https://doi.org/10.26651/allelo.j/2021-55-2-1377 0971-4693/94 Euro 20.00
Allelopathy Journal 55 (2): 151-162 (March, 2022) *International Allelopathy Foundation* 2022
Table: 6, Figs : 4

New centrifuge tube agar profile method to determine the allelopathic effects of plants

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(Received in revised form: January 1, 2022)

ABSTRACT

Generally, the laboratory assessment of allelopathy involves the determination of germination rates and measurements of the roots and shoots elongation of receptor plant species exposed to an allelochemical source (e. g. extracts, leachates, root exudates) or a purified allelochemical. However, these assessments are time consuming and laborious. In the present study, a new centrifuge tube agar profile method (CTAP) was introduced to determine the allelopathic effects of aqueous extracts from 30 plant species on lettuce (*Lactuca sativa* L.). The results demonstrated that the water extract of *Melilotus officinalis* (L.) Pall. had the strongest inhibitory effects on lettuce. Water extract of *M. officinalis* (L.) Pall. was analyzed by GC-MS, and the allelopathic effects of coumarin on 5-plant species (*Lactuca sativa* L., *Raphanus sativus* L., *Raphanus sativus* L., *Lycopersicon esculentum* Mill., *Lactuca sativa* L., and *Brassica campestris* var. chinensis) were determined by CTAP. The exposure of coumarin at 100 mg·L^{-1} for 2 days stimulated the growth of radish (*Raphanus sativus* L.). This study introduces a new method for the rapid determination of allelopathic potential of plants and allelochemicals.

Key words: Agar, allelochemicals, allelopathy, bioassay, coumarin, CTAP, GCMS, *Lactuce sativa*, lettuce, *Melilotus officinalis*, new centrifuge tube agar profile method, *Raphanus sativus*.

INTRODUCTION

Allelopathy is characterized by the release of plant allelochemicals into their surrounding environment, inhibiting the growth and development of own or other plants (3,10,19). This is a well-known phenomenon in agricultural ecosystems and has great significance in plant growth, community succession and plant protection (12,22,32). Plants release allelochemicals in to the environment through leaf leachates, volatilization, root exudation and residue decomposition (8,12). At present, there are more than 100 thousand allelochemicals in plant secondary metabolism and > 2000 plants species produce allelochemicals with herbicidal activity. These chemicals are classified into 14 categories, including phenols, terpenes, alkaloids, organic acids, steroids, etc. (15,18). They could be effectively transformed into bioherbicides, bioinsecticides and biofungicides due to their low toxicity, strong selectivity, easy degradation and high environmental safety (14,18,26). Allelopathy is an important biotechnological tool in the 21st century to protect the human health and promote the sustainable development of agriculture.

Plant metabolites are primary source of bioherbicides (5). Various solvents have been used to extract plant materials to obtain the maximum contents of metabolites (31).

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Allelopathy is often quantified by measuring growth responses (germination rate, seedling growth, and seedling or root length) in Petri dish assays. In Petri dish assays, response of large number of roots and shoots of recipient plants seedlings, exposed to plant extract or allelochemicals are measured. Many studies have tested materials from donor plants mixed with agar, as a direct source of allelochemicals on recipient plants e.g., "Sandwich method" proposed by Fujii *et. al* (9) and the "ground plant tissue powder mixed with agar (PPA)" proposed by Luo (16). In some cases, seeds of acceptor plants were exposed to plant materials mixed with soil (11). However, allelopathic effects cannot be detected in soil tests due to the long time required to see the growth responses (germination rate, plant height and tillering) without replenishment of an allelochemical. This work aimed to introduce a new Centrifuge Tube Agar Profile Method (CTAP), which allows the quick determination of the allelopathic effects of aqueous extracts and allelochemicals on large number of recipient plants.

