml⁻¹) and absorption measured between 200 and 400 nm. Ten micro litre of extracts and 2 µl of standards were injected while the flow rate was kept at 1 ml min⁻¹. A Shimadzu instrument with a Photo Diode Array Detector (PDA) and an elution procedure as described by Vidović et al. (2015) with a C18 column (Phenomenex C18, 250mm × 4.6mm, 5µm diameter), was used to achieve acceptable separation of all compounds. The mobile phase consisted of: A, acetonitrile and B, a mixture of acetic acid- acetonitrile-phosphoric acid-water (10:5:0.1:84:9, by vol.).

2.7 GC-MS analysis

The DCM extracts (10 mg) were dissolved in 1 ml hexane. Analyses was done through GC-MS using a

Shimadzu GC-MS QP-2010 gas chromatography equipped with a DB-5 MS column (30 m length × 0.32 mm diameter × 0.25 µm film thickness) and injecting 1 µl of sample. The GC operating conditions were the following: 5 min at 60 °C, then gradually increased to 280 °C at a rate of 2 °C min⁻¹, and held for 10 min. Helium was used as the carrier gas (1.5 ml min⁻¹ flow rate). Spectra analysis was conducted using the library "National Institute of Standard and Technology (NIST) version 5.0.

2.8 Statistical analysis

The experiments were carried out adopting a completely randomized design with three replications. The results were expressed as means with least significant difference (LSD). Analysis of variance (ANOVA) was performed using SAS 9.3 (Institute Inc., Cary, NC, USA, 2008) statistical programme for data and Tukey-Kramer's LSD procedure for comparison of means. Significance of differences compared to the control groups was determined using the t-test (Steel & Torrie, 1980).

3 RESULTS AND DISCUSSION

3.1 Metabolites

Comparison of the compounds in the MW and DCM leaf extracts of the different temperature treatments of *A. cruentus* plants, are illustrated by TLC in Figure 1. It is clear that temperature played an obvious role in the production of secondary compounds, as clear differences in compounds between the treatments were visible in both the polar and non-polar extracts (Fig. 1A & B). Different compounds with varying R_f values were visible when spraying the TLC's with *p*-anisaldehyde-sulphuric/acetic acid reagent. Colours of compounds range from green, yellow, pink, blue and purple with different R_f values for the polar and non-polar extracts. The diverse coloured compounds with varying R_f values visible in both polar and non-polar extracts on TLC (Fig. 1) may indicate many different compounds, including terpenes, saponins, sugars and flavonoids amongst others (Wagner & Bladt 1996).

In the optimal treatment of the MW extract, 11 compounds were noted, compared to 8 and 5 in the cold and hot treated samples respectively. Prominent spots, including a dark purple (R_f = 0.055), a blue-purple (R_f = 0.49) and a light blue spot (R_f = 0.6) were only present in the optimal extract (Fig. 1A). From these results one can deduce that the stress temperatures, particularly the hot, inhibited the biosynthesis of some of the more polar compounds.

Differences were also visible in the non-polar samples (Fig. 2B), with a noticeable blue coloured compound visible at R_f = 0.83, solely in the hot treatment DCM extract (Fig 1B). Less green pigment, probably chlorophyll was also observed in the hot treatment extract, indicating the effect the hot treatment had on photosynthesis.

Several studies were conducted on the impact of increased temperatures on secondary metabolite production of plants (Morrison & Lawlor, 1999). Phenolic compounds are important and common plant allelochemicals in the ecosystem and the main phenolic compounds are water soluble (Li et al., 2010). Kraujalis et al. (2013) reported on the antioxidant properties and phytochemical composition of amaranth extracts isolated by acetone and methanol-water from plant leaves, flowers, stems and seeds. They found that the methanol-water extract of the leaves possessed the highest antioxidant activities and various phenolic compounds and flavonoids e.g. rutin, nicotiflorin, isoquercitrin, 4-hydroxybenzoic and *p*-coumaric acids were identified as major constituents. In the review article by Mroczek (2015) it is reported that saponins were isolated from a diversity of Amaranthaceae genera and species.

