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Putative volatiles in coelomic fluid of earthworm regulate the growth of *Vigna radiata*

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A R T I C L E I N F O

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A B S T R A C T

Coelomic Fluid (CF) possesses enzymes, nutrients and small molecules that could be essential for plant growth. In this study, the CF was collected from *Eudrilus eugeniae* during normal (T2), cold (T3) and warm (T4) conditions and their growth potential of *Vigna radiata* were evaluated. We measured root, shoot and seedling lengths, vigor index and germination percentage of *V. radiata* pre- and post-treatment of CF and analyzed the data using statistical package. Further, the cold CF was analyzed using Gas Chromatography–Mass Spectrometry (GC-MS) to identify putative volatile compounds. On comparing different treatments, plant growth parameters were significantly higher for cold CF (1:10) treatment than other treatments as evidenced by the increase in root, shoot and seedling length, vigour index and germination percentage, which were significantly lower in other treatments (warm and normal). We identified as many as 9 different volatile compounds, which were already reported as plant growth regulators or possess antifungal, antioxidant, antimicrobial and pesticidal properties. Our results suggest that growth enhancement is due to the significant roles of various volatile compounds along with available nutrients, proteins and enzymes in CF.

1. Introduction

In recent years, organic farming is considerably important in improving soil textures and reducing the adverse effects of chemical fertilizers (Gholami et al., 2018; Gomiero et al., 2008; Ukale et al., 2016). The products of organic crops has tremendous market values in national and global levels (Crowder and Reganold, 2015; Lim et al., 2015). The essence of sustainable agriculture completely relies on improving the soil quality parameters. Vermicompost is one of the essential products, which improves the soil quality and evidenced to induce the growth of diverse plants (Rajiv and Vanathi, 2018). Vermicompost, otherwise, used as alternatives to chemical fertilizers and reduces the burden of diseases in plants (Ansari, 2008).

Vermiwash, the liquid collected from the earthworm culturing unit is also used to improve the growth of the crops and the quality of the soil. Vermiwash helps to maintain the temperature, moisture, pH, organic content of the soil and litter input (Tamizhazhagan et al., 2016). Vermiwash was evidenced to contain beneficial microorganisms, enzymes, proteins, macro, and micronutrients and plant growth compounds such as humic acid, Indole-3-butyric acid and cytokinins (Esakkiammal et al., 2015). For instance, vermiwash increased the availability of macro- and micro-nutrients and enhanced the growth of *Capsicum frutescens* (Varghese and Prabha, 2014). Similarly, vermiwash treatment improved the growth of *Vigna mungo* (Rekha et al., 2018). The yield of paddy was increased significantly upon treatment with extracts of vermiwash (Nath and Singh, 2012). The vermiwash also proved to provide protection against various plant pathogens (Elumalai et al., 2015).

The enzyme composition of vermiwash from *Eisenia fetida, Eudrilus eugeniae and Perionyx excavatus* were identified as cellulase, amylase, acid phosphatase, and alkaline phosphatase. The vermiwash of *E. eugeniae* and *P. excavatus* also contain high concentrations of protease and isozymes. The vermiwash, in addition, possess pesticidal properties perhaps due to the presence of thiocarbamic acid (Pramanik, 2010). Germination on *Vigna*, in which cold vermiwash increased the growth of *Vigna* than warm vermiwash (Chattopadhyay, 2015).

In this study, we collected CF from *E. eugeniae* under three different conditions including, normal, warm and cold. These fluids were eventually used to evaluate the potential to (i) induce the germination on *Vigna radiata* and to (ii) enhance the growth by morphological means. We analyzed the volatile compounds of CF and propose the CF of *E. eugeniae* as an effective medium for sustainable plant growth.

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2. Materials and methods

2.1. Crop selection

Certified seeds of green gram (*Vigna radiata* L. Wilczek. cv CO 6) variety were procured from Tamil Nadu Agricultural University, Coimbatore, India. Seeds were surface sterilized with 1% sodium hypochlorite solution and followed by three times wash with sterile distilled water before use.

2.2. Culturing of earthworms

Eudrilus eugeniae was purchased from Shakthi Vermicompost Industry, Madurai, India and cultured on cow dung, haystack and dried leaves. Moisture in the culturing unit was achieved by spraying water at regular intervals to increase the proliferation of earthworms. The worms were sub-cultured every 45 days.

