Attractant formulations for the management of grape mealy bug *Maconellicoccus hirsutus* (Green)

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ABSTRACT

The pink hibiscus mealy bug *Maconellicoccus hirsutus* (Green) is a serious economic threat to agriculture, forestry and the nursery industries. High incidence of it observed in the grape vineyards resulted in a poor fruit quality, declined crop yields and substantial economic loss. Hence it is necessary to develop an effective formulation for its control. Although information about the sex pheromones of mealy bug is reported, the information about attractant pheromone of mealy bug is not known. To isolate it, colony of mealy bug, *M. hirsutus* was maintained and the crawler mealy bugs were extracted. Bioassay of the crude extract obtained was carried out on the crawlers. Results indicated dose dependant attractant properties. The activity was marginal at low concentration and increased gradually with concentration, went through the maximum and then reduced. Formulations of higher concentration (> 1.5 mg / ml) were repellent.

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Key words: Grape mealy bug, *Maconellicoccus hirsutus,* bioassay, attractant pheromone.

INTRODUCTION

The pink hibiscus mealy bug (PHM) *Maconellicoccus hirsutus* (Green) (Homoptera : Pseudococcidae) is a serious economic threat to agriculture, forestry and the nursery industry. This pest attacks many plants, trees and shrubs (Meyerdirk *et al.*,1998). It is known to attack more than 200 plants, trees and shrubs. It also attacks grapes, an important agricultural crop in India. Mealy bug is a small, soft-bodied insect with a non-flying female and flying male. The intermediate life stages are eggs and three nymphal instars for female and four nymphal instars for male. All the stages are reddish to pink in color and are covered with white mealy wax. The natural wax coating on various stages of these insects provides a natural protection, to some extent, from pesticides (Williams, 1996). The newly hatched mealy bugs, usually referred to as crawlers, are mobile. They settle on host plants and start their development. This lasts for 10-22 days. Although they prefer apical and tender regions of the host, their infestation is found on older plant parts also. The damages caused by mealy bug on grapes are serious. They feed on sprouts developing after pruning and stunt their growth. The growing shoots and the leaves are malformed due to sticky honeydew produced by the pest, predisposing them to mouldy growth and bunching. Heavily infected bunches shrivel and drop (Babu and Azam, 1987). There were high mealy bug incidences on grapes in India in the last few years (Anonymous, 2005). This resulted in poor fruit quality and in turn substantial economic losses to the growers. Attention is, therefore, being focused on methods to control mealy bugs. Literature survey reveals a few methods to control mealy bugs. Application of hot water (Hara and Jacobson, 2005), use of fumigants like methyl bromide (Zettler *et al*., 2002), use of insecticides such as methomyl (Raguraman and Premalatha, 2006), buprofezin (Muthukrishnan *et al.,* 2005) and generic vapor heat treatment (Follet, 2004) are the methods usually practised. Biological control by release of natural enemies like *Cryptolaemus montrouzieri* has been used successfully to reduce large population of *M. hirsutus on* guava in India (Mani and Krishnamoorthy, 2001) and the Caribbean islands (Kairo *et al*., 2000). Similarly the presence of two parasitoids *Anagyrus kamali* Moursi and *Allotropa* sp. near mecrida (Walker) was found to reduce the population density of *M. hirsutus* significantly (Reddy *et al.,* 2009). However, surveys conducted on adaptation of the biocontrol methods revealed that these methods were not adopted by farmers because of the non-availability of bio control agents (Basu, 2010; Gangadhar *et al.,* 2012). Recently

application of fungal isolates for control of pink mealy bug is demonstrated (Ibbara-Cortes *et al.*, 2013). Possibility of developing 'Integrated Pest Management' (IPM) module without any chemical insecticides and releasing of biocontrol agents management for Tukra mealy bug *Maconellicoccus hirsutus* (Green) are reported (Ravikumar *et al.*, 2010). Sex pheromones of mealy bug are also reported. (R)-2-isopropenyl-5-methyl-4-hexenyl (S)-2-methylbutanoate and [(R)-2,2-dimethyl-3-(1-methylethylidene)cyclobutyl] methyl (S)-2-methylbutanoate were identified as the sex pheromones. A synthetic mixture of the two pheromone components was shown to be an extremely potent attractant (Zhang *et al.*, 2004). Use of pheromone-baited traps was made to capture males (Francis *et al.*, 2007). However this method has only a limited application in the control of crawlers. A general attractant would be desired to capture both male and female insects but attractant pheromones for mealy bug have not yet been reported.

