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Chemical behaviour of *Leptosia nina* in host plant selection and the GCMS analysis of *Cardamine hirsuta*

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ABSTRACT

Specialist butterflies are specific in their host selection. *Leptosia nina* (Pieridae), the Psyche butterfly is an insect which feeds on closely related host plants such as plants belongs to Cleomaceae and Capparaceae. The present observation was the ovipositing behaviour of Psyche on *Cardamine hirsuta* (Brassicaceae). The objective of this work was to identify the plant chemical used by the Pysche for their host plant recognition and also the mechanism responsible for the survival of Psyche's caterpillar on C. hirsuta. GCMS analysis of methanolic extracts of C. hirsuta was done to identify the plant chemical. The result demonstrates that the glucosinolate serves as the ovipositing stimulant. The glucosinolates are hydrolyzed by the plant enzyme myrosinase. In plant tissues they occur separately. Both are come together only when the plant tissue was damaged. Hydrolysis of glucosinolate by myrosinase result in the generation of harmful isothiocyanate and several other chemical compounds. So, insect specialized on glucosinolate containing plants have to adapt some mechanism to overcome the toxic effect of isothiocyanate. The counteradaptation involves the breakdown of glucosinolate into nitriles instead of isothiocyanate. This deviation is only because of a protein called Nitrile Specifier Protein (NSP) which is specific to Pierid species. This NSP based counteradaptation on glucosinolate demonstrate the mechanism of coevolution of plant and insect herbivore.

Index terms: *Cardamine hirsuta*, Glucosinolate, *Leptosia nina*, Myrosinase, Oviposition, Phytochemicals.

Abbreviations: GCMS- Gas Chromatography Mass Spectrometer; NSP- Nitrile Specifier Protein.

INTRODUCTION

Cardamine hirsuta is an annual plant native to Europe and introduced worldwide. It is a cosmopolitan weed belongs to the family Brassicaceae. Because of its explosive seed dispersal nature, they are commonly called Hairy bittercress or popping cress (Hay et al. 2014). C. *hirsuta* found on sandy or rocky soil, and it is a common weed in gardens, nurseries and disturbed ground (Rich, 1997; Lihova and Marhold, 2006). C. *hirsuta* is used to study the ecological distribution and evolution in biological invasion (Yastu et al., 2003;).

Coevolution is an approach in which interaction between two major groups of organisms with a close and evident ecological relationship was examined. Butterflies are only one of the major groups of herbivorous organisms coevolving with plants (Ehrlich and Raven, 1964). They require a complex feeding relationship with green plants during their development. Butterflies are very selective in their choice of habitats for food plants for their maximum fitness (Wiklund, 1977; Rausher, 1979; Bonebrake et al., 2010). Ovipositing females prefer suitable habitats where larval growth and development are good and avoid habitats and food plants where growth and development are poor (Gilbert and Singer, 1975). All butterflies show some degree of host plant selectivity. Many butterflies select groups of very closely related plants, where the larvae obtain the entire set of nutrients required for their growth and development (Boppre et al., 1984). The process of host selection by specialist insects is influenced primarily by chemical signal later by visual stimuli and finally by non-volatile chemical stimuli (Hern et al., 1996; Hook and Johnson, 2001). The use of a particular plant as a source of food by the insects involves a metabolic adjustment on their body parts. This tends to restrict their choice of food plants (Thorsteinson, 1960).

Leptosia nina, the psyche is a small butterfly belongs to the family Pieridae. L. nina is commonly called 'Wandering snowflake'. It has weak and erratic flight, and it mostly prefers low heights. These small and delicate adults found mostly on wastelands, nature reserves, grassy patches and urban and residential areas (Tan and Khew, 2012). The larvae of Psyche butterfly feeds on *Capparis rheedi L., Capparis spinosa L., Capparis zeylanica L., Crateva adansonii* belongs to the capparaceae and *Cleome viscosa L., Cleome monophylla L., Cleome rutidosperma* belongs to the family cleomacea (Kunte, 2000). Thus, *Leptosia nina* is an oligophagous insect which feeds on host plants of closely related genera or families.

The present observation is the incidence of *Leptosia nina* on *cardamine hirsuta* in the home garden. Although, three generations of *Leptosia nina* has been observed on C. *hirsuta* plants. Thus, these observation reveals that the Psyche butterfly can successfully transform their larval stage to adult butterfly on C. *hirsuta* plants.

