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FRASS OF SAPROXYLIC-CERAMBYCID LARVAE FROM DEAD TWIGS OF *ACACIA STENOPHYLLA* A. CUNN. EX. BENTH. AND ITS EFFECTS ON GERMINATION AND SEEDLING GROWTH OF *LACTUCA SATIVA* L. VAR. GRAND RAPIDS

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ABSTRACT

The phyo-chemical composition, fungal flora and allelopathic potential of frass from saproxylic- cerambycid larvae of *Chlorophorus annularis* (Fabricius 1787) posted in the wood of dead twigs of *Acacia stenophylla* A. Cunn. Ex. Benth. are described. The frass was found to contain a number of phyto-chemicals – Alkaloids, phenols, sugar, and protein and K and Na. The mycoflora of frass included *Aspergillus flavus* Link Ex. Link, *A. fumigatus* Fresenius, *A. niger* Van Tieghan, *Cladosporium cladosporoides* (Fresn.) G.A. de Vries, *C. sphaerospermum* Penz. and *Trichoderma hamatum* (Bonord.) Bain. The extract of the frass exhibited allelopathic effects against lettuce (*Lactuca sativa* L. var. *Grand Rapids*) seed germination and seedling growth. The results are discussed in the light of available literature.

Key Words: *Chlorophorus annularis*, larval frass, biochemical composition, fungal flora of frass, frass extract, allelopathy, lettuce seed germination and seedling growth.

INTRODUCTION

Oxford English dictionary defines "Frass" as "the excrement of larvae, also refuse left behind by boring insects. "Frass" is derived from German "Frasz" from the root of "fressen" (= fret or to devour). The oldest use of the word is traced back to 1854 and coined by a lepidopterist, H.E. Stainton (Berenbaum, 2003). The word 'frass' is used in several contexts. It may refer to fine masticated material often powdery that phytophagous insects pass as indigestible waste, plant tissues they have processed as well as their physiology permit (Allaby, 2004). Other common examples include the fecal material as coding months (*Cydia pomonella*) leave inside fruit, or as that like that of *Terestia meticulosatis* leave as they bore in the pith of *Erythrina* twigs or dry wood termite droppings or sawdust of carpenter ants or excrement of larvae of Cerambycids or powdery post inside or below the tunnels when saproxylic insects bore in solid or decaying wood (Speight, 1989; Speight *et al.*, 1999; Weis, 2006; Pearcy *et al.*, 2012). In short, it is loose powdery mass due to insects.

During our studies of an Australian Acacia (*Acacia stenophylla* A. Cunn. Ex. Benth.; vernacularly known as River Cooba), a tree growing in Karachi University Campus, the dead twigs of the plant were found infested with cerambycid saproxylic larvae which posted frass inside branches in tunnels in the woody region (Fig.1C). The exit holes were round (Fig. 1A). As we collected frass from the twigs, we found larvae (Fig. 1B) and after some time an adult insect as well from the frass posted in a twig (Fig. 1 D and E). The insect, on the basis of its morphology appeared to be a long-horned beetle,

Chlorophorus annularis (Fabricius 1787) –Vern: Bamboo tiger longicorn; Bamboo longhorn; Bamboo borer

A few larvae, we collected from frass within dead twigs, were around 20-30 (-35) mm in length and milky white to creamy white in colour. The adult was slender around 16 mm in length and 4 -5 mm broad with yellowish and black markings (Fig. 1). The genus *Chlorophorus* Chevrolat, 1863 has c 248 species the World over (omnitexica.com). Beeson (1911) in his book has described this species from India (p. 155). The development of the insect usually takes one or more years depending upon dryness of the wood (Beeson, 1911; Weidner, 1982). The morphology of the insect has been described by Gahan (1906, p.261), Beeson (1911, p. 155), Koon (1999) and Hill (2008). Duffy (1968) gave the key to the larvae of oriental timber beetle. The insect infests bamboo but there are several species reported to be the host of this insect - *Bambusa* spp., *Dendrocalamus strictus*, *Citrus* sp., *Dipterocarpus* sp., *Gossypium* sp. *Indosasa crassiflora*, *Liquidamber* spp., *Phyllostachys reticulata*, *Pyrus malus*, *Saccharum officinarum*, *Shorea robusta*, *Sinobambusa gibbosa*, *Sinocalamus* spp., *Tectona grandis*, *Spondias* sp. *Vitis* sp., *Zea mays*, etc. (USDA, 2000; CABI, 2008; Pierce, ND). *C. annularis* is native to Asia (CABI, 2008) but found in other countries of the World. Friedman *et al.* (2008) reported it from Israel. It was found in Australia (Brisbane) where there was no bamboo (*www.brisbane.insects.com/brisbane_longicorns/cerambycinbae.htm*). It only infests dead wood and probably lacks ability to damage living material. It is thus generally rated as minor pest. It is, however, rated as serious pest of bamboo in India and Japan by USDA Forest Serv. (1985).