MATERIALS AND METHODS

Thirty test weeds (Table 1) were collected near the experimental field, Hunan Academy of Agricultural Sciences, Changsha, China (113° 4′ E, 28° 11′N). After collection, the impurities (soil etc.) were removed, including the rotten parts of plants. Thereafter, the plant materials were dried in oven at 60 ^{o}C to constant weight. The plant materials were ground into powder with a pulverizer, and plant powder was passed through 1 mm mesh screen. Subsequently, the plant powder was sealed into plastic bags and stored at room temperature. The 30-test plants were collected from April to May in 2019, of these 8- were Grasses (Monocots) and 22-were Non-Grasses (Dicots) (Table 1).

Table 1. List of 30- Donor Grasses (Monocots) and Non-Grasses (Dicot) plants)

No	Species	No	Species	No	Species
	Grasses (Monocots)	10	*Carpesium abrotanoides* L.	21	*Ranunculus sceleratus* L.
1	*Alopecurus aequalis* Sobol.	11	*Cerastium glomeratum* Thuill.	22	*Solanum nigrum* L.
2	*Alopecurus japonicus* Sobol.	12	*Cyclospermum leptophyllum* (Persoon) Sprague ex Britton & P. Wilso*n*	23	*Stellaria media* (L.) Cyr.
3	*Beckmannia syzigachne* (Steud.) Fern.	13	*Galium spurium* L.	24	*Torilis scabra* (Thunb.) DC.
4	*Festuca elata* Keng ex E. Alexeev	14	*Hemisteptia lyrata* (Bunge) Fischer & C. A. Meyer	25	*Trigonotis peduncularis* (Trev.) Benth. ex Baker et Moore
5	*Lolium perenne* L.	15	*Humulus scandens* (Lour.) Merr.	26	*Veronica arvensis* L.
6	*Poa annua* L.	16	*Hypericum japonicum* Thunb. ex Murray	27	*Veronica persica* Poir.
7	*Polypogon fugax* Nees ex Steud.	17	*Lapsana apogonoides* Maxim.	28	*Vicia hirsuta* (L.) S. F. Gray
8	*Roegneria kamoji* Ohwi	18	*Lobelia chinensis* Lour.	29	*Viola betonicifolia* J. E. Smith
	Non-Grasses (Dicots)	19	*Melilotus officinalis* (L.) Pall.	30	*Youngia japonica* (L.) DC.
9	*Capsella bursa-pastoris* (L.) Medic.	20	*Plantago depressa* Willd.		

The seeds of common vegetables grown in China (recipient crops) were purchased from the local market. Their seeds germination were > 90 %, and the seeds were stored at 4 ^{o}C for further assays.

The experimental treatments consisted of two factors : (i). Donor weeds spp.-30 (Table 1) and (ii). Recipient spp. : 6 [Lettuce (*Lactuca sativa* L.), White radish (*Raphanus sativus* L.), Red radish (*Raphanus sativus* L.), Cherry tomato (*Lycopersicon esculentum* Mill.), Romaine lettuce (*Lactuca sativa* L.), and Chinese cabbage (Suzhouqing) (*Brassica campestris* var. chinensis)].

The treatments were replicated four times in complete Randomised design.

I. Plants aqueous extracts

Four g powder of each weed were mixed separately with 40 mL of distilled in 50 mL centrifuge tube. This mixture was shaken at 200 rpm for 24 h at 20 ºC to fully extract the allelopathic substances from plants.

II. The centrifuge tube agar profile method (CTAP)

The recipient seeds were disinfected with 0.3 % sodium hypochlorite for 5 min, followed by washing with distilled water. 50 seeds of each test vegetable were placed separately into 9 cm dia petri dishes lined with 2-layers of filter paper. Five mL distilled water was added to each dish. These petri dishes of all test vegetables were placed in a light incubator with night/dark temperatures of 15 ºC /20 ºC (lettuce), 20 ºC /25 ºC (white radish, red radish and cabbage-Suzhouqing) and 25 ºC /30 ºC (cherry tomato) and 12 h photoperiod for seeds germination and seedlings growth. The seeds were considered germinated, when 1-2 mms radicle emerged through the episperm and these were selected for the experiment.