2.3. Coelomic fluid (CF) collection

Coelomic fluid was collected under three conditions from healthy adult earthworms of similar sizes, weighing 1.0 to 1.5 gm. Briefly, 50 casting-removed earthworms were washed with distilled water and kept in tissue paper. Later, the worms were suspended in 50 ml of sterile distilled water (1ml/worm) under mild stirring for 10 min (T2) to collect the fluid. Fifty worms with 50 ml of sterile distilled water in a glass beaker were placed in ice box (4 °C) for 10 min (cold CF-T3), or they were kept in sterile warm water (45 °C) for 10 min (warm CF-T4) to collect CF. The sterile distilled water was served as control (T1). The collected fluids were centrifuged separately for 1 min at 2000 rpm to remove the solid particles. Later this fluid was diluted at a ratio of 1:1 or 1:10 with distilled water and sterilized with 0.2 µm filters to remove microorganisms. The filtered fluids were subsequently stored at 4 °C until further analysis.

2.4. Morphology analysis

Seeds of *Vigna radiata* were placed in Petri plates, and 5 mL of each CF (normal, warm, cold) was added (two concentrations such as 1:1 and 1:10) to the seeds twice daily. After 7 days, the morphological parameters such as root, shoot and seedling lengths, vigour index (G% * Seedling length (cm)) and germination percentage were recorded. The temperature for germination was maintained at 28 ± 2 °C and relative humidity at 95 ± 3% as described earlier (Abdul-Baki and Anderson, 1973).

2.5. GC-MS analysis

The cold CF was used for GC-MS analysis as it enhanced the growth parameters of *Vigna radiata* significantly. The volatile compounds were identified using GC-MS (Clarus 680 GC). The machine was fitted with a fused silica column (Elite- 5MS, 30 m × 0.25 mm i.d × 250 µm d.f). Helium was used as carrier gas at a flow rate of 1 mL/min. The injector temperature was 260 °C and the oven temperature is as follows: 60 °C for 2 min increased to 300 °C at a rate of 10 °C min^{-1} and held for 6 min. The transfer line and ion source temperatures were kept at 240 °C. Electron ionization was done at 70 eV, and *m/z* values were set between 40 and 600 Da. GC-MS NIST library was used to identify the unknown compounds present in the samples.

2.6. Statistical analysis

The experimental data of three trials from four treatments (T1, T2, T3, and T4) with two different concentrations (1:1 and 1:10) were analyzed using Analysis of Variance (ANOVA) method. Completely Randomized Design (CRD) was used for lab-scale experiments in the AgRes statistical package (Version 7.01) (Agres, 1994). Significant differences among various treatments were analyzed by "F" test in ANOVA. The mean values from all the treatments were compared using least square difference at $P < 0.05$. The Standard Error of Difference (SED) and Critical Difference (CD) (0.05%) were compared among treatments, concentrations and their interactions with plant growth parameters (root, shoot and seedling lengths, vigour index and germination percentage).

3. Results and discussion

Earthworms are bioindicators of soil quality and influence plant parameters (Fründ et al., 2011). More research has been done on the effect of vermiwash, vermicompost, vermin tea and coelomic fluid and suggested to regulate the growth of variety of plants (Edwards et al., 2010). The term 'vermiwash' is used interchangeably for two different methods such as stress-induced collection of coelomic fluid and the collection of seepage liquid from a big container that has vermicompost, earthworms, cow dung, soil, and other plant based organic substances. The latter one is a collection of water soluble excretory and secretory products of earthworm, in addition to micronutrients of the soil, cow dung, decomposed organic matter and microbiome (Ismail, 1997; Zambare et al., 2008). It is better to refer the former one as coelomic fluid (CF) to differentiate between the two types as they differ from each other greatly in their composition. Moreover, the presence of microbiome in vermicompost or vermiwash might influence the growth parameters of plants. Here, we collected the CF by three different methods and filtered out the microbiome before the use.