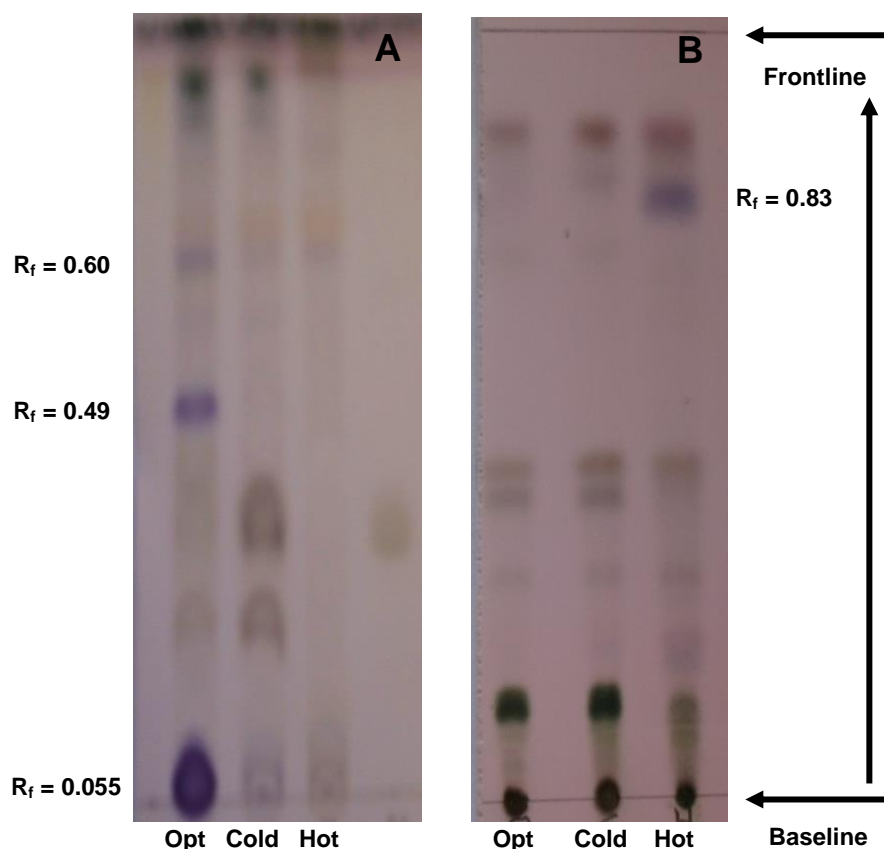


Figure 1: Qualitative TLC profiles of the optimal, cold and hot treated *A. cruentus* MW (A) and DCM (B) leaf extracts. Detection by *p*-anisaldehyde reagent

In this study, the total phenolic and flavonoid content significantly declined in *A. cruentus* plants exposed to hot temperatures compared to plants grown at the optimal temperature (Table 1). The decrease in total phenolic and flavonoid content are in contrast with findings of many authors who reported an increase in production of phytotoxic phenolic compounds in plant tissues exposed to high temperatures and solar radiation (Koeppel et al., 1969; Wender, 1970; Einhelig &

Eckrich, 1984). Rudikovskaya et al. (2008), however reported that low growth temperature decreased the content of some phenolic compounds in pea seedling roots and according to Król et al. (2014), long-term drought stress caused a decrease in particular components of secondary metabolism in the leaves and roots of grapevine. It seems therefore that one cannot expect generalized patterns of phenolic compounds in stress situations.

Table 1: Total phenolic and flavonoid compounds in temperature stressed amaranth leaf material

Treatment	Total phenolic content (mg GAE g D.M ⁻¹ .)	Total flavonoid content (mg QE g D.M ⁻¹ .)
Cold	12.0 b	5.4 a
Optimal	18.8 a	5.6 a
Hot	10.1 b	4.1 b

Analyses by HPLC confirmed the decrease in phenolic compounds in the temperature stressed plants (Fig 2).

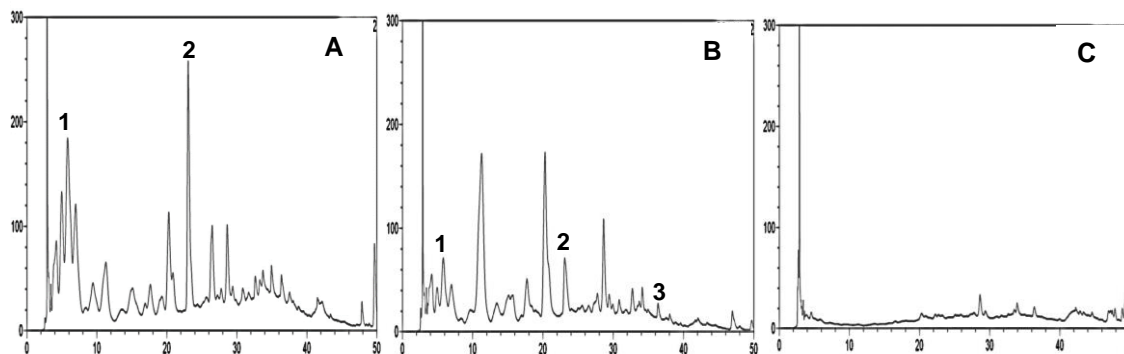


Figure 2: Comparison of HPLC-PDA chromatograms of optimal (A), cold (B) and hot (C) temperature treated *A. cruentus* methanol-water leaf extracts. 1 = Catechin; 2 = Rutin; 3 = Quercetin

