International Journal of Bio-resource and Stress Management 2015, 6(2):182-191 DOI: 10.5958/0976-4038.2015.00031.7

Full Research Article

Effect of Sorghum Allelochemicals on the Mortality and Egg Hatching of Root-Knot Nematode, *Meloidogyne javanica*

Lalit Kumar*, Bansa Singh and Ummed Singh

ICAR-Indian Institute of Pulses Research, Kanpur (208 024), India

Article History

Manuscript No. AR815
Received in 25th June, 2014
Received in revised form 5th February, 2015
Accepted in final form 20th March, 2015

Correspondence to

**E-mail*: lalit1997@ymail.com

Keywords

Allelochemicals, allelopathy, *Meloidogyne javanica*, root exudates, sorghum

Abstract

Root exudates of sorghum plants collected via root exudates trapping system after proper fractionation, give rise a total of five allelofractions of different polarity range i.e., polar to non-polar. The fractions were found to enrich about one major compound in each which was extracted out in purity of 95% by repeatedly following the fractional crystallization and column chromatographic techniques. All these five compounds viz., C, B, A, E and D were converted to three Emulsifiable Concentrate (EC) and two Emulsive Water (EW) formulations. Laboratory bioassay experiments conducted during 2009 and 2010 at IIPR, Kanpur revealed that the Polarity of the compounds used in formulations play key role in imparting toxicity against second stage juveniles (J2) of *Meloidogyne javanica*. EC formulation of fractions C and B (non-polar) was found 100% toxic to the juveniles at 300 and 700 µg ml^{-1} concentrations, respectively at the 24 and 48 hours of incubation. EC and EW formulations of the fractions A and E (medium polarity) showed 100% toxicity at 1100 and 1500 µg ml^{-1} concentrations respectively, at an incubation period of 72 h, while EW formulation of fraction D (completely polar) found almost ineffective in all its test concentrations ranging from 1000-2000 µg ml^{-1}. EC of C (highly non-polar) at 250 µg ml^{-1} also retarded in egg hatching of test nematode up to the extent of 98%. IR, ^{1}HNMR, ^{13}CNMR of C and B showed flavanol skeleton as a basic chemical moiety in their chemical structures, however, both differ structurally in respect of their functional substituents and the position of attachment at flavanol skeleton

1. Introduction

The root-knot nematodes (*Meloidogyne species*) are sedentary endoparasites and most damaging agricultural nematode pest, attacking a wide range of crops. Second stage juveniles hatched from the eggs in egg masses laid by females on the infected roots initiate the penetration causing the infection. Nematode infestation is considered to be a prime constraint in realizing optimum yield potential of cultivated crops causing approximately 15-20% yield losses (Sasser and Frackman, 1987). Cultivation of resistant varieties, cultural practices such as crop rotation, cover cropping, green mannuring, organic amendments, chemical nematicides etc. have been advocated for management of nematode infestation but the application of nematicides provides short term measures as chemicals are reported to induce resistance in target species and also possess severe environmental hazards (Taylor, 2000). Since most of the management practices have their own limitations and not yielded desirable results hence need was always felt to develop some of the alternative control options. Now a days allelopathic approaches is being emerging as an effective tool for successful management of nematodes as the exudates of numerous plants or extracts of their various parts were found to contain nematicidal or nematostatic compounds (Devine and Jones, 2003; Rodger et al., 2003; Ruhm et al., 2003). In this respect, in several field experiments, sorghum when grown under crop rotation or as a mixture of crops was reported to curtail nematode populations tremendously (Fay and Duke, 1977; Colbran, 1979; Mc Sorley et al., 1994a and 1994b). Though the effect of this crop is blamed to be of its non-host or poor host properties and release of hydrocyanic acid after degradation of dhurin but practically no effort has been made so far to isolate and assess the allelopathic potential of root exudates released by intact live plants during their life cycle which can be more potent inhibitor and at the same time may also be exploited effectively and easily as natural nematicides. Further generated information may also be utilized as a lead for development of more potent nematicides. With this view present study was

undertaken to isolate and chemically characterized the root exudates from intact live plants of sorghum and to assess the effect of isolated compounds against second stage juveniles and egg hatch of *Meloidogyne species* nematodes.

2. Materials and Methods

Our own developed, root exudates trapping system was employed to collect root exudates of intact live plants of sorghum (Kumar, 2004; Kumar and Varshney, 2008)., approximately hundred liters of root zone water was collected periodically from 50-60 plants of sorghum grown in 20 sets of root exudates trapping systems till 120-150 days (Figure 1). Initially, entire collected root zone water was slowly reduced to 15-20% of the original volume by evaporation under fan at room temperature and then the concentrated root zone was transferred to a separating funnel and partitioned with equal amount of ethyl acetate. In this process allelocompounds got fractionated into two major groups viz., polar (water layer) and non-polar (ethyl acetate layer). Different allelocompounds present in both the layers were recovered by fractionally crystallizing the layers in suitable polarity of solvents as per the fractionation scheme represented in Figure 2.