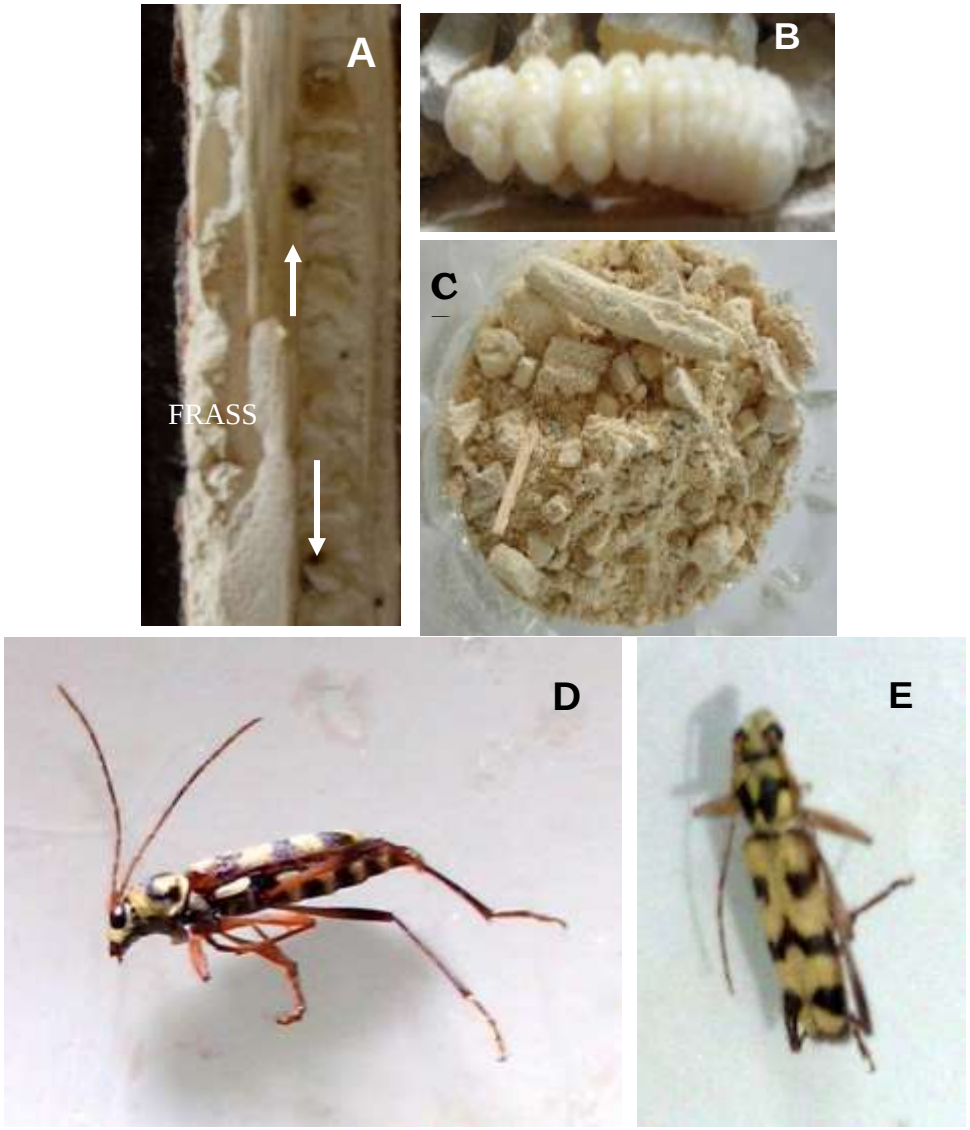

Fig. 1. The holes due to cerambycid infestation of stem and branches of *Acacia stenophylla*, broken to show the frass accumulated in the wood tunnels by the insect (A), Larva of the insect – c 35 mm in length (B), frass taken out of the branches (C) and adult of a beetle (*Chlorophorus annularis)* obtained from inside of dead twig of *Acacia stenophylla* and immediately photographed (D); Insect lateral view (D) and dorsal view (E).