III. Allelopathic effects

(i). Plant aqueous extracts: One mL of plant aqueous extract was transferred into 5 mL graduated centrifuge tube (scale divided in 0.5 ml intervals). Then, the tube was placed in 60 ºC water bath for insulation. Later, 2.0 % agar solution was prepared with distilled water. Before the agar cool down, 1 mL of 2.0 % agar solution was added into a 5 mL centrifuge tube containing 1 mL of plant aqueous extract and thoroughly mixed to get the final agar concentration of 1.0 %. Thus the plant aqueous extract concentration was 50 g L^{-1}. After mixing, the centrifuge tube was kept flat on the test bench. After the agar was completely hardened, an agar profile developed in the centrifuge tube. Then, the centrifuge tube was opened, the agar surface was pressed lightly with forceps, and the agar profile was rotated below the scale surface of the centrifuge tube with forceps. This step also removed the water mist in the centrifuge tube. The lettuce seeds were placed into the centrifuge tube with the pointed end facing down (root site) by forceps. Three seeds were put into a centrifuge tube in a flat row to keep them close to the agar surface. The pointed end position was unified at the scale line of "4 mL" corresponding to the scale of the centrifuge tube. The centrifuge tube was inserted vertically into the centrifuge tube shelf and moved into the light incubators. The centrifuge tube was taken out after 2 days, and the scale line of the centrifuge tube was kept at eye level. Each line interval of 0.5 ml of the scale is hereafter referred as a grid, and the number of grids required to cover full length of roots and shoots were recorded.

(ii). Coumarin: Coumarin (99 % concentration) was purchased from Shanghai Macklin Biochemical (Shanghai, China), and dissolved in a small amount of ethanol for further assays. Then, 1 % agar water solution was prepared and a certain amount of coumarin was added to prepare the final concentration of 400 and 100 mg·L^{-1}, respectively. Later, 2 mL of different concentrations of agar coumarin solution was added to the 5 mL graduated centrifuge tube, and the control was 2 mL of 1 % distilled water agar solution. The centrifuge tube was kept on the desk. After the agar hardened, 2 or 3 germinated seeds of all test vegetables were transferred into the centrifuge tube. All seeds were grown for 2 days in the light incubator. The follow-up operation steps were the same as above.

III. GC-MS analysis

The allelopathic substances in the aqueous extract of *M. officinalis* (L.) Pall. were determined by GC- MS analysis using an Agilent 7890 gas chromatography coupled with a time-of-flight mass spectrometer. A DB-5MS (30 m×250 μm×0.25 μm) capillary column was utilized in this system, and the injection volume was 1 μL aliquot of sample in spitless mode. The initial temperature was kept at 50 °C for 1 min, then raised to 310 °C at a rate of 10 °C min^{-1} and kept for 8 min at 310 °C. The injection, transfer line, and ion source temperatures were 280, 280 and 250 °C, respectively. The energy was -70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 12.5 spectra per second after a solvent delay of 6.25 min. The flow rate was 1 mL · min^{-1}. Under these chromatographic conditions, the results of GC-MS were used for metabolite annotation with the cut-off for annotation set to 0.7.

IV. Statistical analysis

Each treatment was repeated four times, and all experiments were conducted in triplicate. The mean root and shoot lengths was calculated by one-way analysis of ANOVA using SPSS 26.0 software. The significance was determined by Duncan's new complex range method ($P < 0.05$).

Allelopathic effects of coumarin and aqueous extracts from 30 weeds were compared as per the response index (RI) formula and the comprehensive allelopathic effect (CE) formula (27,28). RI was calculated as under:

$$RI=T/C-1$$

Where, C : Control root length or shoot length, and T : Treated root length or shoot length.
CE was calculated as under :

$$CE= (RI_1+RI_2...RI_n)/n$$

Where, RI_1, RI_2, and RI_n are RI of n different test indexes of plants with the same receptor under the same treatment.