Some chemical compounds are "common" to all plants, which may be important for plant survival in general and may not be involved in interaction with specific butterflies. Certain other chemical compounds are "common specific" to plants which contribute to plant herbivore relationship. The common specific compounds present in the host plants of Pieridae include benzylglucosinolate, glucosinalbate, gluconasturtin, singirin, glucohirsutin, glucoibervin, gluconapin, 2R-3R-fustin, liquiritigenic, fisetin and crysophenol (Muto-Fujita et al., 2017).

The present study focuses on, which compound is responsible for the completion of Psyche's life cycle on C. *hirsuta* plant. For that, the analytical method Gas chromatography mass spectrometry (GCMS) was used. GCMS combines the features of gas chromatography and mass spectrometry to identify the compounds present in the test sample.

MATERIALS AND METHODS

COLLECTION OF MATERIAL

Ripe yellow pods of C. *hirsuta* were collect from Homi Bhabha Centre for Science Education, Mumbai. The pods burst open and sawn in the home garden for germination. After few weeks, the plants were grown up to maturity and started to flowering and seed setting. When their pods became fully ripened, the seeds were collected and sawn in another tray. Likewise, 3 more generations of the plant were domesticated.

INCIDENCE OF PSYCHE

Caterpillars are metamorphoses to a butterfly. During the culturing of Cardamine plants, some caterpillars were found on the plants. This felt curious to know which butterfly's caterpillar was that. For that, life cycle of the caterpillar was observed. From the observation it was found that the butterfly emerging from the caterpillar's pupa was Psyche.

PREPARATION OF LEAF EXTRACTS

The fresh leaves of C. *hirsuta* were collected and oven dried at 40°C. The sample was crushed into powder form by using mortar and pestle. Then the powdered material was stored in clear and air tight containers. Aqueous extracts were prepared by mixing powdered material with extraction solvent methanol, shake well for 20 minutes and then filtered by using Whatsman No:1 filter paper for further analysis.

GAS CHROMATOGRAPHY MASS SPECTROMETRY (GCMS)

Gas chromatography mass spectrometry is an analytical method which separates chemical compounds that are chemically stable and identifies the components based on the molecular weight. GCMS integrate the properties of gas chromatography (GC) and mass spectrometry (MS) to distinguish different compounds present in the test sample. Gas chromatography (GC) examine and isolate the components that can vaporized without disintegration. Mass spectrometry (MS) experimentally assort the ions based on their mass to charge ration and ionize atoms, molecules, ions, molecular fragments and other chemical species. The principle of GCMS is the separation of mixture into individual substances by heating. These heated gas pass through a column with the help of an inert gas, such as helium. The chemicals which are less volatile move slower than more volatile chemicals. As a result, mixture will separate into individual compounds. These separated compounds flow into mass spectrometer from the column. Mass spectrometer identifies the compounds based on their mass. The procedure followed was of Dandekar et al.

GCMS ANALYSIS: GCMS analysis of bioactive compounds from the leaf extract of selected plant was done at Centre for Analytical Instrumentation Kerala (CAI-K), KFRI, Thrissur. The column used for the analysis was Rxi 5SilMS with an inner diameter of 0.25 mm, 30 m length and a film thickness of 0.25 µm. The flow rate of carrier gas was 1 ml/min. A sample volume of 1 µl were introduced into the instrument. During chromatographic run the injector temperature was set at 260°C. In the instrument the column oven temperature was set at 80°C and the ion source temperature was at 200°C. Chromatogram was evaluated by using the software embedded in the GCMS. Relative quantities of the chemicals present in the leaf extracts were expressed as percentage based on peak area produced in the chromatogram.

OBSERVATION OF COMPONENTS: Elucidation of mass spectrum of GCMS was conducted using the databases of NIST 11 and WILEY 8. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST 11 and WILEY 8 libraries.

RESULT

The GCMS analysis of methanolic extracts of *Cardamine hirsuta* L. confirmed the presence of different chemical classes of volatile compounds. GCMS analysis of C. *hirsuta*'s leaves reveals the presence of a total of 23 phytochemical constituents (Table: 1). Thus, the chromatogram of methanolic extracts of C. *hirsuta* showed 23 peaks (Fig: 1). Based on the peak area, retention time (RT) and molecular weight, the phytochemical compounds present in the extract were identified. The first compound identified with least retention time (9.364) was Benzyl nitrile and the last compound which took maximum retention time (49.709) to identify was Campestrol.