Flavonoids are not usually seen as allelopathic compounds but they have other roles in plants such as attractants to pollinators, protection against ultraviolet light (Li et al., 1993) and as an anti-inflammatory, anti-allergic and anti-viral activities (Miller, 1996). Some flavonoids do however have allelopathic properties such as quercetin (Inderjit & Dakshini, 1995), catechin (Bais & Kaushik, 2010; Chobot et al., 2009) and rutin (Basile et al., 2000), which has been found in both *A. hybridus* and *A. cruentus*. From our HPLC results, catechin and rutin were identified in the optimal and cold treated amaranth MW leaf litter extracts (Fig 2A & B), while a small amount of quercetin was detected in only the cold treated sample (Fig 2B). The heat treated sample contained a reduced amount of unidentified compounds (Fig 2C), indicating the role temperature play on the biosynthesis of flavonoids and the possible consequence on allelopathy.

The influence of temperature on the expressed compounds in the different DCM extracts were clearly visible after analyses through gas chromatography and

mass spectrometry (GC-MS). Major compounds made up a total composition of 75.69 % (9 compounds), 90.44 % (7 compounds) and 91.89 % (9 compounds), of the optimal, cold and heat treated samples respectively (Table 2). Neophytadiene and hexadecanoic acid were the only compounds present in all three extracts, although the concentrations of these compounds varied substantially between the treatments (Table 2). The highest concentration of neophytadiene (27.53 %) was found in the cold treated sample, while hexadecanoic acid (13.52 %) was maximum in the heat treatment extract. Squalene, trans-phytol and the phytosterol, stigmasta-7,22-dien-3-ol were present in only cold and heat treated samples.

Gamel et al. (2007) found high squalene concentrations in oil fractions of *A. caudatus* L. and *A. cruentus*, while Shah (2005) reported on the presence of stigmasta-7,22-dien-3-ol (α -spinasterol) in *A. spinosus* L.. According to Szakiel et al. (2010), lower soil temperatures triggered an increase in levels of steroidal furostanol and spirostanol saponins.

Table 2: GC-MS results of compounds present in optimal, cold and hot temperature treated DCM leaf extracts of *A. cruentus*

Retention time (min)	Compound name*	Optimal area %	Cold area %	Hot area %
63.315	16-Heptadecenal	13.22	-	-
64.054	Neophytadiene	9.03	30.70	4.44
65.296	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-	5.19	3.72
66.193	trans-Phytol	-	10.04	6.01
70.621	Hexadecanoic acid (Palmitic acid)	11.01	3.29	13.52
77.243	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	-	-	7.82
78.458	9,12,15-Octadecatrienoic acid (α -Linolenic acid)	-	27.40	29.68
79.695	Octadecanoic acid (Stearic acid)	-	-	2.30
81.156	Tetrahydrofurano[6a,7a-b]-5-oxa-8-thiaphenanthrene	9.86	-	-
97.733	Hexahydrothunbergol	7.07	-	-
102.751	bis-Naphthylfuran	5.47	-	-
103.841	Methyl ester of decyclotrenudine	5.02	-	-
103.992	(-)-18-Noramborx	4.84	-	-
104.067	Benzyl methyl ether	10.17	-	-
104.920	Squalene	-	3.73	6.22
121.002	Stigmasta-7,22-dien-3-ol (α -Spinasterol)	-	10.09	12.36
Total		75.69	90.44	91.89

Area (%) of compound = height of peak x width of peak at $\frac{1}{2}$ height \leq x Total area⁻¹

* Identification by Library: NIST 05. LIB

3.2 Phytotoxicity

Germination

Phytotoxic activity of *A. cruentus* extracts may be ascribed to a wide range of biologically active phytochemicals such as phenolic acids, flavonoids and fatty acids which are known for their phytotoxic and allelochemical activities. When these compounds are released into the soil by leaf litter decomposition there may be a change in both the physical and chemical

properties and therefore affecting the organization and growth of plant communities. At different concentrations both the MW and DCM extracts of the different temperature treatments, significantly inhibited germination of both lettuce and pepper ($LSD_{(T \leq 0.05)} = 1.88$) (Table 3). The polar MW extract was more effective in lowering germination percentages in both pepper and lettuce than the non-polar DCM extract (Table 3).