VERTEX-70 Infra Red Spectrometer and 500 MHz JEOL make DELTA 2 NMR spectrometer was used. ^{1}HNMR, spectra were recorded on δ ppm (0-10) scale with end of sweep at 0 ppm by using pulse programme x_pulse 6.625(US) with number of scan 16. In both the cases samples were analyzed at ambient temperature using deuterated chloroform ($CDCl_3$) as solvent.

For bioassay 10% emulsifiable concentrate (EC) and emulsion concentrate (EW) formulations were prepared by using uniformly the tween-80 (10%) as emulsifier and cyclohexanone (80%) and water (80%) as a solvent for EC and EW formulations, respectively. Non-polar compounds recovered from the further fractionation of non-polar fraction named as C, B and A were converted into EC formulations whereas, polar compounds recovered from polar fraction and named as D and E were converted into EW formulations. Both the formulations were emulsified thoroughly by agitating the mixtures at 45±2 °C for an hour by using REMI mechanical stirrer.

Egg masses of the root-knot nematode (*Meloidogyne javanica*) were collected from the infected chickpea roots and further cultured on chickpea plants to maintain regular population of the juveniles during experimentation. Experiment was conducted on freshly hatched second stage juveniles in 50 mm diameter petriplates of approximately 15ml capacity. Initially 9ml distilled water was poured in each test petriplates then required quantity of test EC formulation was added in accurate amount to get the desire concentration in final volume of 10ml and mixed well. One ml of freshly hatched second stage juveniles of *M. javanica* suspension containing approximately 300-400 in number was added to each petriplate and kept at 25±1 °C for 24, 48 and 72 h. All the treatments were replicated thrice along with a set of control containing formulation auxiliaries viz. cyclohexanone and tween-80 similarly prepared. After specified period of incubation, the treated suspension in petriplates was stirred properly and 1ml suspension was transferred to another petriplate. It was diluted 10 times with distilled water to reduce the concentration of chemical much below to its toxic level. To observe the revival possibility if any, observations on live and dead nematodes were recorded after 24 h of dilution by using stereoscope microscope. Dead nematodes appeared straight, while live nematodes retained the characteristic sigmoid shape and exhibited movement (Sethi and Prasad, 1982). The per cent mortality was worked out from the average of three replications in each case and converted to natural mortality according to Abbott's formula (Abbott, 1925). Effect of most active formulation was also observed on egg hatch of *Meloidogyne javanica*. Replicated bioassay tests for egg hatch were conducted by using different concentrations viz., 50, 100, 150, and 200 ppm of test EC formulation. Five egg masses having 600-700 hundred eggs in each were kept in each petriplate. A separate set of three petriplates containing five egg masses in each with formulation auxiliaries was kept as control. Observations on egg hatch were recorded after incubation period of 1, 3, 5, 7, 9, 11, 13, 15 and 18 days.