Insect frass is known to be biologically active substance. On the basis of concentrations of various elements in frass, it has been suggested to have a role in micronutrient cycling (Chen and Forschlor, 2016). Insect canopy herbivory and frass deposition affect soil nutrient dynamics (Frost and Hunter, 2004). There are also few reports on allelopathic potential of insects' frass rain in eucalypts (Trenbath and Fox, 1976, 1977; Trenbath and Silander, 1978, 1979; Trenbath, 1978; Silander *et al.*, 1983, 1985). It was shown that insect frass due to browsing of eucalypts by leaf-chewing insects suppresses germination, growth and survival of herb layer species. Plant species vary in tolerance and thus structure and composition of associated herbaceous under storey plant communities were markedly affected by frass fall (Silander *et al.*, 1983). Trenbath and Fox (1976, 1977) observed that bare zones commonly develop under certain individual mature trees of *Eucalyptus globulus* ssp. *bicostata* in parks in Canberra. This pattern was suggested to be associated with the presence of a foliaceous-foliovorous chrysmelid beetle, *Chrysophtharta m-fuscum* and to the frass rain. The aqueous extract of foliage showed little allelopathic activity whereas beetle frass severely inhibited the germination of mustard (*Sinapis alba* L.) in seed bioassay. That is to say that beetle provided a means of release and transport of phytotoxins from the foliage *of E. globulus* ssp. *bicostata.* Trenbath and Silander (1978) investigated the Phytotoxicity of frass from another Chrysomelid beetle, *Poropsis*

atomaria feeding on leaves of *E. bicostata*. This frass was tested with seeds of *Sinapis alba, Trifolium repens* and *Festuca rubra* var. *fallax*. Mustard and Fescue proved to be most sensitive and radicle extension was almost completely inhibited Trenbath and Silander (1980) concluded that frass can have a significant effect on the patterning of under storey species. Kagata and Ohgushi (2012) described positive and negative effects of frass due to leaf-eating larvae of *Memestra brassicae* feeding on fertilized and unfertilized *Brassica rapa* L. var. *perrviridis* due to differential N-content in frass and its deposition on to soil.

In view of the above, in present studies, a preliminary attempt has been made to determine composition of frass of *C. annularis* larvae, posted in the dead twigs of *A. stenophylla*, and investigate its fungal flora and the possible phytotoxic effects on lettuce.

MATERIALS AND METHODS

Phytochemical screening

Preparation of frass ethanolic extract: 100g frass was extracted with 2.00L ethanol for one week. Yellow extract was filtered and evaporated at room temperature to semi-solid substance that weighed c 6.0g. This frass extract served for qualitative analysis for various phytochemicals (Harborne, 1973; Vishnoi, 1979; Sofowora, 1993; Trease and Evans, 2002).

Biochemical Estimation

Proteins: The frass sample (0.5 g) was grounded in liquid nitrogen and homogenized in 5 mL of ice chilled potassium phosphate buffer (pH = 7, 0.1 M) containing 1mM EDTA and 1% PVP (w/v). The homogenate was filtered through a muslin cloth and then centrifuged at 21,000 g at 4 °C for 20 min in refrigerated centrifuge. The supernatant was separated and stored at -20 °C. The protein contents were determined by using Bradford Assay reagent method (Bradford, 1976). The protein content was determined against Bovine Serum Albumin as standard and the protein content was calculated from a following best-fitted standard curve equation.

Proteins ($\mu g.mL^{-1}$) = -3.29196 + 114.2755 OD ± 5.3436

(t = 16.76, F = 280.93, P < 0.0001, R^2 = 0.9723)

The concentration of protein contents was expressed in $mg.g^{-1}$ dry weight.

Total sugars: The frass sample was boiled in 80% ethanol at boiling water bath. It was homogenized in 80% ethanol and centrifuged at 4000 g for 10 minutes. The supernatant was separated and the residue was again extracted with 80% ethanol. Both supernatants were combined and then the volume was made up to desired level by distilled water. The extract was used for the determination of total sugars by the method of Fales (1951). The total sugars were determined against glucose as standard and the total sugars were calculated from a following best-fit standard curve equation.

Total sugars ($\mu g.mL^{-1}$) = $228.462.\ OD^{0.97275}$ ± 0.04455

(t = 49.28, F = 2428.32, P < 0.0001, R^2 = 0.9967)

The concentration of total sugars was expressed as $mg.g^{-1}$ dry weight.

Phenols: Soluble phenols were determined by the method of Singleton and Rossi (1965). The frass material was homogenized in 80% methanol and centrifuged. To 1 mL of diluted extract 5 mL of Folin-Ciocalteu reagent (1:9 ratio in distilled water) and 4 mL of 7.5% Na_2CO_3 were added. The absorbance was recorded at 765 nm after incubation of 30 minutes at 25 °C. The soluble phenols concentration in frass was determined against Gallic acid and calculated from a following best-fit standard curve equation.