Table 3: Germination percentage of pepper and lettuce seeds exposed to increasing concentrations of MeOH-H₂O

Extract [mg ml ⁻¹]	Germination % of pepper				Extract [mg ml ⁻¹]	Germination % of lettuce			
	temperature treatment					temperature treatment			
MW	Optimal	Cold	Hot	Ave	MW	Optimal	Cold	Hot	Ave
0	100	100	100	100	0	100	100	100	100
0.5	29	24	29	27	0.5	24	29	31	28
2	29	24	33	28	2	24	24	36	28
Ave	52	49	54		Ave	49	51	56	
DCM					DCM				
0	100	100	100	100	0	100	100	100	100
0.5	49	29	56	44	0.5	69	67	42	59
2	40	22	47	36	2	42	24	36	34
Ave	63	50	68		Ave	70	64	59	

LSD_(T ≤ 0.05) = 1.88. n = 96

Growth

Both the hypocotyl and seminal root were significantly inhibited when exposed to extracts of all the temperature treatments, however the cold stress treatment was the most detrimental (Table 4 & 5). The results also confirmed that root elongation was more sensitive to allelochemicals than stem (hypocotyl) elongation in both species. Furthermore, both polar and non-polar extracts, at 0.5 and 2 mg ml⁻¹, significantly reduced the growth of lettuce (Table 4) and pepper (Table 5). Allelopathy influences plant succession through root exudation, leaching and volatilization when the plant dies and starts to decompose (Rice 1984; Weston 2005; Minorsky 2002; Bertin et al. 2003). The most frequently reported morphological effects from allelochemicals on sensitive plants is the inhibition or retarded seed germination and retarded development of shoots and roots (Ghafarbi et al. 2012). It has been cited in literature that allelopathy was involved in many natural and manipulated ecosystems and that they play a role in the evolution of different plant communities (Ding et al., 2007). Abiotic stresses can lead to morphological, physiological, biochemical and molecular changes within the plants, and therefore has an impact on plant growth (Wang et al., 2003). Climate change and temperature play a role in the synthesis of

allelochemicals, which can affect the growth processes of neighbouring plants (Li et al., 2010). Results by Amini (2009, 2012), proved that root exudates of *A. retroflexus* had inhibitory effects on shoot length of both crop (wheat) and vegetable (common bean) plants. Aqueous extracts from the leaves, roots and stems of *A. retroflexus* L. had inhibitory effects on the hypocotyl growth of maize (Konstantinović et al., 2014). Dhole et al. (2013) noticed that seed germination and seedling growth of maize were inhibited when using aqueous extracts from the root, stems and leaves of *A. tricolor* L..

Phenolic compounds take part in the regulation of seed germination and work together in regulating the growth of plants. The role of several phenolic compounds e.g. lignin, salicylic acid, flavonoids and phytoalexins play important roles in plant resistance, taking part in defence responses during biotic and abiotic stress (Kulbat, 2016). It is therefore possible that the reduction in phenolic compounds caused by temperature stress, could play a role in the germination and growth of lettuce and pepper seeds. Furthermore, it is clear from both literature and our results, that concentration plays a major role in the severity of allelopathic effects on different plants (Qasem, 1995; Obaid & Qasem, 2005).

Table 4: Hypocotyl and seminal root lengths of lettuce seeds exposed to increasing concentrations of MeOH-H₂O and DCM leaf extracts of *A. cruentus* grown at optimal, cold and hot temperatures

Extract [mg ml ⁻¹]	Hypocotyl length of Lettuce (mm)				Seminal root length of Lettuce (mm)			
	Temperature treatment				Temperature treatment			
MeOH-H ₂ O	Optimal	Cold	Hot	Ave	Optimal	Cold	Hot	Ave
0	28.77±9.07	28.77±9.07	28.77±9.07	28.77 a	26.31±11.2	26.31±11.2	26.31±11.2	26.31 a
0.5	26.77±16.5	29.15±17.8	31.69±11.4	29.03 a	17.54±12.5	13.23±7.25	15.08±6.45	15.28 b
2	20.62±12.8	6.31±9.46	13.08±7.99	13.34 b	8±7.43	3.15±3.31	3.85±2.44	5 c
Ave	25.39	21.41	24.51		17.28	14.23	15.08	
LSD _(T≤0.05)	T = ns	C = .23	T×C =		T = ns	C = .39	T×C = ns	
DCM								
0	35.48±11.7	35.48±11.7	35.48±11.7	35.48 a	27.63±12.4	27.63±12.1	27.62±11.8	27.63 a
0.5	28.85±6.27	17.31±9.29	9.77±4.64	18.64 b	19.62±5.04	15.77±9.49	8±4.16	14.46 b
2	26.77±7.93	7.08±8.75	25.38±7.79	19.74 b	14.08±5.99	9.08±8.84	16.69±6.71	13.28 b
Ave	29.79	20.54	23.54		19.49	17.82	17.44	
LSD _(T≤0.05)	T = 4.89	C = 4.89	T×C = 4.89		T = ns	C = .66	T×C = 4.66	

Different letters along the column indicate significant differences at T ≤ 0.05 (Tukey test). Significant differences within each vegetable; n = 13.