RESULTS AND DISCUSSION

ALLELOPATHIC EFFECTS

(I). Plant aqueous extracts

The first step in the development of bioherbicides based on allelopathy is the identification of plant extracts with promising phytotoxic activity on recipient plants (13,23,25). We tested the allelopathic effects of 30 weeds aqueous extracts on lettuce using the Centrifuge Tube Agar Profile Method (CTAP). The root length of lettuce was 2.90 grids and the shoot length was 0.39 grid in control (distilled water). The 30 weeds aqueous extracts significantly inhibited the root length of lettuce at concentration of 50 g·L^{-1} ($P < 0.05$) (Table 2). The RI values obtained for shoots and roots are summarized in Table 3. They show that all aqueous extracts exerted a stronger inhibitory effect on root growth than on shoot.

Table 2. Effects of aqueous extracts of weeds on the Root and shoot lengths of lettuce after 2- days.

No.	Root length*	Shoot length*	No.	Root length*	Shoot length*
CK	2.9±0.196 a	0.39±0.065 a	15	0.228±0.039 hijkl	0.128±0.019 hi
	Grasses (Monocots)		16	0.605±0.055 cde	0.303±0.04 abcde
1	0.663±0.081 c	0.328±0.062 abcd	17	0.158±0.013 jkl	0.1±0.016 i
2	0.943±0.113 b	0.28±0.044 bcde	18	0.683±0.094 c	0.345±0.05 abc
3	0.123±0.019 kl	0±0 j	19	0.015±0.01 l	0±0 j
4	0.215±0.012 ijkl	0.125±0.025 hi	20	0.678±0.092 cd	0.215±0.02 efgh
5	0.235±0.017 hijkl	0.178±0.019 fghi	21	0.365±0.039 fghi	0.283±0.034 bcde
6	0.228±0.049 hijk	0.22±0.004 efgh	22	0.65±0.108 cd	0.225±0.018 efg
7	0.635±0.085 cde	0.263±0.026 cdef	23	0.635±0.068 cde	0.298±0.018 abcde
8	0.495±0.063 cdef	0.233±0.02 defg	24	0.583±0.059 cde	0.298±0.032 abcde
	Non-Grasses (Dicots)		25	0.235±0.037 hijkl	0.248±0.031 cdefg
9	0.593±0.061 cde	0.375±0.028 ab	26	0.363±0.047 fgh	0.155±0.006 ghi
10	0.51±0.079 cdefg	0.26±0.023 cdef	27	0.125±0.025 kl	0±0 j
11	0.22±0.032 hijk	0.23±0.027 defg	28	1.038±0.197 b	0.265±0.029 cdef
12	0.338±0.051 ghij	0.205±0.023 efgh	29	0.538±0.059 cdef	0.24±0.03 defg
13	0.488±0.051 defg	0.278±0.025 cdef	30	0.45±0.063 efg	0.275±0.018 cdef
14	0.695±0.088 c	0.203±0.029 efgh			

(i). Grass (Monocot) weeds: The aqueous extract of *B. syzigachne* (No. 3) totally inhibited the growth of lettuce, and the CE of *B. syzigachne* was -0.98, which was lowest in the 8-monocot weeds. There was no significant difference in the shoot length of lettuce between the treatment of aqueous extracts of *A. aequalis* (No. 1) and control treatment, and the shoot length was 0.328 grid in the aqueous extracts of *A. aequalis*, and its CE was highest in all 8-monocot weeds, which was -0.465. The other aqueous extracts of monocot weeds significantly inhibited the root and shoot lengths of lettuce.

Table 3. Inhibitory allelopathic effects of 30-weeds aqueous extracts on lettuce seedlings