3. Results and Discussion

3.1. Recovery of allelocompounds and chemistry

Figure 1: Sorghum grown in root exudates trapping system

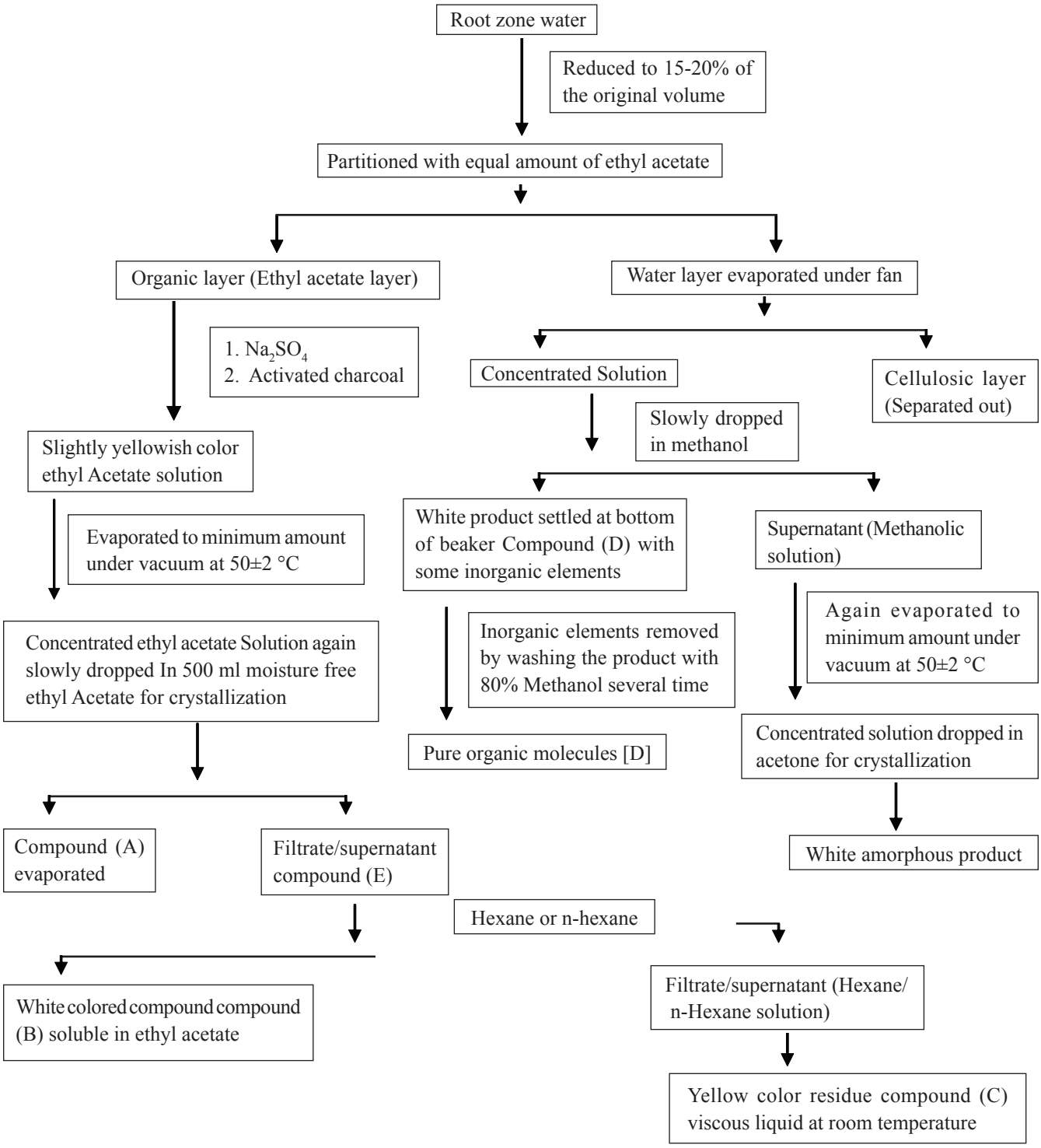

Figure 2: Partitioned and fractional crystallization technique adopted for extraction and fractionation of sorghum root exudates.

By repeatedly following the proposed fractionation scheme the allelochemicals were successfully fractionated into five different groups of compounds of distinct polarity viz., A, B, C (non-polar i.e. organic layer), D and E (polar, aqueous layer) with more than 95% purity as a single component as recorded by HPLC. Three compounds i.e. C, B and A with R_F values 0.86, 0.616 and 0.313 were detected on TLC as major component of non-polar fraction i.e. ethyl acetate layer. Whereas, three compounds under the R_F zones viz., 0.86 and 0.527 (acetone: methanol 65: 35) A & E and 0.360 (methanol: water 80:20) D were detected clearly in polar fraction (water layer). During fractional crystallization of ethyl acetate layer