Phenols ($\mu g.mL^{-1}$) = 1.62724+94.5284 OD - 17.19352 $(OD)^2$ ± 0.3425

(t = 35.57 & -4.17, F = 8786.10, P < 0.0001 & 0.0051, R^2 = 0.9996)

The concentration of total phenols was mentioned in $mg.g^{-1}$ dry weight.

Na and K content: Na and K in the frass were determined according to the method of Chapman and Pratt (1961). Frass was dried at 60 ˚C for 48 h. The dried frass (100 mg) was transferred to porcelain crucible. The crucible was placed in a muffle furnace at 550 ˚C for 6 h. The ash was dissolved in 5 mL of 2 N HCl. After 20 min the solution was diluted with deionized water. This solution was filtered through a Whatman No. 1 filter paper and the concentrations of Na^+ and K^+ ions were determined with flame photometer. The best-fit standard curve equations were as follows:

Na (ppm) = $0.016135.X^{1.879824}$ ± 0.04433

(t = 49.528, F = 2453.01, P < 0.0001, R^2 = 0.9968)

K (ppm) = $0.244346.X^{1.314603}$ ± 0.04433

(t = 29.47, F = 868.54, P < 0.0001, R^2 = 0.9909)

Where X = Reading on the flame photometer.

The concentration of Na and K ions were expressed as $meq.g^{-1}$ dry weight.

Isolation of Fungi

a) Direct plate method: Powder frass (0.01g) was dispersed in 1 mL sterile distilled water in a sterilized petri dish. Approximately 10 mL melted and then cooled agar was poured in the dish and mixed. The frass particles were distributed throughout the medium by rotating the dish. The petri dishes were incubated at room temperature 25 ^{o}C. Fungi growing the plates were identified (Wareup, 1950).

b) Serial dilution method: powder frass (0.2g) was suspended in 18 mL sterilized distilled water which gave a dilution of 1:10. Serial dilutions of 1:100, 1:1000 and 1:10000 were prepared and 1mL aliquot from 1:1000 dilution was poured in a petri dish containing penicillin @ 20,000 units/L and streptomycin @ 200 ug /L), and approximately 10 mL of potato dextrose agar medium . The test replicated three times. The dishes were incubated at room temperature. The number of colonies produced by the fungus was multiplied by the dilution factor to obtain total number of propagules per g of the frass (Waksman and Fred, 1922).

Preparation of frass extract stock solution for phytotoxic test: 10g of frass was added to 100 mL distilled water and left for over night and following day the solution was filtered with filter paper. It was referred to as stock solution from which different dilutions were made e.g., 12.5, 25, 50, 75 and 100% stock solution.

Frass phytotoxicity: The test species in allelopathic investigation was lettuce (*Lactuca sativa* var. Grand Rapids). Ten seeds of lettuce were placed on filter paper in Petri plates (5cm diameter) already autoclaved. Five mL of the frass extract was added to each plate. The experiment was with four replicates. Distilled water was used as positive control. Seed germination was performed in a growth chamber at 28 ^{o}C in dark. The experiment was run for 10 days. Germination was recorded daily. On alternate day, the extract in the petri plate was replaced with fresh extract. At the end of the extract, the seedlings were collected and their root and shoot length was measured. Seedlings were wrapped in paper and dried in oven at 80 ^{o}C for 24h. They were weighed individually. The data was analyzed statistically. Germination velocity was estimated on the basis of Woodstock (1976). The germination data was subject to ANOVA to elucidate the effects of extract concentration and incubation time period. The mean difference significance was tested at $p < 0.05$.

RESULTS AND DISCUSSION

Composition of frass: The qualitative phytochemical analysis of frass of *C. annularis* larvae recovered from dead twigs of *Acacia stenophylla* indicated the presence of alkaloids, flavanoids, soluble sugars, proteins and phenols in the frass (Table 1).