We have recently demonstrated that pheromone based attractant formulations could be developed for the aquatic stages of insects *Chironomus ramosus* larvae (Naik *et al.*, 2006). Their cuticular extract was found to possess attractant properties. We decided to investigate attractant formulations for mealy bugs on the similar lines. As the first step, it was decided to obtain the whole body methanolic extract of crawlers, which would also contain cuticular extract, and to use it for the bioassay.

MATERIALS AND METHODS

Methanol (HPLC grade), ethyl acetate (AR), acetone (AR), formic acid (AR) were purchased from Qualigens Fine Chemicals, Mumbai, India. Pre-coated silica gel 60 F_{254} TLC aluminium sheets were purchased from E. Merck.

Rearing of crawlers

The authentic culture of fully grown mealy bugs, *M. hirsutus*, was collected from National Research Centre for Grapes, Pune, India. Insects were reared using standard method (Roltsch, 2014) with slight modifications in which they were allowed to grow on pumpkin (*Cucurbita maxima* Duch. ex Lam. (*Cucurbitaceae*) in a ventilated cubical wooden cabinet with 45 cm length on each side. Rearing conditions were 25^0C, photoperiod 0 L: 24D. The crawlers were collected with a small camel's hair brush.

Extraction of the crawlers

The crawlers (2.5 g) were individually collected and cleaned with a camel's hair brush to remove the waxy coating on them as much as possible and dropped into HPLC grade methanol (5 mL) maintained at -70^{o}C. They were kept at -70^{o}C for five minutes and then crushed with a glass rod. The mixture was filtered. A small part of the filtrate was submitted for TLC, GC-MS, and HPLC analyses and the rest was concentrated *in vacuuo*. Residue obtained was directly used for the bioassay.

Thin Layer Chromatography (TLC) analysis

A part (5 µL) of the test solution was applied on a precoated silica gel 60 F_{254} plate (E. Merck) of uniform thickness of 0.2 mm. The plate was run in the solvent system ethyl acetate: acetone: formic acid (5: 4.8: 0.2) up to distance of 8 cm. It was dried and kept in an iodine chamber to visualise the spots.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

GC-MS analyses were carried out on a Shimadzu QP – 5000 spectrometer fitted with a Supelcowax – 10 capillary column having 0.32 mm ID, 30 m length and 30 µm film thickness. Helium was used as the carrier gas. Injector SPL – 17 in a split mode; split ratio 28, 200^0C; column temperature programme: 80°C (5 min), 80-200°C / 50°C / min, 200°C (60 min). Mass spectral conditions: mass spectra were recorded at 70 eV. Mass range 45–350, scan time 0.5 sec, solvent cut 4 min, start time 5 min. NIST library was installed for reference.

High Performance Liquid Chromatography (HPLC) analysis

HPLC analyses were carried out on a ZORBAX, Eclipse, XDB-C8, 4.6 mm×150 mm, 5 µm column using Agilent 1100 high performance pump and Agilent 1100 variable wavelength UV detector (254 nm) using methanol : water (80 : 20, by volume) at a flow rate of 1 mL/min.

Bioassay of the crude extract

Bioassay was performed using a protocol used for the bioassay of pheromonal extract on *Chironomus*

larvae (Naik *et al*., 2006)) with suitable modifications. A Petri dish (diameter 11.5 cm) was used for the bioassay. Inside of the Petri dish was covered with a Whatman filter paper circle. The circle was divided into two semicircular halves (Figure 1).