in hexane, compound B and A were crystallized and hence recovered by filtration. Compound C remained soluble in hexane phase (filtrate) was recovered by evaporation as brown color viscous liquid at room temperature. Both A and B were successfully separated out from each other by using chromatographic columns. Rest of the two compounds viz., D (R_F 0.360) and E (R_F 0.527) present in water layer were recovered and separated out by following the repeatedly fractionation. Compound E was found completely soluble in methanol and water whereas, compound D was found completely soluble in water only. Two compounds viz., A and B were successfully purified to maximum extent by using preparative TLC and instrumentally analyzed to illustrate their chemical structures. IR, 500 MH_z ^{1}HNMR and ^{13}CNMR spectrum as illustrated in figure 3 and 4 for the compounds of C and B respectively, showed characteristic signals in favor of the flavonol skeleton as a major chemical moiety in their chemical structures. Thus, IR Cm^{-1} signals located at 3439.62 (brs. O-H str.), 2958.30, 2926.94 & 2854.80 (sym and asym bending vibrations in aromatic and aliphatic bonds), 1729.05 (C=O str.), 1600.19, 1580.86, 1463.53 (C=C str. in aromatic rings), 1286.37, 1122.24 and 1073.74 (str. C-O-C bonds), 963.21 and 942.84 (bending vibrations of substituted aromatic rings) in case of compound C and IR Cm^{-1} 3492.30 (brs. chelated O-H str.), 3015.49, 2918.83 and 2821.66 (sym and asym bending vibrations in aromatic and aliphatic bonds), 2821.66, 2709.94, 2647.60 and 2544.24 (hump, may be due to the aldehydic or acidic groups), 1922.89, 1661.20 (C=O str.), 1588.59, 1513.26 and 1424.10 (C=C str. in aromatic rings), 1394.95, 1321.67 and 1284.46 (bending vibrations in alkane and alkenes), 1254.51, 1169.39, 1108.39 and 110.96 (str. C-O-C bonds), 914.54 (bending vibration of substituted aromatic rings) in case of compound B were structurally identified them as closely related flavanol structures, concluded by comparison of their spectral data with published standard values as well as the spectral data of already known compounds of sorghum. Comparison of IR spectrum of B with that of C though showed similarities at many places but differing only between IR region of 2800-2500. Unlike C in case of B, appearance of a hump by retaining four extra peaks at 2821.66, 2709.94, 2647.60 and 2544.24 Cm^{-1} is a clear indication of presence of either the CHO or the COOH group. This helps in concluding that both of them differ from each other in respect of functional substituents and their position of attachment at the flavanol skeleton. ^{1}HNMR and ^{13}CNMR spectrums of both of the compounds i.e. C and B fully supported the views of IR analysis and also showed the pattern as structurally assigned and expected as flavanol structures. Thus double duplet signals located at δ 7.4 and 7.2 in case of compound C under ^{1}HNMR which crosses 13 different signals between δ 145-107 ppm under ^{13}CNMR was evidently ascribable to different H and C of flavanol and attached aromatic rings. Other signals located at much up field viz., δ 3.7, 2.4, 1.8, 1.4, 1.2 and 0.8 ppm in the ^{1}HNMR spectrum of C indicated the substitution of highly saturated aliphatic chains that seems to be accompanied with some functional groups at different places of flavanol and other aromatic rings. ^{1}HNMR and ^{13}CNMR analysis of compound B also favored in flavanol skeleton in the chemical structure of the molecule. In this case also, three multipletes at δ 7.8, 7.6 and 7.4 ppm under ^{1}HNMR spectrum which crosses 13 different signals between δ 171-111 ppm under ^{13}CNMR was evidently, ascribable to different H and C of flavanol and attached aromatic rings. Signal at δ 171 can evidently, be assignable to the carbonyl carbon of attached acidic or aldehydic functional group. Likewise, other signals located at much up field of ^{1}HNMR and ^{13}CNMR spectrums of compound B indicated the substitution of highly saturated aliphatic chains accompanied with some functional groups at different places of flavanol and other aromatic rings. Present analytical results in favor of flavanol skeleton in the chemical structures of both of the isolated compounds are in good agreement with several other findings by which sorghum was reported as a major source of 3-deoxyanthocyanidins (contained flavanol skeleton), structurally related to the anthocyanin pigments which either act as a seed pigments and as phytoalexins responding to pathogen attack (Joseph et al., 2004; Chun et al., 2007; Liyi et al., 2009). Apart from this a high level of accumulation of 3-deoxyanthocyanidins a phytoalexins in germinated seedlings of sorghum which act as a defense mechanism against pathogen attack is also reported by Lo et al. (1996); Hipskind et al. (1996).

Though both of the isolated compounds were evaded to retain flavanol as a basic chemical moiety in their chemical structures like others viz., apigeninidin, 7-methoxyapigeninidin, 5, 7-dimethoxyapigeninidin, luteolinidin etc. reported previously in different parts of sorghum but in comparison to these known compounds the attached substituents on the flavanol moiety in these two isolated compounds are seems to be different. Because appearance of some of the signals just down to 0 and at slight negative side of TMS signal on δ ppm scale of ^{1}HNMR in the spectrum of both of the compounds firmly indicated either the presence of cyclopropane as a substituent or may be by the H of those substituted group/ groups occupying much hindered position in the molecules. Other signals also, those located at up field in the ^{1}HNMR and ^{13}CNMR spectrums of both of the compounds do not match with the expected signals of known and already reported flavanol derivatives of sorghum. Hence, analytical results revealed that the compounds released by intact live plants of sorghum via their roots are new and may be the products of secondary metabolic reactions (schimic acid path way) of already reported flavanoid compounds. To confirm this hypothesis and elucidate the exact chemical structures of compounds there is a need to analyze the compounds by ^{1}HNMR in its different modes viz., by increasing field strength, double resonance, lanthanide shift reagents etc. and by some

other techniques i.e. UV, MS, X-RAY, etc. currently work is taking place in this direction.