No	RI (Response Index)*			No	RI (Response Index)*			No	RI (Response Index)*		
	Root length	Shoot length	CE**		Root length	Shoot length	CE**		Root length	Shoot length	CE**
	Grasses (Monocots)			10	-0.82	-0.33	-0.575	21	-0.87	-0.28	-0.575
1	-0.77	-0.16	-0.465	11	-0.92	-0.41	-0.665	22	-0.78	-0.42	-0.6
2	-0.68	-0.28	-0.48	12	-0.88	-0.47	-0.675	23	-0.78	-0.24	-0.51
3	-0.96	-1	-0.98	13	-0.83	-0.29	-0.56	24	-0.8	-0.24	-0.52
4	-0.93	-0.68	-0.805	14	-0.76	-0.48	-0.62	25	-0.92	-0.37	-0.645
5	-0.92	-0.54	-0.73	15	-0.92	-0.67	-0.795	26	-0.88	-0.6	-0.74
6	-0.92	-0.44	-0.68	16	-0.79	-0.22	-0.505	27	-0.96	-1	-0.98
7	-0.78	-0.33	-0.555	17	-0.95	-0.74	-0.845	28	-0.64	-0.32	-0.48
8	-0.83	-0.4	-0.615	18	-0.76	-0.12	-0.44	29	-0.81	-0.38	-0.595
	Non-Grasses (Dicots)			19	-0.99	-1	-0.995	30	-0.84	-0.29	-0.565
9	-0.8	-0.04	-0.42	20	-0.77	-0.45	-0.61				

RI*: Response Index CE**: Mean Allelopathic Effects

(ii). Non-Grass (Dicot) weeds: The aqueous extract of *L. apogonoides* (No. 17), *M. officinalis* (No. 19) and *V. persica* Poir (No. 27) drastically inhibited the growth of root and shoot of lettuce in the twenty-two dicot weeds and their CE were -0.845, -0.995 and-0.98 respectively. The aqueous extracts of *A. aequalis*, *C. bursa-pastoris* (No. 9), *H. scandens* (No. 15), *H. japonicum* (No. 16), *L. chinensis* (No. 18) and *T. scabra* (No. 24) had no significant inhibitory effects on the shoot length of lettuce (P = 0.058), but all treatments inhibited the roots of lettuce. The CE of *C. bursa-pastoris* (No. 9) was -0.420 (Table 3), which was higher than other treatments. This indicated that the inhibitory effects of aqueous extracts of *C. bursa-pastoris* on lettuce were the weakest. Extract of *M. officinalis* had the strongest inhibitory effect with a CE value of -0.995. The lettuce seeds exposed to the water extract of *M. officinalis* became darker, the radicle did not grow, and the root turned yellow and brown (No. 19) (Figure 1).

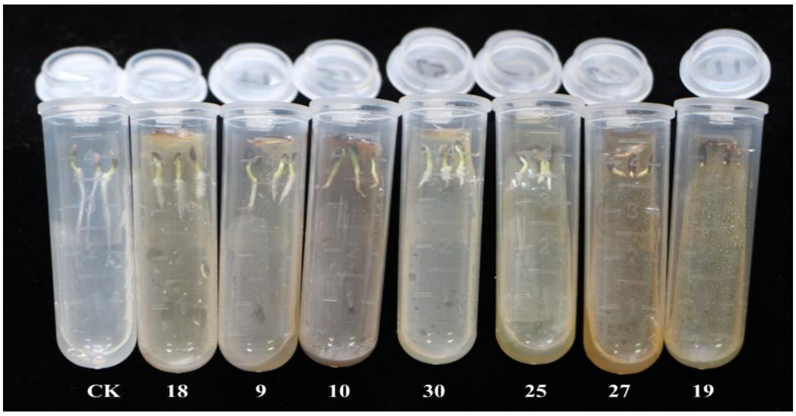

Figure 1. The allelopathic effect of weeds aqueous extract assessed by CTAP
CK: The control,18: *L. chinensis,* 9: *C. bursa-pastoris,* 10: *C. abrotanoides,* 30: *Y. japonica,* 25: *T. peduncularis,* 27: *V. persica,* 19: *M. officinalis*

Figure 2. *Melilotus officinalis* crop

M. officinalis (Figure 2) possesses a strong adaptability to the environment and is often used as green manure in arid areas for prevention of wind erosion and sand fixation (4,24). It is allelopathic plant and inhibits the seed germination and seedling growth of *Lolium multiflorum* Lamk., *P. annua* L. and *Vicia hirsuta* (L.) S. F. Gray (29). Its aqueous extracts are rich in organic acids, fatty acids, phenols, saccharides, sterols, amino acids, vitamins and other compounds (2,7). Its aqueous extracts contained 30 primary compounds (Table 4). The relative content of each substance was obtained by the peak-area normalization method. The major constituent was L (-)-Malic acid which accounted with 12.82 %, followed by lactic acid (5 %) and succinic acid (5 %). Other constituents were found in lower percentages. Coumarin, the main allelochemical usually associated to the allelopathic effect of *M. officinalis* (30), was found 0.34 %. Hence, we suppose that coumarin was the major compound responsible for the observed inhibitory effects.