The influence of three different CF was analyzed in improving the germination of *Vigna radiata*. We found cold and normal CF (T3 & T2) increased the germination percentage of *Vigna radiata,* compared to control (T1 (89%)) and warm CF (T4) (97–98%). The critical difference (CD) among the four treatments with two different concentrations (1:1 and 1:10) was observed (Table 1). Warm CF had been shown to increase the rate of germination and the growth of two cereals, *Cyamopsis tertagonoloba* and *Trigonella foenum-graecum* (Suthar, 2010). Moreover, coelomic fluid has been shown to enhance the germination in *Vigna radiata*, *Lepidium sativum* and *Raphanus sativus* (Chattopadhyay, 2015). However, the collected coelomic fluid or vermiwash would have had considerable microbial population as reported earlier (Gopal et al., 2010). Rhizospheric bacteria has been shown to increase the growth of plants either by promoting the bioavailability of nutrients or by producing the growth hormones like Indole-3-acetic acid (Ghosh et al., 2015; Kumari et al., 2018). The fluid we used was free from microbes as it was passed through 0.2 µm filter, before the use. Hence our results suggest that coelomic fluid, that is free from microbes, is sufficient enough to enhance the seed germination of *V. radiata*. It excludes the possible role of microorganisms of CF in seed germination.

Table 1
Effect of CF treatments on germination percentage of *Vigna radiata* -T1 to T4 represent different treatments (T1-control (water), T2-normal CF, T3-cold CF and T4-warm CF) and *** means significantly different. NS represents not significant.

Treatments	Germination Percentage		
	C1 (1:1)	C2 (1:10)	Mean
T1	89.33	89.33	89.33
T2	100	100	100
T3	100	100	100
T4	98.666	97.333	98
Mean	97	96.66	96.83
	Treatment	**Concentration**	**Interaction between Treatment and Concentration**
SED	0.94	0.66	1.33
CD (0.05%)	1.99***	1.41 (NS)	2.82 (NS)

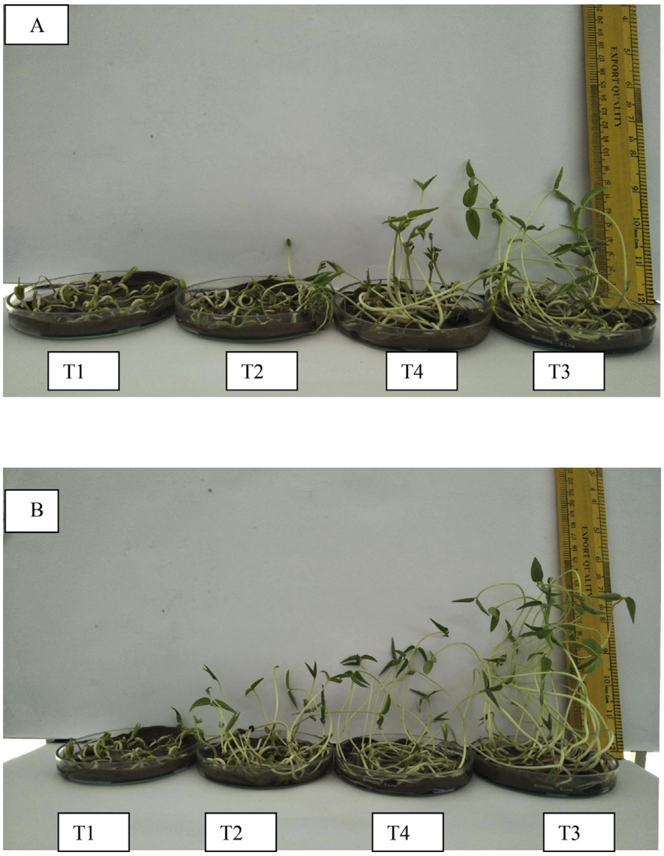


Fig. 1. Effect of CF on growth of *Vigna radiata*. T1- Control (water), T2- Normal CF treatment, T3- Cold CF treatment and T4- Warm CF treatment). **1A** and **1B** represents 1:1 and 1:10 dilution respectively. Photographs were taken after 7 days of treatments.