3.2. Effect on 2nd stage juveniles of root-knot nematode

The nematicidal activity of all the five formulations, viz., A, B, C, D and E of sorghum root exudates varied according to the nature of compound, doses and the incubation period. Results revealed in table 1, clearly indicated that formulations especially developed from the non-polar compounds viz.,

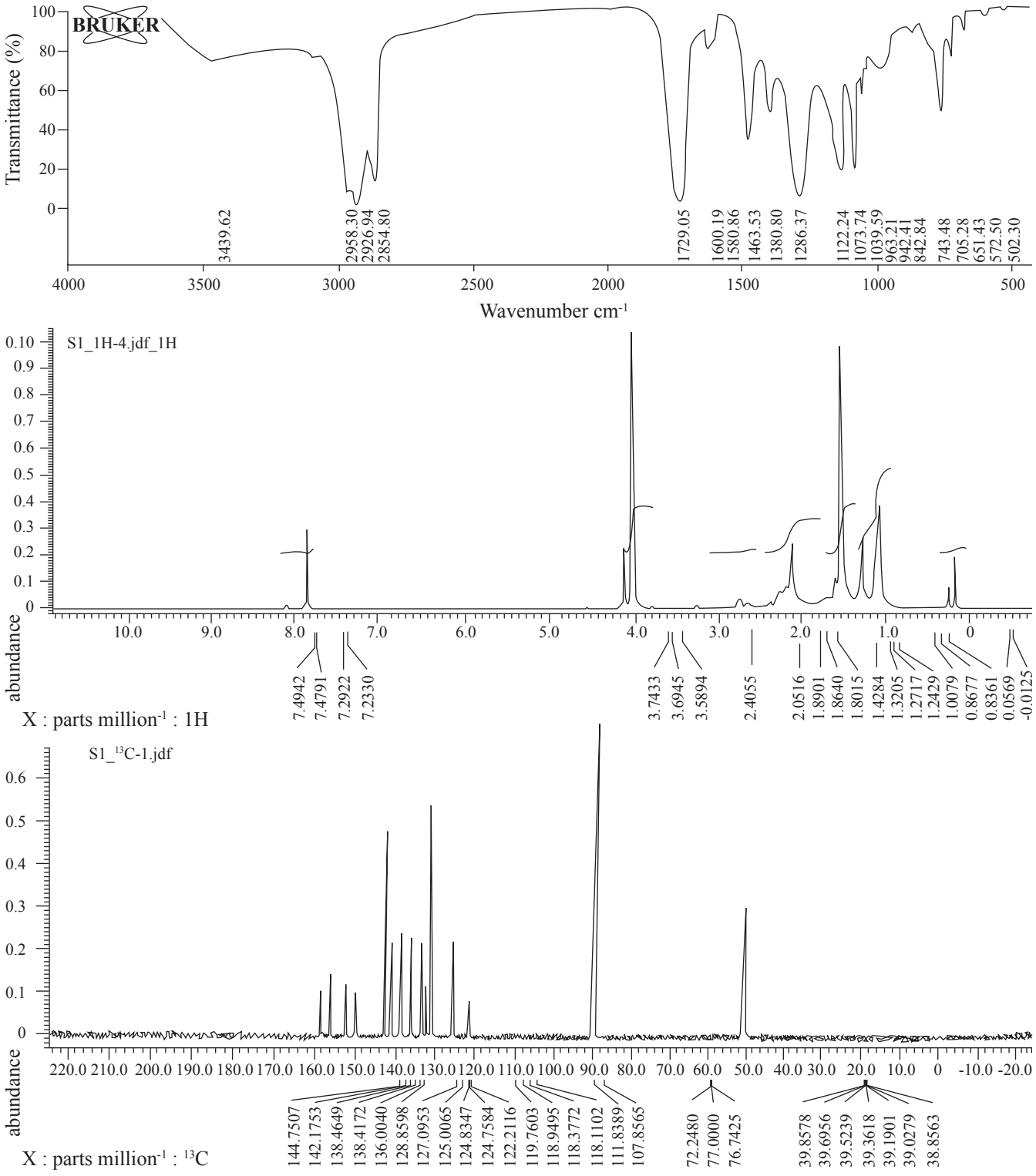

Figure 3: IR, [1]HNMR and [13]CNMR spectrum of compound C

Table1: Efficacy of formulations developed from allelofractions of sorghum on the 2nd stage juveniles (J2) of Meloidogyne javanica