Table 4. Compounds identified from water extract of *M. officinalis* by GC-MS.

Name	CAS Number	Molecular formula	Molecular weight （Da）	Relation content （%）	Retention time （min）
L (-)-Malic acid	97-67-6	$C_4H_6O_5$	134.09	12.82	13.23
Lactic acid	50-21-5	$C_3H_6O_3$	90.08	7.18	7.44
Succinic acid	110-15-6	$C_4H_6O_4$	118.09	6.99	10.94
Ethyl 2-benzylacetoacetate	20-79-1	$C_{13}H_{16}O_3$	103.12	4.59	13.72
L-Alanine	56-41-7	$C_3H_7NO_2$	89.09	4.51	7.99
D (-)-Fructose	57-48-7	$C_6H_{12}O_6$	180.16	4.16	17.49
Alpha-Sophorose	534-46-3	$C_{12}H_{22}O_{11}$	342.30	3.9	25.19
Citric acid monohydrate	5949-29-1	$C_6H_{10}O_8$	210.14	3.81	17.02
D (+)-Glucose	50-99-7	$C_6H_{12}O_6$	180.16	3.46	17.99
D-Leucrose	7158-70-5	$C_{12}H_{23}O_{11}$	343.30	3.25	25.54
Inositol	87-89-8	$C_6H_{12}O_6$	180.16	3.13	19.65
1,2-Propanediol,3,3'-oxybis-	56-81-5	$C_3H_8O_3$	92.09	2.98	10.36
Myo-Inositol,1,2-anhydro-	6090-95-5	$C_6H_{10}O_5$	162.14	2.96	18.36
L-Pyroglutamic acid	98-79-3	$C_5H_7NO_3$	129.11	2.51	13.63
Fumaric acid	110-17-8	$C_4H_4O_4$	116.07	2.43	11.42
Malonic acid	141-82-2	$C_3H_4O_4$	104.06	2.24	9.39
D- (+)-Cellobiose	550-33-4	$C_{10}H_{12}N_4O_4$	252.23	1.71	24.90
Glycolic acid	79-14-1	$C_2H_4O_3$	76.05	1.46	7.61
L-Asparagine	70-47-3	$C_4H_8N_2O_3$	132.12	1.13	13.42
Palmitic acid	57-10-3	$C_{16}H_{32}O_2$	256.42	0.94	19.28
L-Proline	147-85-3	$C_5H_9NO_2$	115.13	0.87	10.7
2,3,4-Trihydroxybutansure	7306-96-9	$C_4H_8O_5$	136.10	0.79	14.03
Ribose	24259-59-4	$C_5H_{10}O_5$	150.13	0.77	15.39
Xylitol	87-99-0	$C_5H_{12}O_5$	152.15	0.76	16.24
2-Hydroxypyridine	142-08-5	C_5H_5NO	95.10	0.46	15.61
Stearic acid	57-11-4	$C_{18}H_{36}O_2$	284.48	0.42	7.11
2,3-Dihydroxypropanoic acid	6000-40-4	$C_3H_6O_4$	106.08	0.35	21.06
Coumarin	91-64-5	$C_9H_6O_2$	146.14	0.34	11.11
Maleimide	541-59-3	$C_4H_3NO_2$	97.07	0.29	7.94
L-Valine	72-18-4	$C_5H_{11}NO_2$	117.15	0.28	9.55
Uracil	66-22-8	$C_4H_4N_2O_2$	112.09	0.24	11.24

CAS: Chemical Abstracts Service

(II). Coumarin

Here all 5-recipient vegetables were dicots. Coumarin is a phenolic derivative showing allelopathic effects on seed germination and seedling growth of many weeds and crops (1,6,17,20). Few studies have reported the allelopathic effects of coumarin on vegetables, hence, we tested CTAP on seeds of five vegetable species with coumarin as allelochemical. The embryos of all the vegetables were seriously inhibited, and the radicles did not grow more at the concentration of 400 mg·L^{-1}. At this concentration, the CE of 5 vegetables was lower than -0.970, and the inhibition rates of shoot and root were > 95 %, indicating that coumarin completely inhibited the growth of vegetables (Table 5, Figure 3, 4).