The sugar content was 47.11± 6.94 mg.g^{-1} (D. wt.), protein 4.57 ±0.88 mg.g^{-1} (D. wt.) and phenols 13.89 ± 0.80 mg.g^{-1} (D. wt.) of frass (Table 2). K and Na concentrations in frass were 0.4113 ± 0.0444meq.L^{-1} and 0.9050 ± 0.1028 meq.L^{-1}, respectively, which corresponded to 0.1604mg.g^{-1} and 0.2082 mg.g^{-1} D. wt. of frass, respectively. *A. stenophylla* is a potassiophillic plant and K / Na in its leaves has been estimated to be 10.21 ± 1.59 (Sahito *et al.*, 2013). From the data of Khan and Sahito (2015) K / Na ratio in bottom ash of stem wood of this species amounted to be 17.46 ± 0.638. It is clear that K / Na ratio in frass (0.7704) is comparatively very much lower than that in leaves or the bottom ash of stem wood of *A. stenophylla*. Such a difference in K / Na ratio of frass, leaves and stem wood may probably be attributed to proportionately larger absorption of K than that of Na from the wood substrate in the larval gut.

Table 1. Phytochemical analysis of Frass.

ALK	ANTH	PH.BT	SAPON.	STER	TRIT	FLAV.	Sol. S.	PROT
+	-	-	-	-	-	+	+	+

Acronyms: ALK, Alkaloids; ANTH, Anthroquinones; PH.BT, Phlobatannins; SAPON, Saponins; STER, Steroids, TRIT, Tritrerpenoids; FLAV, Flavanoids; Sol. S, Sugars; PROT, Proteins.

Table 2. Mineral and biochemical analyses of frass.

Source	Statistics	Na (meq/L)	K (meq/L)	Phenols (mg.g^{-1}DW)	Sugar (mg.g^{-1}DW)	Protein (mg.g^{-1}DW)
Frass	Mean	0.9050	0.4113	13.8880	47.1085	4.5750
	SE	0.1028	0.0444	0.8008	6.94388	0.8848

Table 3. Number of Conidia (per mL) of the fungal Species of Frass as determined by serial dilution method on PDA.

Fungi	Replicates		
	1	2	3
Aspergillus flavus Link ex Link	69000	63000	74000
A. fumigatus Fres	1000	1000	2000
A. niger Van Tieghan	4056	0	0
Cladosporium cladosporoides (Fresn.) G.A. de Vries	0	2000	1000
Cladosporium sphaerospermum Penz.	0	4000	3000
Trichoderma hamatum (Bonord.) Bain	0	0	1000

Table 4. Mycological detection in frass by direct plate method.

Fungi	Replicates		
	1	2	3
Aspergillus flavus	+	+	+
Aspergillus niger	+	+	+
Aspergillus fumigatus	+	-	+

Frass mycoflora: Six fungal species belonging to four genera were isolated from frass powder by dilution technique. *Aspergillus flavus* showed very high number of conidia per mL. Other fungi had comparatively much lower number of conidia (Table 3). By direct plating method only Aspergilli could be isolated of which *A. flavus* and *A. niger* were the frequent species (Table 4). *C. annularis* has been reported not to be a vector of any micro-organisms and not associated with any organisms (http://caps.ceris.purdue.edu/dmm/1978). Our studies of *C. annularis* larval frass, however, indicated a number of fungi present in the frass. Many fungi species are known to inhabit the woody portion (xylem) of tree stems (Leal *et al.*, 2010). Frass is known to contain abundant amoebae, bacteria and fungi. Dawar *et al.* (2015) have isolated several fungal species from wood including Aspergilli (*A. flavus, A. fumigatus and A. niger*) and *T. hamatum* isolated in the present studies. These Aspergilli have also been isolated from paper and fabric, the cellulosic materials (Dawar *et al.*, 2015). They are common in our environment and could have entered the frass through holes in the wood. The tunnels inside wood provide a passage way and the frass and the softened wood to form a substrate for bacteria (Smith and Smith, 2007). The wind-blown frass may disseminate bacteria and fungi to healthy trees (Iton, 1961). The significance of these fungi in frass chemical breakdown within larval gut or outside gut needs to be investigated.

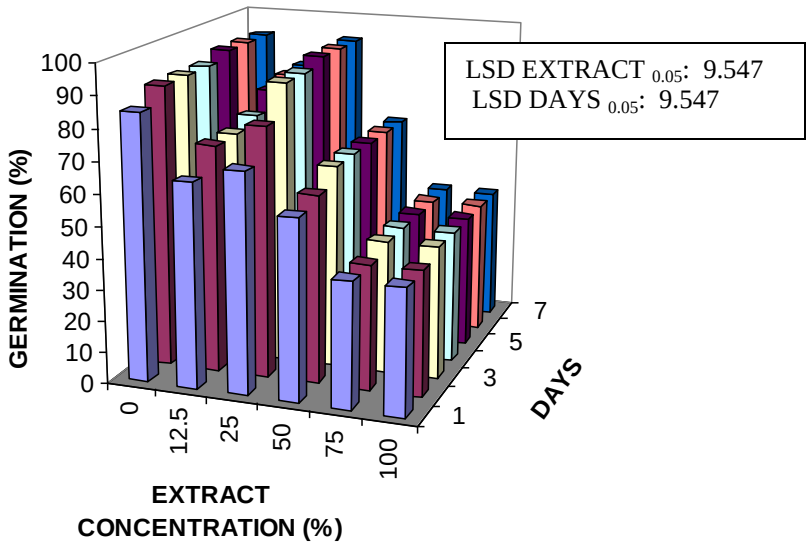

Fig. 2. Germination of *L. sativa* seeds under influence of frass extract.