Exposure time (hrs.)	% mortality of 2nd stage juveniles (J2) at different concentrations of developed formulations and time exposures																				
	Concentrations µg ml^{-1}																				
	150	200	250	300	350	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500	1600	1800	2000	Mean
Effect of EC formulation of fraction C																					
24	7	33	70	89	100																60
	(12.3)	(35.1)	(57.0)	(71.5)	(90.0)																(53.2)
	27	55	88	99	100																74
48	(31.0)	(47.7)	(69.8.)	(87.8)	(90.0)																(65.3)
	36	80	98	100	100																83
72	(36.6)	(63.5)	(83.6)	(90.0)	(90.0)																(72.8)
	23	56	85	96	100																
Mean	(26.7)	(48.8)	(70.1)	(83.7)	(90.0)																
CD (p=0.05)	Time=3.2; Concentration= 4.1; Time×concentration=7.1																				
Effect of EC formulation of fraction B																					
24		4	-	8	-	19	34	55	63	76	97										45
		(9.4)		(16.3)		(25.2)	(35.8)	(47.9)	(52.3)	(61.2)	(81.8)										(41.3)
48		15	-	22	-	45	60	78	89	100	100										64
		(22.5)		(27.9)		(41.9)	(50.8)	(62.2)	(63.0)	(90.0)	(90.0)										(56.0)
72		19\	-	33	-	75	89	97	100	100	100										77
		(25.7)		(35.2)		(67.8)	(62.6)	(80.6)	(90.0)	(90.0)	(90.0)										(67.7)
Mean		13	-	21	-	46	61	77	84	92	99										
		(19.2)		(26.4)		(45.0)	(49.7)	(63.6)	(68.4)	(80.4)	(87.3)										
CD (p=0.05)	Time=2.4; Concentration=3.9; Time×Concentration=6.7																				
Effect of EC formulation of fraction A																					
24									15	19	30	43	76	96							47
									(22.6)	(25.8)	(33.0)	(41.0)	(60.7)	(80.6)							(43.9)
48									35	50	68	79	95	100							71
									(36.2)	(45.0)	(55.6)	(62.3)	(77.6)	(90.0)							(61.1)
72									56	71	87	91	100	100							84
									(48.5)	(57.7)	(69.1)	(73.5)	(90.0)	(90.0)							(71.5)
Mean									35	47	62	71	90	99							
									(35.8)	(42.8)	(52.5)	(58.9)	(76.1)	(86.9)							
CD (p=0.05)	Time×2.5; Concentration=3.6; Time×Concentration×6.2																				
Effect of EW formulation of fraction E																					
24												6	8	18	40	56	61				32
												(11.2)	(16.3)	(25.0)	(39.2)	(48.5)	(51.3)				(31.9)
48												17	22	35	55	73	95				50
												(23.9)	(27.6)	(36.2)	(47.9)	(59.2)	(80.0)				(45.8)

continue...

	Concentrations µg ml^{-1}																	
	150	200	250	300	350	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500	1600	1800	2000	Mean
72												28	35	48	69	91	100				62
												(32.1)	(36.2)	(43.9)	(56.2)	(73.5)	(90.0)				(55.3)
Mean												17	22	34	55	73	85				
												(22.4)	(26.7)	(35.0)	(47.8)	(60.4)	(73.7)				
CD (p=0.05)	Time×3.7; Concentration=5.3; Time×Concentration×9.2																				
Effect of EW formulation of fraction D																					
24												3	-	3	-	4	-	4	5	5	4
												(9.4)		(7.4)		(11.0)		(11.5)	(12.5)	(13.3)	(10.9)
48												4	-	4	-	5	-	6	6	6	5
												(10.9)		(11.3)		(12.5)		(13.7)	(13.3)	(13.8)	(12.6)
72												4	-	5	-	7	-	7	8	9	7
												(11.9)		(13.3)		(14.9)		(15.3)	(16.4)	(17.1)	(14.8)
Mean												4	-	4	-	5	-	5	6	7	
												(10.7)		(10.7)		(12.8)		(13.5)	(14.1)	(14.7)	
CD (p=0.05)	Time×NS.0; Concentration NS; Time×Concentration×NS																				