Table 5: Hypocotyl and seminal root lengths of pepper seeds exposed to increasing concentrations of MeOH-H₂O and DCM leaf extracts of *A. cruentus* grown at optimal, cold and hot temperatures

Extract [mg ml ⁻¹]	Hypocotyl length of pepper (mm)				Seminal root length of pepper (mm)			
	Temperature treatment				Temperature treatment			
MeOH-H ₂ O	Optimal	Cold	Hot	Ave	Optimal	Cold	Hot	Ave
0	20.23±15.3	20.23±15.3	20.23±15.3	20.23 a	13.92±5.02	13.92±5.02	13.92±5.02	13.92 a
0.5	2.69±4.55	5.62±3.91	11.31±7.78	6.54 b	1.15±2.54	3.31±3.28	9.85±8.71	4.77 b
2	1.08±1.80	3.46±2.26	5.15±1.99	3.23 b	0.46±0.88	1.08±0.76	3.46±4.74	1.67 c
Ave	8	9.77	12.23		5.18	6.10	9.08	
LSD _(T≤0.05)	T=ns	C=4.35	T×C=ns		T=2.29	C=2.29	T×C=2.29	
DCM								
0	16.08±6.21	16.08±6.21	16.08±6.21	16.08 a	24.38±11.4	24.38±11.4	24.38±11.4	24.38 a
0.5	6.31±2.56	4.31±5.75	2.46±1.61	4.36 b	12.77±3.68	2.85±3.74	1.92±0.86	5.85 b
2	3.69±2.66	4.31±5.02	8.77±7.33	5.59 b	5±5.74	6.46±8.90	5.85±6.01	5.77 b
Ave	8.69	8.23	9.10		14.05	11.23	10.72	
LSD _(T≤0.05)	T = ns	C = 2.78	T×C = 2.78		T = ns	C = 3.87	T×C = 3.87	

Different letters along the column indicate significant differences at T ≤ 0.05 (Tukey test). Significant differences within each vegetable; n = 13.

Many studies have been done on allelopathy of the polar extracts of amaranth, but no information is available on the non-polar compounds. This is the first report on the *in vitro* phytotoxicity of a DCM extract of grain amaranth. The characteristics of allelochemicals are

important and play a significant role in their fate in the environment. For example, the mobility of compounds within the soil are influenced by their water solubility; the vapour pressure can impact their volatilization and their chemical structure can affect their affinity with the

soil surface (Souza Filho & Alves 2002). The outcome of all these complex interactions results in compounds

with allelopathic properties which can be of potential agronomic use.

4 CONCLUSION

Temperature influenced the chemical composition of *A. cruentus* and *in vitro* bioassays proved the negative impact of the extracts on germination and growth of vegetables. This demonstrated that the environment for the cultivation of *A. cruentus* is important and that more than one compound were responsible for allelopathy, thus both polar and non-polar compounds were involved. Furthermore, with increased concentrations of extracts a decrease in germination and seedling development occurred. Consequently, if more plant residues are left behind in the soil, the growth of the

next crop will often be affected with a subsequent decline in yield. It was also clear that vegetables displayed diversity in reaction towards the temperature treatments and type of extract. This information proves that a holistic understanding of the influence of abiotic environmental factors on the production of metabolites in various plant parts are of importance. The release of potentially phytotoxic compounds from *A. cruentus* leaf litter into soil deserves further investigation as well as the purification of extracts to determine unidentified natural compounds with herbicidal activity.