It was noticed after seven days that the growth of seedlings was increased significantly with 1:10 dilution of CF compared to 1:1 dilution (Fig. 1A and 1B). To analyze the difference quantitatively, the growth parameters such as shoot length and root length were measured and compared with three treatments of CF and control. The increase in the shoot length of *Vigna radiata* was clearly visible after 7 days with 1:1 dilutions of cold CF, when compared with control, normal or warm CF. It indicates that cold and warm stress greatly induced the earthworms to secrete potential compounds in CF. Cold CF promoted the shoot better than warm CF. The shoot length was increased in the order of T2, T4 and T3 from control (Fig. 2A and Table 2). It suggests that warm temperature might have affected the optimal activity of enzymes or association of enzymes with small molecules that were necessary for the growth enhancement. The enhancement of shoot growth was better than control when all three types of CF were diluted 10 times with sterile distilled water. The pattern of increasing order among T2, T3 and T4 treatments remained same (Fig. 2B and Table 2). It clearly shows that the concentration of CF plays a crucial role in the growth and development of seedlings.

Interestingly, the root growth was also increased by different treatments of CF. It followed similar pattern that, 1:1 dilution of cold CF enhanced root growth better followed by warm CF and normal CF than control (Fig. 3A). Treatments of T2, T3, and T4 yielded the root length by 5, 11 and 7.17 cm, respectively (Table 3). It was compared with application of 1:10 diluted CF. The root lengths were significantly increased by cold CF (T3) (16.5 cm) followed by warm and normal treatments (9.33 cm and 8.66 cm respectively), whereas root length of the control (T1) was only 1.5 cm (Table 3; Fig. 3B). Analysis of shoot and root lengths with statistical software showed that the SED and CD are significantly different (Tables 2 and 3). Conclusively, 1:10