Values given in parenthesis () are angular transformations

C and B exhibited severe impact on mortality of second stage juveniles of *Meloidogyne javanica* in comparison to the formulations developed from polar compounds i.e. D and E. Maximum juvenile mortality was achieved with the formulation developed from highly non-polar compound i.e. C followed by the formulations developed from B, A, E. Whilst, the EW formulation developed from D (highly polar) was found practically ineffective, as it could not produced significant mortality even at highest concentration of 2000µg ml^{-1} and at maximum time of exposure (72h). Thus the polarity of allelocompounds of sorghum root exudates determines the toxicity against second stage juveniles of *Meloidogyne javanica*. Formulation of C was found detrimental to the juveniles even at extremely low concentrations ranging from 150-350 µg ml^{-1}. The activity of formulation was found highly correlative with time of exposure and the concentration of the formulation. At lowest concentration (150 µg ml^{-1}) of the formulation, compare to control, approximately 7-10% mortality in juveniles was observed after 24 h of exposure but there was a progressive and highly significant increase in the mortality with both i.e. the increase in exposure period as well as the concentration of formulation. The formulation at same concentration resulted approximately about 25 and 35% nematode (J_2) mortality after exposure periods of 48 and 72 h, respectively. Quick and absolute mortality was observed at highest concentration viz., 350 µg ml^{-1} at this concentration not a single nematode was found to survive even after 24 h of exposure. Even at 250 µg ml^{-1} the formulation was found quite effective by producing the kill of around 98% after an exposure period of 72 h. Formulation of B also exhibited significant impact on the mortality of *M. javanica* juveniles. The formulation was observed toxic with different capabilities between the concentrations ranged from 200 to 1000 µg ml^{-1}. The formulation at higher concentrations viz. 800 and 1000 µg ml^{-1} was found absolute detrimental to nematodes after exposure periods of 48 and 72 h. Whereas, at comparatively low concentrations viz., 600 and 700 µg ml^{-1} it also observed quite effective by being producing, 97 and 100% juvenile mortality, respectively, after 72 hrs exposure. In this case also concentration of formulation and the exposure period played a significant role in enhancing the toxicity of test formulation. Formulations developed from the compounds A and E were found moderately toxic to the second stage juveniles of *M. javanica* since both the compounds retained > 80% as polar character. Nonetheless, fraction A is comparatively non-polar in nature therefore, its formulation was found little bit more efficacious as compare to E, formulation of A caused around 95 and 100% mortality at 1100 µg ml^{-1} concentration after exposure periods of 48 and 72 hrs respectively, over control, whereas, up to 91% mortality was achieved at 1000 µg ml^{-1} after an exposure period of 72 hrs. But in case of formulation

E 1400 and 1500 µg ml^{-1} concentration of the formulation was required to bring out the same juvenile mortality i.e. 91and 100% respectively, after an exposure period of 72 hrs. However, in both of the cases a good correspondence between the formulation concentrations and time of exposures with the activity of formulations was observed. Highly non-significant results were obtained with the formulation developed from compound D constituted entirely the polar compounds with some inorganic elements released by sorghum plants. Formulation was found completely ineffective in all the test concentrations viz., 1100 to 2000 µg ml^{-1} and at any time of exposure therefore, declared here as an ineffective compound for a practical point of view of nematode control.

3.3. Effect on egg hatch

Out of the total five formulations only one, exclusively developed from the highly non polar fraction (C) was observed