Table 5. Effects of coumarin concentrations on root and shoot lengths (expressed in grids) of Recipient vegetables.

Test vegetables	Coumarin dose (mg·L^{-1})					
	0		100		400	
	Root length*	Shoot length*	Root length*	Shoot length*	Root length*	Shoot length*
Cherry tomato	7.47±0.524 a	2.23±0.12 a	5.07±0.549 b	1.97±0.233 a	0.1±0 c	0±0 b
Chinese cabbage	4.17±0.219 a	1.73±0.067 a	3.47±0.291 a	1.47±0.12 a	0.1±0 b	0±0 b
White radish	3.47±0.338 a	2.23±0.133 a	3.53±0.273 a	2.2±0.208 a	0.17±0.033 b	0±0 b
Red radish	2.50±0.379 a	1.93±0.24 a	1.6±0.265 a	2.03±0.176 a	0.1±0 b	0±0 b
Romaine Lettuce	3.13±0.376 a	1.73±0.167 a	2.37±0.26 a	1.67±0.203 a	0.03±0.033 b	0±0 b

* Lengths are expressed in grid units. One grid is the distance in the line-scale of the graduate centrifuge tube represented for a volume of 0.5 ml. The means values ± SE are shown, and different letters indicate significant difference between treatments ($p < 0.05$).

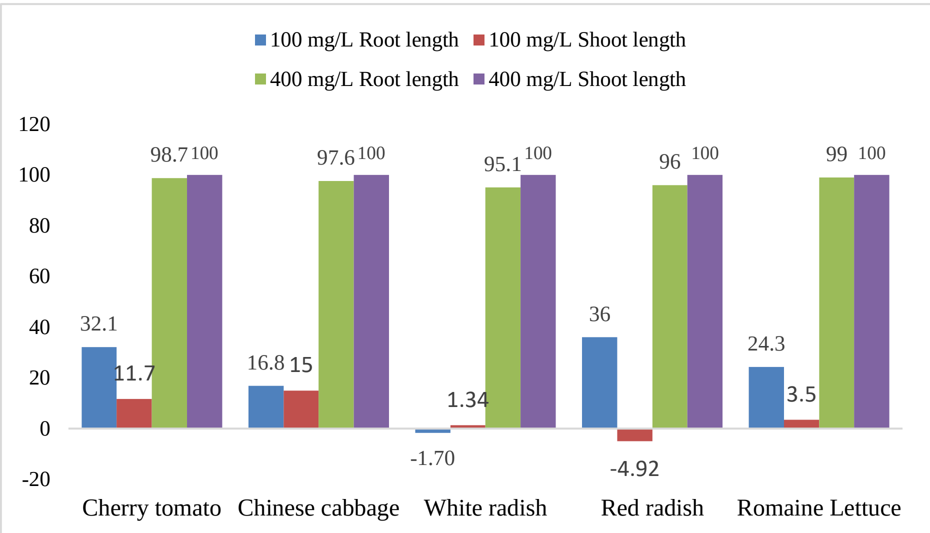

Figure 3. The inhibitory rate of coumarin doses of 100 and 400 mg/L on test vegetables roots and shoot lengths

Figure 4. The allelopathic effects of coumarin doses (0, 100 and 400 mg·L^{-1}.) on test vegetables

The root length of cherry tomato was 5.07 grids with treatment of coumarin at 100 mg·L^{-1}, which was significantly lower than 7.47 grids in control. In contrast, there was no significant difference between the other treatments and their controls (Table 5). At 100 mg·L^{-1} coumarin, the root length of white radish was stimulated to 3.53 grids, which was higher than control (3.47 grids), and its inhibition rate was -1.70, indicating that the roots were stimulated with this treatment (Table 5, Figure 3). While, the root lengths of other vegetable were inhibited (Table 6). At this dose, there was no significant difference in the shoot length between all vegetables and their controls. The shoot length of white radish was 2.03 grids at 100 mg·L^{-1} coumarin dose, which was slightly higher than control (1.93 grids) (Table 5). The CE of white radish was 0.005, indicating that the growth of white radish was promoted by coumarin at 100 mg·L^{-1} after 2 days, and the other four vegetables were inhibited (Table 6).