Effect of frass extract on germination and seedling growth of lettuce: The allelopathic studies, in the present work, were carried out with Lettuce (*Lactuca sativa* var. Grand Rapids. The lettuce seed germination declined significantly and gradually with the frass extract concentration from 12.5% S (stock extract) to 100% S (Fig. 2). ANOVA OF the data indicated that frass extract significantly influenced germination ($F=38.98$, $p < 0.0001$) but the

time period of incubation had no any significant influence on germination (F=1.98, p < 0.0733, NS). Likely, there was no interaction between extract concentration and time period of incubation (F = 0.4043, p < 0.9974, NS). There was significant reduction in germination velocity with frass extract concentration (Fig. 3).

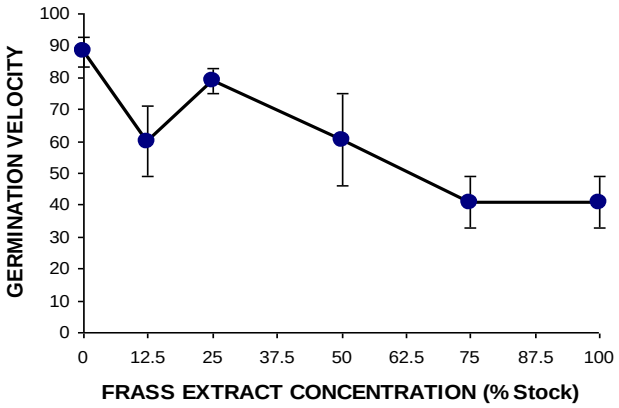

Fig. 3. Germination velocity of lettuce seeds under various concentrations of frass extract.

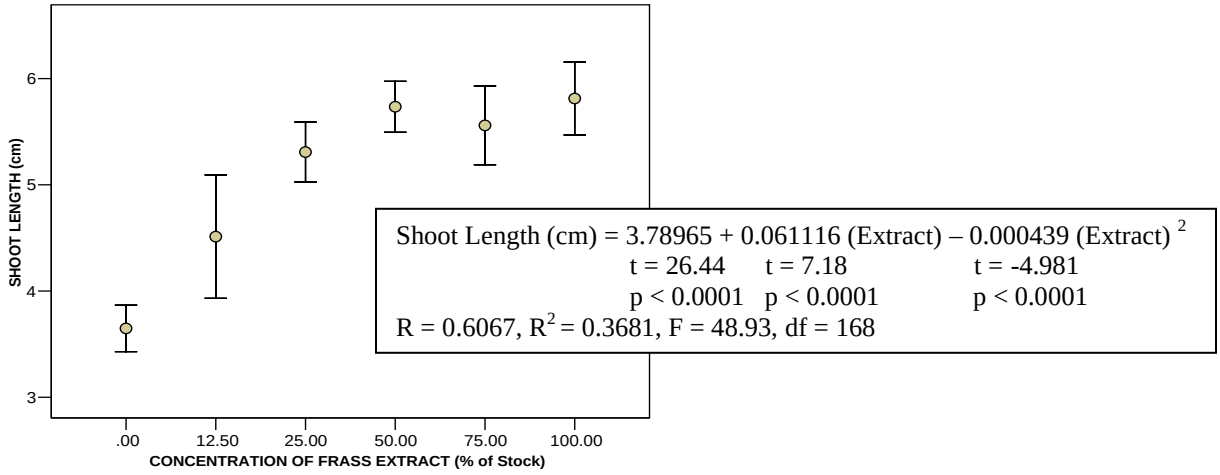

Fig. 4. Shoot length of lettuce seedlings (cm) under various concentrations of Frass extract (% of Stock).

Frass extract significantly promoted the shoot length (Fig. 4) in quadratic way. The behaviour of root growth to frass extract was, however, was quite opposite and declined (Fig. 5) in curvilinear fashion. The seedling dry weight declined significantly with frass extract but related poorly with frass extract (r = - 0.474; F=6.08) and frass extract could account for only 22.4% of the seedling growth (Fig.6). The cummulative biomass of seedlings declined with frass extract (Table 5) but more at high concentrations of the extract.