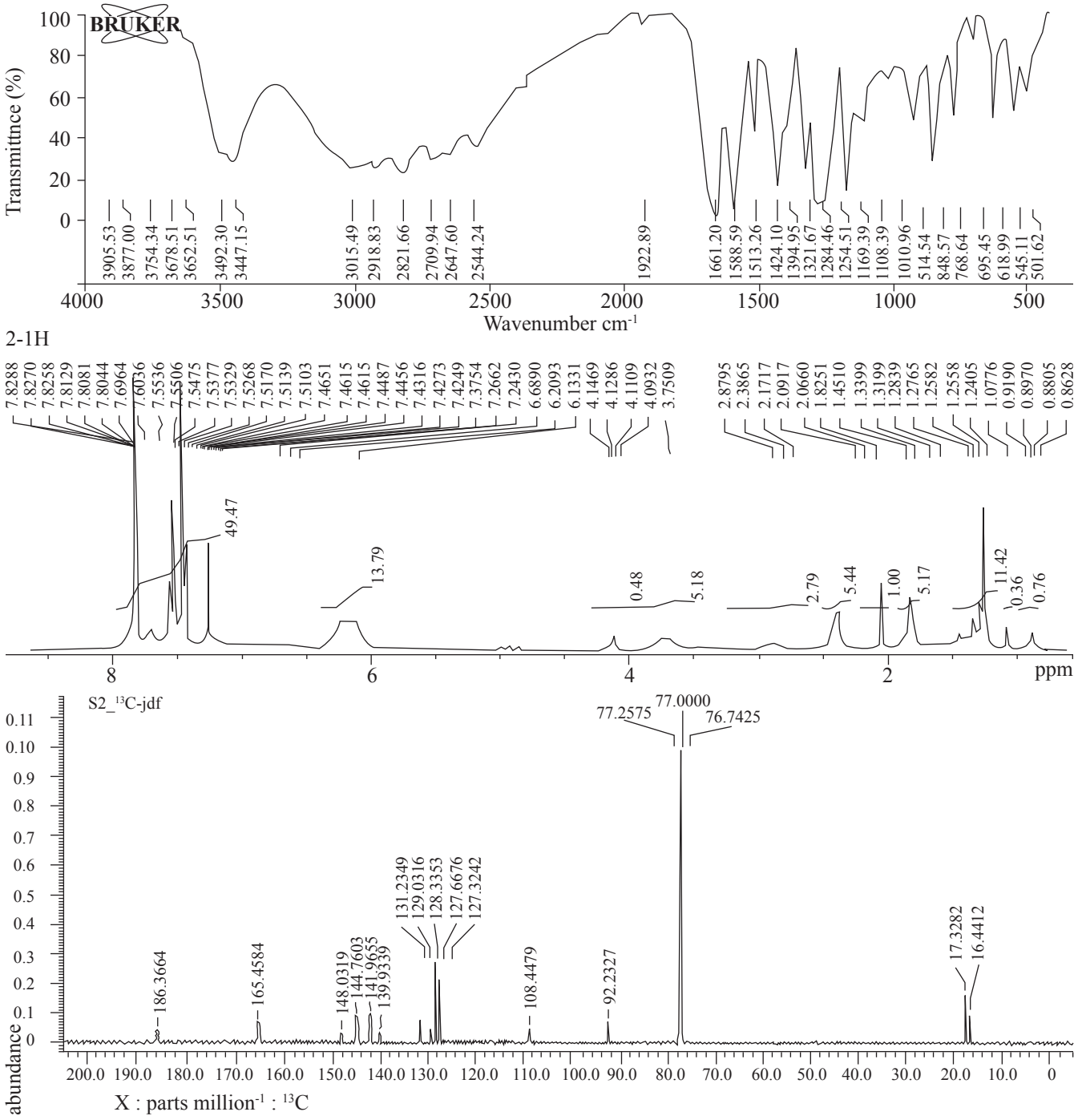

Figure 4: IR, ^{1}HNMR and ^{13}CNMR spectrum of compound B

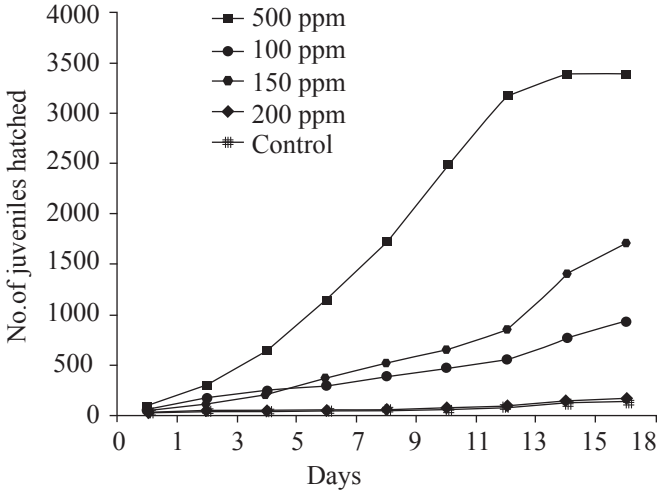

Figure 5: Effect of non polar EC formulation (C) of sorghum on egg hatch of *Meloidogyne javanica*

considerably effective in retarding the egg hatch properties of the test nematode species. Results, as compare to control, revealed a grave impact of the test formulation on egg hatch in all the test concentrations of the formulation (figure 3). In case of control egg hatch started immediately after first day of treatment which increased progressively with time and reached to the maximum in 15 days. Whereas, 100% egg hatch was not observed in none of the test formulation concentrations i.e. 50, 100, 150 and 200 µg ml^{-1} even after 18 days of exposure. A positive linear correlation (r 0.91) between reductions in egg hatch with the concentration of formulation was observed. In comparison to hatching in control, approximately 55 and 80% reduction, in egg hatch was noticed at 50 and 100 µg ml^{-1} concentrations of the formulation. Whereas, at higher concentrations of 150 and 200 µg ml^{-1} were found extremely effective in retarding egg hatch of the test nematode up to an extent of more than 98%. Though the test formulation at its lower concentrations of 50 and 100 µg ml^{-1} showed the retardation effect in egg hatch only when the egg masses continuously remained under exposure of chemical but as and when the treated egg masses were transferred to distill water magnitude of egg hatching increased. In contrary, at higher concentration i.e. 150 µg ml^{-1} and above of the formulation caused permanent damage to the exposed egg masses as there was no further egg hatching noticed after transferring the egg masses in distilled water for longer period.

4. Conclusion

The allelochemicals of sorghum has immense ability to suppress nematodes. The non-polar group of compounds was found effective in killing second stage juveniles and retarding egg hatch, which would lead to curtail the nematode population in field. Hence, allelochemicals of sorghum could be treated as potential strategy for effective control and management of *Meloidogyne* spp and possibly, other nematodes infesting agricultural and other important crops. The benefits of findings can be harnessed by including the sorghum crop in crop rotations.

5. Acknowledgement

The authors acknowledge the support received from the ICAR-Indian Institute of Pulses Research for carrying out the study. Authors are privileged to convey our gratitude to Dr. Prem Chand convener central EPR facility IIT, Kanpur for providing valuable support in analysis of the compounds. We are also extremely thankful to Mr. N. Ahmad and Dr. Veena Singh for analysis of the compounds via NMR and IR.

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