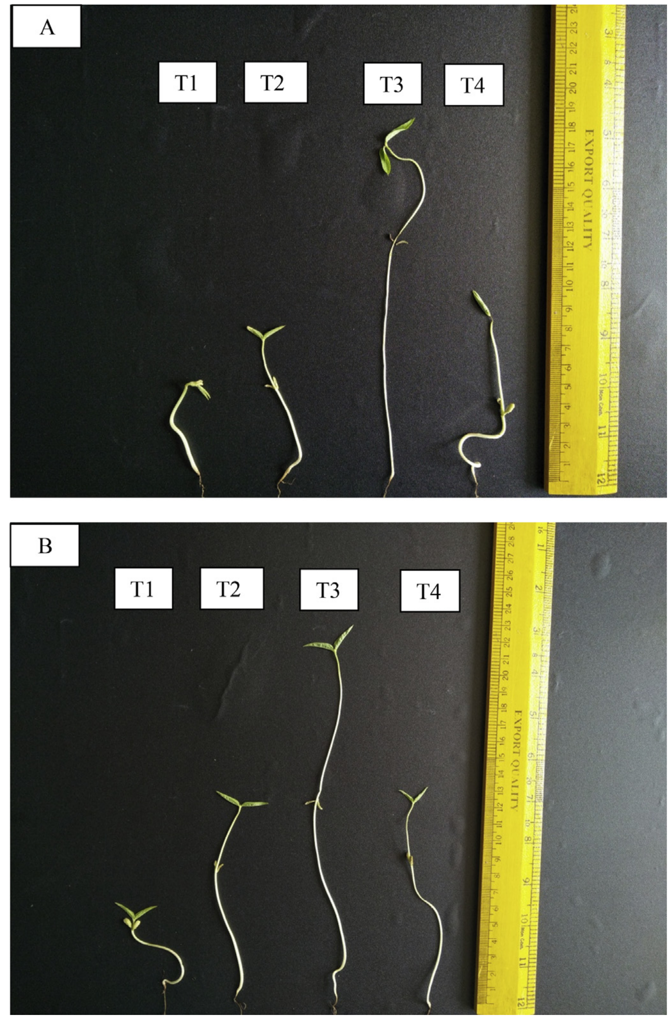


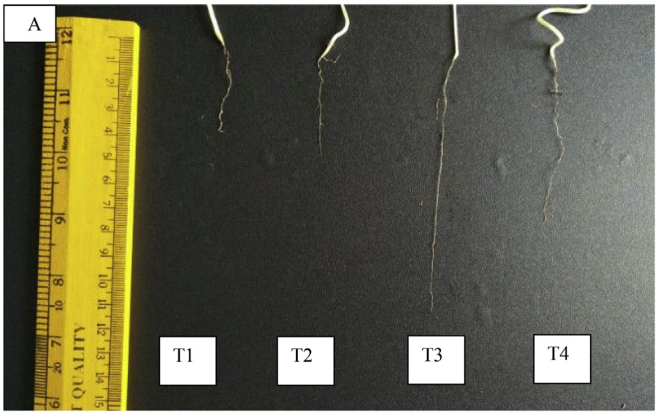
Fig. 2. Effect of CF on shoot length of *Vigna radiata*. T1- Control (water), T2- Normal CF treatment, T3- Cold CF treatment and T4- Warm CF treatment). **2A** and **2B** represents 1:1 and 1:10 dilution respectively. Photographs were taken after 7 days of treatments.

Table 2
Effect of CF treatments on shoot length of *Vigna radiata*- T1 to T4 represent different treatments (T1-control (water), T2-normal CF, T3-cold CF and T4-warm CF) and *** means significantly different.

Treatments	Shoot length (cm)		
	C1 (1:1)	C2 (1:10)	Mean
T1	6.50	6.50	6.50
T2	8.16	13.66	10.91
T3	21.50	24.50	23.00
T4	12.66	14.83	13.75
Mean	12.20	14.87	13.54
	Treatment	**Concentration**	**Interaction between treatment and concentration**
SED	0.18	0.13	0.26
CD (0.05%)	0.39***	0.27***	0.55***

concentration of all the treatments increased the shoot or root length significantly more than its corresponding 1:1 concentration treatments (Tables 2 and 3). Among four treatments, cold CF (both concentrations) showed better growth.

Micronutrients released from vermiwash were documented to contain essential elements like organic carbon, N, P, K, Na, Ca and Mg to increase the growth of the plant (Chattopadhyay, 2015; Hatti et al.,



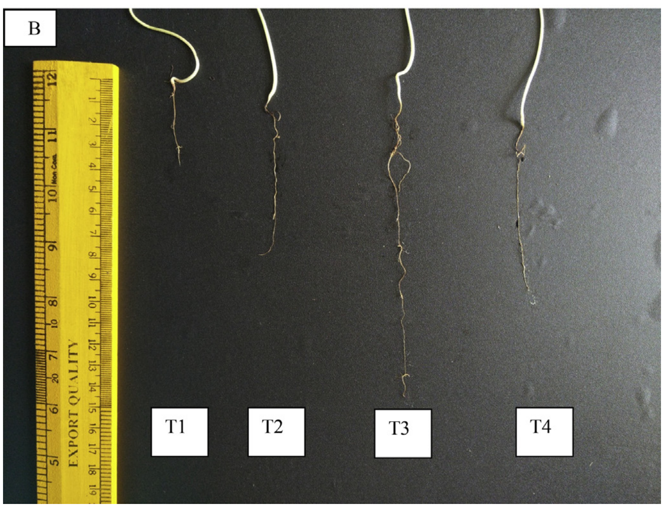


Fig. 3. Effect of CF on root length of *Vigna radiata*. T1- Control (Water), T2-Normal CF treatment, T3- Cold CF treatment and T4- Warm CF treatment. **3A** and **3B** represents 1:1 and 1:10 dilution respectively. Photographs were taken after 7 days of treatments.

Table 3
Effect of CF treatments on root length on *Vigna radiata* -T1 to T4 represent different treatments (T1-control (water), T2-normal CF, T3-cold CF and T4-warm CF) and *** means significantly different.

Treatments	Root length (cm)		
	C1 (1:1)	C2 (1:10)	Mean
T1	4	4	4
T2	5	8.66	6.83
T3	11	16.50	13.75
T4	7.16	9.33	8.25
Mean	6.79	9.62	8.20
	Treatment	**Concentration**	**Interaction between Treatment and Concentration**
SED	0.20	0.14	0.28
CD (0.05%)	0.43***	0.30***	0.61***