Table 6. Allelopathic effects of coumarin doses on seedlings growth of Recipient vegetables

Test vegetables	Root length		Shoot length		CE: Comprehensive Allelopathic Effect	
	Coumarin (mg·L^{-1})					
	100	400	100	400	100	400
Cherry tomato	-0.32	-0.99	-0.12	-1.00	-0.220	-0.995
Chinese cabbage	-0.17	-0.98	-0.15	-1.00	-0.160	-0.990
White radish	0.02	-0.95	-0.01	-1.00	0.005	-0.975
Red radish	-0.36	-0.96	0.05	-1.00	-0.155	-0.980
Romaine Lettuce	-0.24	-0.99	-0.04	-1.00	-0.140	-0.995

The application of CTAP

The extraction and identification of plant allelochemicals is the basis for screening of allelopathic plants and biological pesticides (18,21). Agar is a good fixative to determine the plant allelopathy and used in the sandwich and 24-well plate methods. The CTAP used agar to produce the agar profile, was conducive to the vertical growth of the recipient plant. Compared to these methods, the CTAP has the advantage of scale line and the transparency of the centrifuge tube, hence, the growth of the recipient could be seen directly. The scale line of tube was used as "ruler" to measure the length of root and shoot. This measurement method is convenient for the investigators to read the shoot and root lengths easily and quickly, and to effectively differentiate the allelopathic effects. Additionally, the "ruler" on the centrifuge tube provided more reliable data than eye measurement. Certainly, the extract method for donor plants could be modified based on the need of investigators and the number of plant materials. The plant powder and the plant tissue can be added directly into the agar. To determine the allelochemicals, many expansive chemicals at high concentration are used (Some chemicals cost > $ 1000 for 1-5 mg). A small amount of materials is difficult to meet the requirements of allelopathy screening, leading to the loss of some potentially useful allelochemicals. Due to the limited amount of agar added to the centrifuge tube, the CTAP introduced in this study requires less allelopathic material, and this method could also be improved with different receptors. For instance, the 5 mL centrifuge tube could be replaced by another graduated tube. The CTAP was also suitable for the receptors such as *L. perenne* L., *Echinochloa crus-galli* (L.) P. Beauv., *Setaria viridis* (L.) Beauv. and other small seeds. However, due to the limited cross-sectional area of the centrifuge tube, this method was not suitable for large seeds such as *Xanthium strumarium* L., *Glycine* max Sieb. et Zucc., *Glochidion puberum* (L.) Hutch. Overall, the CTAP was appropriate for the preliminary screening of many samples due to the low calibration accuracy of the scale line in the centrifuge tube than the ruler.

CONCLUSIONS

We developed a new method Centrifuge Tube Agar Profile Method (CTAP) to determine the allelopathic effects of plants. This method required less quantity of allelopathic material, and reduced the time to measure the root and shoot growth of recipient plants by using the scale line of the centrifuge tube. However, this method was not suitable to study large seed because of limited cross sectional area of centrifuge tube. Notably, the method could be improved by changing the size of the centrifugal tube or using other tubes with scales. The CTAP method could do the preliminary screening of large number of samples quickly with less allelopathic material.

ACKNOWLEDGEMENTS

This article was supported by the Changsha Municipal Natural Science Foundation, Grant/Award Number (kq2014168), China Agriculture Research System of MOF and MARA (CARS-16-E19), Training Programme for Excellent Young Innovators of Changsha (kq 2009081).

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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PUBLISHER NOTE