Test solutions were prepared by dissolving 2 mg, 1 mg, 0.5 mg, 0.33 mg, 0.16 mg and 0.05 mg of the residue individually into 1 mL of HPLC grade methanol. The test solution was then applied to the half of the filter paper marked 'T'. The other half of the filter paper marked 'C' was coated only with HPLC grade methanol. The Petri dish was kept under the fluorescent tube light and was uniformly illuminated. Crawlers (20 numbers) of mealy bug were released at the centre of the Petri dish and were allowed to migrate to the zones of their choice. The migration was found to take place in about 25 minutes in pilot experiments. Hence all further bioassays were carried out for 25 minutes. The number of crawlers migrating towards the zone 'T' coated with the test solution, and those migrating towards the zone 'C' coated with methanol, in 25 minutes were counted. Bioassay of each test solution was run 10 times. Average numbers of the crawlers migrating to either of the zones was counted. Difference (Δ) between the number of crawlers migrating towards the zone 'T' and migrating towards the zone 'C' was taken as a measure of attractiveness of the particular formulation.

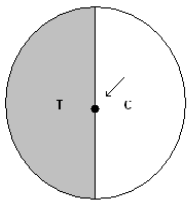

Fig.1.Schematic design of the bioassay set up showing half of the glass Petri dish coated with the crude extract (T) and the other half left uncoated (C). The arrow indicates the 'center of the dish', from where the crawlers were released for the bioassay.

Statistical Analysis

The data generated from the bioassay were statistically analysed with Microsoft Excel 2003. The significance of the observation made was determined with the Kruskal-Wallis test and Mann-Whitney test using SPSS version 11.0.

RESULTS AND DISCUSSION

Extraction of crawlers and its TLC analysis

The methanolic extract of the crawlers finally yielded a brownish residue (0.16g, 6.4%). Its TLC chromatogram indicated three spots suggesting the presence of three groups of compounds.

GC-MS analysis of methanolic extract

No compound was detected in the GC-MS analysis indicating the absence of any volatile chemical constituent in the extract.

HPLC analysis

The HPLC chromatogram (Figure 2) showed the presence of four peaks of which one was major (d); one was of intermediate concentration (b) while two (a and c) were minor signals.

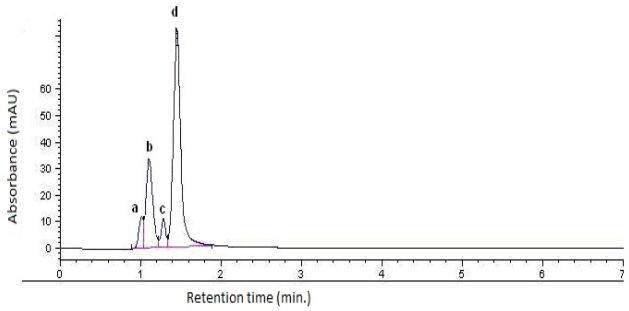

Fig. 2. HPLC chromatogram of the crude whole body extract of crawlers.

Bioassay

It was found that the majority of the crawlers preferred the zone 'T' of the Petri dish coated with the test solution. The results of average numbers of crawlers migrating to zones 'T' and 'C' were recorded (Table 1). It was observed that the number of crawlers migrating towards test formulations was significantly more than those visiting control upto the concentration of about 1.5 mg/ml, indicating attractant nature of the test formulations. Formulation of the higher concentration showed repellent properties (Figure 3).

As compared to the control, formulations of concentrations lower than 1.5 mg/ml of the whole body extract (Sr. No. 1 to 6, Table 1) showed

statistically significant difference in the number of crawlers migrated to the test and control formulations (χ^2= 102.711, p= 0.0001).

Table 1. Average number of crawlers of mealy bugs that migrated towards the zone (T) coated with their whole body extract and those to the zone 'C' coated with methanol.