5 REFERENCES

- Allemann, I., Cawood, M.E., Allemann, J. (2017). Influence of altered temperatures on allelopathic properties of *Amaranthus cruentus* L. *Acta agriculturae Slovenica*, 109(2), 465-471. doi:10.14720/aas.2017.109.2.29
- Alemayehu, F.R., Bendevis, M.A., Jacobsen, S.E. (2015). The potential for utilizing the seed crop Amaranth (*Amaranthus* spp.) in East Africa as an alternative crop to support food security and climate change mitigation. *Journal of Agronomy and Crop Science*, 201, 321-329. doi:10.1111/jac.12108
- Amini, R., An, M., Pratley, J., Azimi, S. (2009). Allelopathic assessment of annual ryegrass (*Lolium rigidum*): Bioassays. *Allelopathy Journal*, 24(1), 67-79.
- Amini, R., Movahedpour, F., Ghassemi-Golezani, K., Mohammadi-Nasab, A.D., Zafarani-Moattar, P. (2012). Allelopathic assessment of common amaranth by ECAM. *International Research Journal of Applied & Basic Sciences*, 3(11), 2268-2272.
- Bais, H.P., Kaushik, S. (2010). Catechin secretion & phytotoxicity. *Communicative & integrative Biology*, 3(5), 468-470. doi:10.4161/cib.3.5.12559
- Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Cobiainchi, R.C., Vuotto, M.L., Ferrara, L. (2000). Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia* 7, Suppl., S110-S116. doi:10.1016/S0367-326X(00)00185-4
- Bertin, C., Yang, X., Weston, L.A. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil*, 256, 67-83. doi:10.1023/A:1026290508166
- Bishop, G.J., Yokota, T. (2001). Plants steroid hormones, brassinosteroids: current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. *Plant and Cell Physiology*, 42(2), 114-120. doi:10.1093/pcp/pce018
- Cai, Y., Sun, M., Corke, H. (1998). Colorant properties and stability of *Amaranthus* betacyanin pigments. *Journal of Agricultural and Food Chemistry*, 46(11), 4491-4495. doi:10.1021/jf980457g
- Chobot, V., Huber, C., Trettenhahn, G., Hadacek, F. (2009). (±)-Catechin: Chemical weapon, antioxidant, or stress regulator? *Journal of Chemical Ecology*, 35(8), 980-996. doi:10.1007/s10886-009-9681-x
- Connick, W.J., Bradow, J.M., Legendre, M.G. (1989). Identification and bioactivity of volatile allelochemicals from Amaranth residues. *Journal of Agricultural and Food Chemistry*, 37, 792-796. doi:10.1021/jf00087a049
- Dhole, J.A., Lone, K.D., Dhole, G.A., Bodke, S.S. (2013). Allelopathic effect of aqueous and ethanolic extracts of some common weeds on seed health of *Triticum aestivum* L. (Wheat). *International Journal of Current Microbiology and Applied Sciences*, 2(6), 254-260.
- Ding, J., Sun, Y., Xiao, C.L., Shi, K., Zhou, Y.H., Yu, J.Q. (2007). Physiological basis of different allelopathic reactions of cucumber and fig leaf gourd plants to cinnamic acid. *Journal of*