2010). Vermicompost has also been reported to have micronutrients, which indicates that micronutrients play an important role in plants growth (Manyuchi et al., 2013). Root length of *Vigna radiata* was increased possibly due to the presence of high amount of micronutrients as suggested earlier (Chattopadhyay, 2015). Our results suggest that the type and the concentration of micro and macronutrients are one of the major reasons for the differences in the growth among four treatments. Cold CF showed maximum growth promotion as that may contain essential nutrients intact than other CF. It should be noted that the presence of plant hormones such as indole-3-acetic acid and gibberellic acid or hormone-like substances has been shown in the vermicompost and vermiwash (Gopal et al., 2010; Tomati et al., 1988). The hormone or hormone-like substances might be synthesized from the microbial community in the vermicompost or vermiwash (Gopal et al., 2010). As our CF does not have microbes, we hypothesize that CF may have hormone-like compounds or other molecules that could serve as precursors or inducers of hormone biosynthesis in *Vigna radiata*. The synergistic effect of hormone-like substances, enzymes and the nutrients could be the reason for the growth enhancement.

Table 4
Effect of CF treatments on seedling length of *Vigna radiata*. T1 to T4 represent different treatments (T1-control (water), T2-normal CF, T3-cold CF and T4-warm CF) and *** means statistically different.

Treatments	Seedling length (cm)		
	C1 (1:1)	C2 (1:10)	Mean
T1	10.50	10.50	10.50
T2	13.16	22.33	17.75
T3	32.50	41	36.75
T4	19.83	24.16	22
Mean	19	24.50	21.75
	Treatment	**Concentration**	**Interaction between treatment and concentration**
SED	0.31	0.22	0.44
CD (0.05%)	0.66***	0.46***	0.93***

Similar to other parameters, the seedling length was also compared among the treatments to reveal the role of CF in supporting the plant growth. The seedling length was also significantly higher in T3 treatment compared to T4 and T2 treatments with respect to both concentrations (1:1 and 1:10). Particularly, the mean seedling length of T2 and T4 treatments were 40.84% and 52.27%, respectively (Table 4), which was higher than T1 treatment. The seedling length was improved in 1:10 concentration than 1:1. Earthworms play a major role in plant seed germination and seedling growth (Suthar, 2010). Vermiwash was foliar sprayed for 40 days and shown to increase the yield (Hatti et al., 2010). The vermiwash was evidenced to increase the growth of marigold, and cowpea (Sivasubramanian and Ganeshkumar, 2004). The seedling growth of *Linnum usitassimum* was also found to be maximum when treated with vermicompost and foliar spray of vermiwash (Ansari, 2008; Makkar et al., 2017). However, we show here that diluted cold CF alone could enhance the seedling length. In order to quantitatively represent the relative effect of CF and its two different concentrations, vigour index was calculated. It was calculated based on the germination percentage and seedling length (Table 5). As T3 treatment showed higher germination percentage and seedling length, the vigour index was also higher for T3 than any other treatments in both

Table 5
Effect of CF treatments on vigour index of *Vigna radiata* - T1 to T4 represent different treatments (T1-control (water), T2-normal CF, T3-cold CF and T4-warm CF) and *** means significantly different.

Treatments (T)	Vigour Index		
	C1 (1:1)	C2 (1:10)	Mean
T1	938	938	938
T2	1316.666	2233.333	1775
T3	3250	4100	3675
T4	1956.666	2351.333	2154
Mean	1865.333	2405.666	2135.50
	Treatment	**Control**	**Interaction between treatment and control**
SED	30.260	21.397	42.795
CD (0.05%)	64.151***	45.361***	90.723***