Extract Concentration (mg/ml)	No of crawlers migrated to zone T (N_T)	No of crawlers migrated to zone C(N_C)	Average attractiveness (Δ) (N_T -N_C)
0	10	10	0
0.05	12	8	4
0.16	14	6	8
0.33	13	7	6
0.5	11	8	3
1	10	7	5
2	6	10	4

Further examination of pairwise differences among control and formulations of the whole body methanolic extract by Mann-Whitney test revealed significant increase in the differences from concentration 0.05 mg/mL to 0.16 mg/mL ($p< 0.05$) after which there is significant decline from concentration 0.16 mg/mL to 2 mg/mL ($p< 0.05$).

Pink mealy bug is a destructive pest on grapes. Application of insecticides can control it to some extent but the waxy coating on their body puts a serious limitation on it (Williams, 1996). Further, applications of insecticides can induce the problems of residue on grapes (Cabras and Angioni, 2000) which is detrimental to their acceptability in the international market Methods of biological control are only partially successful since population of mealy bugs is likely to build up to high levels before their natural enemies brought them under control. In this situation, application of attractants appears more promising. With the attractant formulations crawlers can be attracted for trapping. Literature survey indicates that the glands responsible for the release of attractant pheromone in crawlers are not yet studied. Mealy bugs, like *Chironomus* larvae, are suspected to release the attractant pheromone from their cuticle. In the present study the total extract of crawlers was, collected by immersing them in methanol. The TLC analysis showed it to be a mixture of three types of compounds. However, it was very surprising to note from the GC-MS analysis that the extract did not contain any volatile chemical constituent. Its HPLC analysis clearly indicated presence of a major, a peak of an intermediate concentration and two minor peaks indicating presence of four constituents. In the bioassay, migration of crawlers to the zone 'T' coated with the extract was observed which indicated attractant nature of the extract.

When mealy bugs were dipped in methanol and crushed at lower temperature, the cuticle also got extracted yielding the cuticular pheromone in the mixture. This pheromone constituted a part of total methanolic extract. The attractant nature of the total extract supported this. It can be seen from the plot of attractiveness against concentration of the formulation (Figure 3) that the response of crawlers to the formulations of the extract was dose dependant. This type of variations in properties further confirms the pheromonal nature of the extract. Formulations of the lower concentrations, around 0.05 mg/ml, possessed lower attractiveness. Attractiveness then increased with the formulation upto the concentration 0.16 mg/mL. Further increase attractiveness. It is noteworthy that the formulation of concentration 2 mg/mL possessed repellent properties.

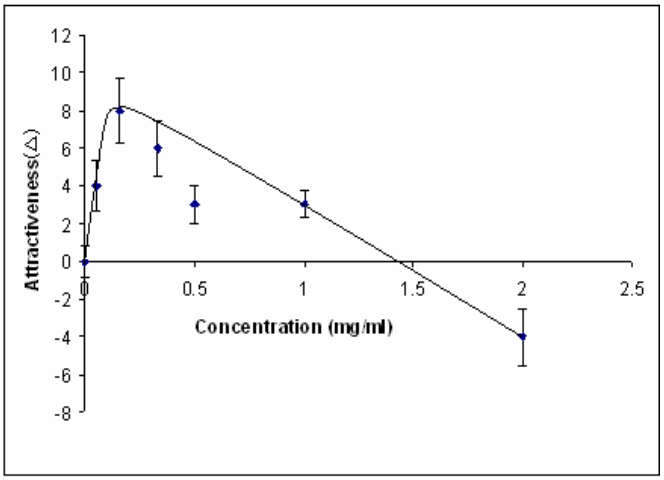

Fig.3. Graph of average attractiveness Δ, of the formulation towards mealy bugs against the concentration of the formulation. Standard deviation is shown at each point, χ^2 = 102.711, p = 0.0001 (Kruskal Wallis test).

Thus the formulations for attracting crawlers of mealy bug *Maconellicoccus hirsutus* (Green) were developed using the whole body extract of crawlers themselves. The dependence of attractant properties on the concentration of the formulations was also demonstrated. Availability of such easily accessible formulations is important for grape growers involved in organic farming.

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