- Experimental Botany, 58(13), 3765-3773. doi:10.1093/jxb/erm227
- Einhellig, F.A., Eckrich, P.C. (1984). Interactions of temperature and ferulic acid stress on grain sorghum and soybeans. *Journal of Chemical Ecology*, 10, 161-170. doi:10.1007/BF00987653
- Einhellig, F.A. (1987). Interactions among allelochemicals and other stress factors of the plant environment. *ACS Symposium Series*, 330, 343-357. doi:10.1021/bk-1987-0330.ch032
- Einhellig, F.A. (1996). Interactions involving allelopathy in cropping system. *Agronomy Journal*, 88, 886-893. doi:10.2134/agronj1996.00021962003600060007x
- Fujii, Y., Parvez, S.S., Parvez, M.M., Ohmae, Y., Iida, O. (2003). Screening of 239 medical plant species for allelopathic activity using the sandwich method. *Weed Biology and Management*, 3, 233-241. doi:10.1046/j.1444-6162.2003.00111.x
- Gamel, T.H., Mesallam, A.S., Damir, A.A., Shekib, L.A., Linszen, J.P. (2007). Characterization of Amaranth seed oils. *Journal of Food Lipids*, 14, 323-334. doi:10.1111/j.1745-4522.2007.00089.x
- Gershenson, J. (1984). Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Advances in Phytochemistry*, 18, 273-320. doi:10.1007/978-1-4684-1206-2_10
- Ghafari, S.P., Hassannejad, S., Lotfi, R. (2012). Allelopathic effects of wheat seed extracts on seed and seedling growth of eight selected weed species. *International Journal of Agricultural Research*, 4(19), 1452-1457.
- Gobbo-Neto, L., Lopes, N.P. (2007). Medicinal plants: factors of influence on the content of secondary metabolites. *Quimica Nova*, 30(2), 374-381. doi:10.1590/S0100-40422007000200026
- Gouvea, D.R., Meloni, F., Ribeiro, A.B.B., Lopes, J.L.C., Lopes, N.P. (2012). A new HPLC-DAD-MS/MS method for the simultaneous determination of major compounds in the crude extract of *Lychnophora salicifolia* Mart. (Brazilian arnicão) leaves: Application of chemical variability evaluation. *Analytica Chimica Acta*, 748, 28-36. doi:10.1016/j.aca.2012.08.027
- Hill, E.C., Ngouajio, M., Nair, M.G. (2007). Allelopathic potential of hairy vetch (*Vicia villosa*) and cowpea (*Vigna unguiculata*) methanol and ethyl acetate extracts on weeds and vegetables. *Weed Technology*, 21, 437-444. doi:10.1614/WT-06-167.1
- Inderjit, A.U., Dakshini, K.M.M. (1995). On laboratory bioassays in allelopathy. *The Botanical Review*, 61, 28-44. doi:10.1007/BF02897150
- Kobayashi, K. (2004). Factors affecting phytotoxic activity of allelochemicals in soil. *Weed Biology and Management*, 4, 1-7. doi:10.1111/j.1445-6664.2003.00112.x
- Koeppe, P.E., Rohrbaugh, L.M., Wender, S.H. (1969). The effect of varying UV intensities on the concentration of scopolin and caffeoylquinic acids in tobacco and sunflower. *Phytochemistry*, 8, 889-896. doi:10.1016/S0031-9422(00)85879-3
- Konstantinović, B., Blagojević, M., Konstantinović, B., Samardžić, N. (2014). Allelopathic effect of weed species *Amaranthus retroflexus* L. on maize seed germination. *Romanian Agricultural Research*, 31, 315-321.
- Kraujalis, P., Venskutonis, P.R., Kraujaliene, V., Pukalskas, A. (2013). Antioxidant properties and preliminary evaluation of phytochemical composition of different anatomical parts of Amaranth. *Plant Foods for Human Nutrition*, 68, 322-328. doi:10.1007/s11130-013-0375-8
- Król, A., Amarowicz, R., Weidner, S. (2014). Changes in the composition of phenolic compounds and antioxidant properties of grapevine roots and leaves (*Vitis vinifera* L.) under continuous of long-term drought stress. *Acta Physiologiae Plantarum*, 36, 1491-1499. doi:10.1007/s11738-014-1526-8
- Kulbat, K. (2016). The role of phenolic compounds in plant resistance. *Biotechnology and Food Sciences*, 80(2), 97-108.
- Li, J., Ou-Lee, T.M., Raba, R., Amundson, R.G., Last, R.L. (1993). Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. *The Plant Cell*, 5(2), 171-179. doi:10.1105/tpc.5.2.171
- Li, Z-H., Wang, O., Xiao, R., Cun, D.P., Jiang, D-A. (2010). Phenolics and plant allelopathy. *Molecules*, 15, 8933-8952. doi:10.3390/molecules15128933
- Maiyo, Z.C., Ngure, R.M., Matasyoh, J.C., Chepkorir, R. (2010). Phytochemical constituents and antimicrobial activity of leaf extracts of three Amaranthus plant species. *African Journal of Biotechnology*, 9(21), 3178-3182.
- Mensah, J.K., Okoli, R.I., Ohaju-Obodo, J.O., Eifediyi, K. (2008). Phytochemical, nutritional and medical properties of some leafy vegetables consumed by Edo people of Nigeria. *African Journal of Biotechnology*, 7(14), 2304-2309.