The effects of frass extract of *C. annularis* larvae on lettuce seed germination and seedling growth may be attributed to the phytochemical nature of the frass which contained flavonoids, alkaloids, phenols etc. Various types of phenolic acids, terpines, terpenoids, glycosides, alkaloids, flavonoids, saponins, steroids and tannins have been reported to be the potent allelochemicals (Rice, 1974; .Mandava, 1985; Blum, 1996; Inderjit *et al.*, 1999 a, b; Khan and Shaukat, 2006 a and b). Saponins have considerable significance because of their allelopathic effects (Fons *et al.*, 2003). Saponins are known to inhibit seed germination and seedling growth (Mirchaim *et al.*, 1970; Khan and Shaukat, 2006a and b). Phenolic compounds are potent allelochemicals and may inhibit germination and seedling growth (Massart, 1957; Naqvi, 1976; Evenari, 1961; Shaukat *et al.*, 1985; Khan and Shaukat, 1990, 2006a and b) and may influence soil nutrient and microbial ecology (Inderjit and Asakawa, 1998).

The saproxylic insects comprise a diverse species-rich biodiversity dependent on the kind of the dead wood and old trees (Grove, 2002). This association has complex chemical ecology (Allison *et al.*, 2004). The process of wood decomposition may take place in three phases: wood invasion by primary saproxylics which use intact wood, decomposition due to primary saproxylics which may be joined by secondary saproxylics which use products of the

primary saproxylics as food or feed on other saproxylics and humification of wood when saproxylics are replaced by soil organisms which feed on bacteria and micro-fungi and play a dominant role in humifying the wood (Dajoz, 1980; Frankland *et al.,* 1982; Ulyshen, 2014). Complex biotic interactions occur in the process which may be extremely slow in arid areas. The saproxylic larvae devour wood and have ability to digest cellulose and bring other chemical changes resulting in the formation of frass – their excretory product. We found that *C. annularis* larvae fed on wood and deposited frass in tunnels as powdery material in dead twigs of *A. stenophylla*. This frass is phyto-chemically complex and bears number of allelochemicals like flavonoids, phenols, alkaloids, proteins, etc. and harbour several fungal species.

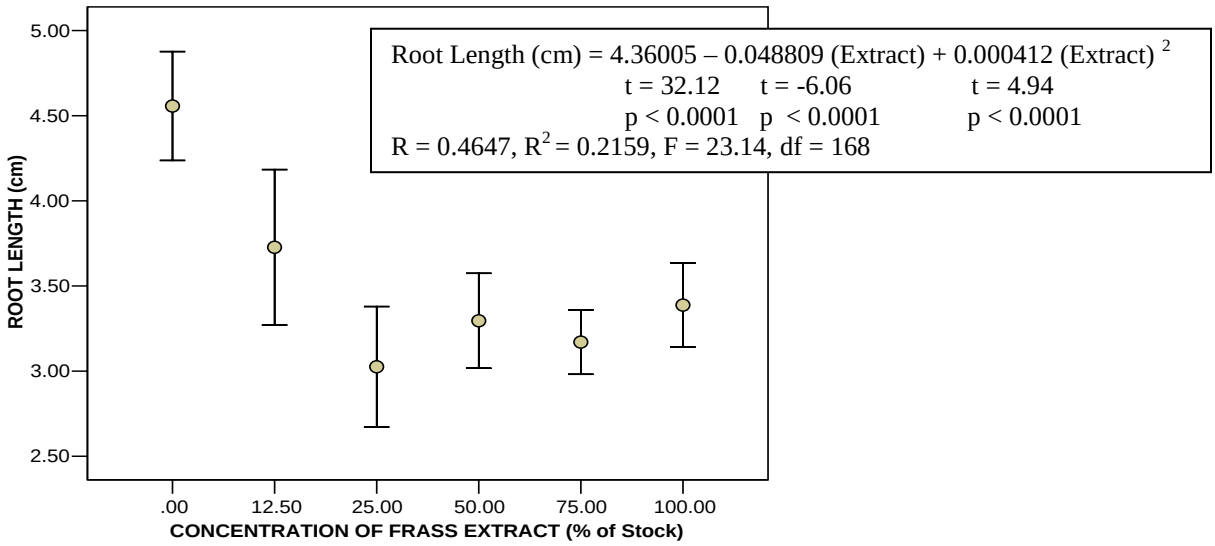

Fig. 5. Root length of lettuce seedlings (cm) under various concentrations of Frass extract (% of Stock).