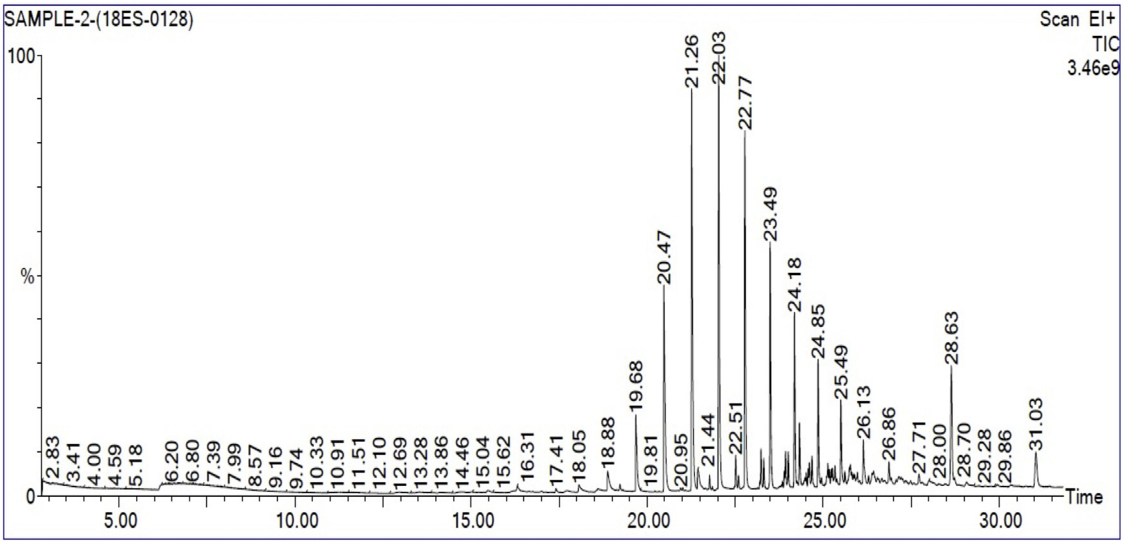


Fig. 4. GC-MS chromatogram of cold CF from *E.eugeniae*.

Table 6
Volatile compounds identified in cold CF using GC-MS analysis.

S. No	Retention Time	Compound name	Reported properties
1	22.03	Heneicosane	Plant growth regulator and an antimicrobial agent
2	21.25	Hexanedioic acid, bis (2-ethylhexyl) ester (fatty acid)	Antifungal, antioxidant, and nematicide
3	22.77	Tetratetracontane	Antioxidant and antimicrobial
4	24.185	Hentriacontane	Antioxidant and antimicrobial
5	25.49	Tritetracontane	Antioxidant and antimicrobial
6	24.18	Squalene	Antioxidant, Antitumor and Immunostimulant
7	26.86	Heptacosane	Antimicrobial
8	22.50	Hexatriacontane	Antioxidant and antimicrobial
9	26.13	Thiocarbomic acid	Pesticidal

concentrations. The maximum vigour index based on seedling length was recorded in cold treatments of two different concentrations as 3250 for 1:1 and 4100 for 1:10 respectively, over other treatments. The mean vigour index of warm CF treatments showed higher values than normal treatments. The vigour index from (T3) cold treatment was found to be maximum than warm CF treatment (T4) followed by T2 and T1 treatments. The seedling vigour with respect to vermiwash foliar application had a remarkable yield in cowpea radish and rice (Naik et al., 2014; Thangavel et al., 2003).

Among four treatments, T3 treatment (both concentrations) showed better outcome in morphological and other growth parameters. Therefore, the cold CF was subjected to GC-MS analysis to identify the plant growth promoting volatile compounds (Fig. 4). We found that CF mainly contains hydrocarbons and fatty acids such as heneicosane, hexadecanoic acid, bis (2- hexyl) ester, tetratetracontane, hentriacontane, hexatriacontane, tritetracontane, squalene, heptacosane, and thiocarbamic acid. The biological significance of these compounds is listed in Table 6. Compounds like heneicosane, shown to have plant growth regulator activity, and others having antioxidant property, could have influenced on growth of seedlings (Shorning et al., 1999). The results suggest that cold CF contains different volatile compounds with properties of promoting growth, pesticidal, antimicrobial or antioxidant.

4. Conclusions

We conclude that cold stress method is better to collect CF as it may not be changing the optimal activity of proteins, enzymes, nutrients and small molecules in the liquid. The concentration of CF is playing crucial role in determining the growth of seedlings. Passage of CF with 0.2 µm filter before the use indicates that the growth enhancement is not due to any beneficial microbial interaction with the plants. We hypothesize that growth enhancement of *V. radiata* is due to the synergistic act of volatile compounds along with nutrients, hormone-like compounds, enzymes and proteins. More biochemical characterization of CF and gene expression studies in seedlings would reveal the molecular mechanism and helpful to decipher the role of cold CF in promoting plant growth.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101070.

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