- Miller, A.L. (1996). Antioxidant flavonoids: structure, function and clinical usage. *Alternative Medicine Review*, 1(2), 103-111.
- Minorsky, V. (2002). Allelopathy and grain crop production. *Plant Physiology*, 130, 1745-1746. doi:10.1104/pp.900057
- Morrison, J.I.L., Lawlor, D.W. (1999). Interactions between increasing CO₂ concentration and temperature on plant growth. *Plant, Cell & Environment*, 22, 659-82. doi:10.1046/j.1365-3040.1999.00443.x
- Mroczek, A. (2015). Phytochemistry and bioactivity of triterpene saponins from Amaranthaceae family. *Phytochemistry Reviews*, 14, 577-605. doi:10.1007/s11101-015-9394-4
- Nana, F.W., Hilou, A., Millogo, J.F., Nacoulma, O.G. (2012). Phytochemical composition, antioxidant and xanthine oxidase inhibitory activities of *Amaranthus cruentus* L. and *Amaranthus hybridus* L. extracts. *Pharmaceuticals*, 5(6), 613-628. doi:10.3390/ph5060613
- Obaid, K.A., Qasem, J.R. (2005). Allelopathic activity of common weed species on vegetable crops grown in Jordan. *Allelopathy Journal*, 15(2), 221-236.
- Oboh, G., Raddatz, H., Henle, T. (2008). Antioxidant properties of polar and non-polar extracts of some tropical green leafy vegetables. *Journal of the Science of Food and Agriculture*, 88, 2486-2492. doi:10.1002/jsfa.3367
- Qasem, J.R. (1995). The allelopathic effect of three *Amaranthus* spp. (pigweeds) on wheat (*Triticum durum*). *Weed Research*, 35(1), 41-49. doi:10.1111/j.1365-3180.1995.tb02015.x
- Pifrung, M.C. (2006). *The Synthetic Chemists Companion*. Wiley. Online Library. <http://www.onlinelibrary.wiley.com/book/10.1002/9780470141045> (15/09/2013).
- Ramakrishna, A., Ravishankar, G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, 6(11), 1720-1731. doi:10.4161/psb.6.11.17613
- Rhoads, D.M., Umbach, A.L., Subbaiah, C.C., Siedow, J.N. (2006). Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiology*, 141, 357-366. doi:10.1104/pp.106.079129
- Rice, E.L. (1984). *Allelopathy*. 2nd ed. Orlando, Florida, USA: Academic Press; p. 67-68.
- Rudikovskaya, E.G., Fedorova, G.A., Dudareva, L.V., Makarova, L.E., Rudikovskii, A.V. (2008). Effect of growth temperature on the composition of phenols in pea roots. *Russian Journal of Plant Physiology*, 55(5), 712-715. doi:10.1134/S1021443708050178
- Shah, A.H.A. (2005). Spinoside, new coumaroyl flavone glycoside from *Amaranthus spinosus*. *Archives of Pharmacal Research*, 27(12), 1216-1219.
- Singleton, V.L., Rossi, Jr J.A. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16, 144-158.
- Souza Filho, A.P.S., Alves, S.M. (2002). *Allelopathy: Basic principles and general aspects*. EMBRAPA Amazônia Oriental: Belém, Brazil, p. 206.
- Steel, R.G.D., Torrie, J.H. (1980). *Principles and procedures of statistics*, 2nd ed. New York: McGraw-Hill; p. 633.
- Steffensen, S.K., Rinnan, Å., Mortensen, A.G., Laursen, B., de Troiani, R.M., Noellemeyer, E.J., Janovska, D., Dusek, K., Délano-Frier, J., Taberner, A., et al. (2011). Variations in the polyphenol content of seeds of field grown *Amaranthus* genotypes. *Food Chemistry*, 129, 131-138. doi:10.1016/j.foodchem.2011.04.044
- Stintzing, F.C., Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology*, 15, 19-38. doi:10.1016/j.tifs.2003.07.004
- Szakiel, A., Paczkowski, C., Henry, M. (2010). Influence of environmental abiotic factors on the content of saponins in plants. *Phytochemistry Reviews*, 10, 471-491. doi:10.1007/s11101-010-9177-x
- Tang, C-H., Cai, W-F., Kohl, K., Nishimoto, R.K. (1995). Plant stress and allelopathy. In: Inderjit AU, Dakshini KMM, Einhellig FA, editors. *Allelopathy, Organisms, Processes and Applications*. American Chemical Society: Washington, DC; p. 142-157.
- Trezzi, M.M., Vidal, R.A., Balbinot, Junior A.A., Bittencourt, H.H., da Silva Souza Filho, A.P. (2016). Allelopathy: driving mechanisms governing its activity in agriculture. *Journal of Plant Interactions*, 11(1), 53-60. doi:10.1080/17429145.2016.1159342
- Vidović, M., Morina, F., Milić, S., Jovanović, S.V. (2015). An improved HPLC-DAD method for simultaneously measuring phenolics in the leaves of *Tilia platyphyllos* and *Ailanthus altissima*. *Botanica Serbica*, 39(2), 177-186.
- Vincken, J.P., Heng, L., De Groot, A., Gruppen, H. (2007). Saponins, classification and occurrence in

- the plant kingdom. *Phytochemistry*, 68, 275–97. doi:10.1016/j.phytochem.2006.10.008
- Wagner, H., Bladt, S. (1996). *Plant Drug Analysis: A thin layer chromatography atlas*. 2nd ed. Springer-Verlag Berlin Heidelberg, Germany; p. 384. doi:10.1007/978-3-642-00574-9
- Waller, G.R. (1987). *Allelochemicals: role in agriculture and forestry*. ACS Symposium Series, 330, American Chemical Society, Washington, D. C. doi:10.1021/bk-1987-0330
- Wang, W., Vinocur, B., Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, 218, 1-14. doi:10.1007/s00425-003-1105-5
- Wender, S.H. (1970). Effects of some environmental stress factors on certain phenolic compounds in tobacco. *Recent Advances in Phytochemistry*, 3, 1-29.
- Weston, L.A. (2005). History and current trends in the use of allelopathy for weed management. *Hort Technology*, 15(3), 529-534.
- Zhishen, J., Mengcheng, T., Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64, 555-559. doi:10.1016/S0308-8146(98)00102-2