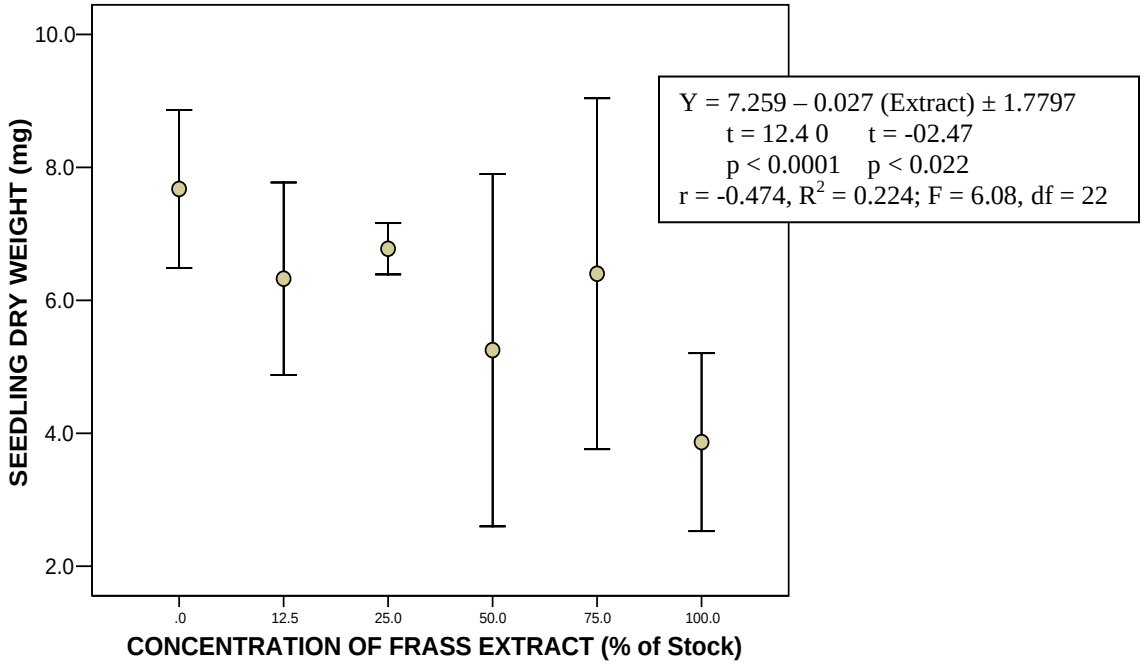

Fig. 6. Seedlings biomass of lettuce (mg) per plate under various concentrations of frass extract (% of Stock). There were four replicates to each treatment.

Table 5. Cummulative dry biomass of lettuce seedlings (per treatment set) as influenced by the frass extract. Each treatment was composed of four replicates.

Extract Concentration (% of Stock)	Cummulative Seedling biomass (mg)
0	33.40
12.5	30.40
25	31.60
50	24.80
75	29.10
100	16.70

Saproxylic insects, on the basis of elemental concentrations of the frass, have also been suggested to have their role in micro-nutrient dynamics and cycling (Chen and Forschlor, 2016). Our studies supported the view of chemical changes taking place in elemental composition during the process of frass formation due to insects' larvae. According to our studies, K / Na ratio in larval frass of *C. annularis* was found to be much lower than that in stem wood of *A. stenophylla.* It indicated proportionately larger absorption of K from the wood substrate in the larval gut than that of Na.

In eucalypt ecosystem, prominent among the herbivore insects are particularly the leaf-eating insects (Penfold and Willis, 1961) showing diverse interactions of herbivory (Landsberg and Cork, 1977). The well-documented allelopathic influence of *Eucalyptus globulus* ssp. b*icostata* (*E. bicostata*) on underlying vegetation were reported to be due to frass rain from the foliovorous insects such as *Chrysophtharta m-fuscum* (Trenbath and Fox, 1976; 1977) and *Poropsis atomaria* (Silander, 1978; Silander *et al.,* 1983). We know nothing about the ecological significance of the frass due to saproxylic- cerambycid larvae of insects (including *C. annularis),* the frass of which remain packed inside the twigs of the host plant (*Acacia stenophylla* in present case) and may only be released on breakage and decay of the twigs. It is, however, clear that larval frass of *C. annularis* formed inside dead twigs of *A. stenophylla* bear allelopathic principles and exert its effects on lettuce seed germination and seedling growth.

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