The compounds present in the leaf of C. *hirsuta* were Benzyl nitrile, β-Eudesmol Viridiflorol, 2-Hexadecene, 3,7,11,15-tetramethyl-[R-[R*,R*-(E)]], Phytol acetate, (2E)-3,7,11,15-Tetramethyl-2-hexadecene, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, (E)-Phytol, α -Copaene-11-ol, Hedycaryol, 3-Methyl-2-(3,7,11-trimethyldodecyl) furan, Methyl palmitate, α-Bisabolol, Rosifoliol, cis-Z-alpha-Bisabolene epoxide, Linoleic acid methyl ester, α-Linolenic acid methyl ester, Palmitaldihyde, Diallyl acetal, 4-t-Butyl-2-[4-nitrophenyl]phenol, Squalene, Vitamin E, Campestrol. Of these, Benzyl nitrile is the hydrolysis product of benzyl glucosinolate. Benzyl glucosinolate is one of the common specific compounds of pieridae. Thus, the compound that contribute for the completion of Psyche's lifecycle on C. *hirsuta* may be benzyl nitrile.

The life cycle of Psyche was completed in four stages. *Leptosia nina* laid eggs singly on the underside of the leaf. The eggs were hatch out within 2-3 days. The early instar first eats the empty egg shell and then moves on to the leaf lamina. By the intake of leafy diet, their body gets light green in colour (Fig.1. A). A fully grown caterpillar is green in colour. Their body length reaches upto 1.5-2 cm (Fig.1. B). The body surfaces are dotted with many shallow tubercles, which gives a speckled appearance to the body. Besides these, many moderately long fine setae emerged from tubercles are found laterally on the body. The body of later instar shortened and changes to a bright green colour. Then the caterpillar ceases feeding and comes to a resting state on the leaf stalk or stem of the host plant. Here, the caterpillar enters to a cradled pre-pupatory pose (Fig.1.C). Pupation takesplace on the next day itself. The green pupa has a very short and pointed cephalic horn, a slight dorsal protrusion and a large wing case tapering into a keel (Fig.1. D). After about three days, yellowish streaks appeared on the wing case. After 4-5 days, the pupal skin turns translucent. On the next day itself, the adult butterfly emerges from the pupal case (Fig.1. E, F).

Table 1: The phytocompounds detected in the methanolic leaf extracts of C. *hirsuta* by GCMS analysis

Peak	Retention Time (min)	Area	Area%	Height	Height%	Name of compound	Base m/z
1	9.364	12427769	7.31	433381	0.91	Benzyl nitrile	117.10
2	22.475	6264950	3.69	1754500	3.69	*β*-Eudesmol	59.05
3	26.016	2222138	1.31	694322	1.46	Viridiflorol	59.10
4	26.196	443640	0.26	218895	0.46	2-Hexadecene, 3,7,11,15-tetramethyl-, [R - [R, R*-(E)]]	70.10
5	26.333	20759431	12.21	8362492	17.60	Phytol, acetate	68.05
6	26.456	2126010	1.25	666390	1.40	(2E)-3,7,11,15-Tetramethyl-2-hexadecene	70.10
7	26.837	3163027	1.86	1225764	2.58	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	82.10
8	27.208	6287404	3.70	2500604	5.26	(E)-Phytol	82.10
9	27.449	51831373	30.49	14976914	31.52	*α*-Copaene-11-OL	59.05
10	27.859	3436521	2.02	1116178	2.35	Hedycaryol	59.05
11	27.933	2100489	1.24	406051	0.85	3-Methyl-2-(3,7,11-trimethyldodecyl) furan	95.05
12	28.130	2058153	1.21	705050	1.48	Methyl palmitate	74.05
13	28.875	970500	0.57	299753	0.63	*α*-Bisabolol	109.10
14	28.966	9236531	5.43	2545112	5.36	Rosifoliol	59.05
15	29.196	3070115	1.81	721234	1.52	cis-Z-*α*-Bisabolene epoxide	109.10
16	31.311	1141653	0.67	434107	0.91	Linoleic acid, Methyl ester	67.05
17	31.418	3605683	2.12	1067756	2.25	*α*-Linolenic acid methyl ester	79.10
18	31.629	12107224	7.12	4044857	8.51	Phytol, acetate	71.05
19	32.181	1765865	1.04	623993	1.31	Palmitaldehyde, Diallyl acetal	84.10
20	39.620	16480754	9.69	2790254	5.87	4-t-Butyl-2-[4-nitrophenyl] phenol	256.10

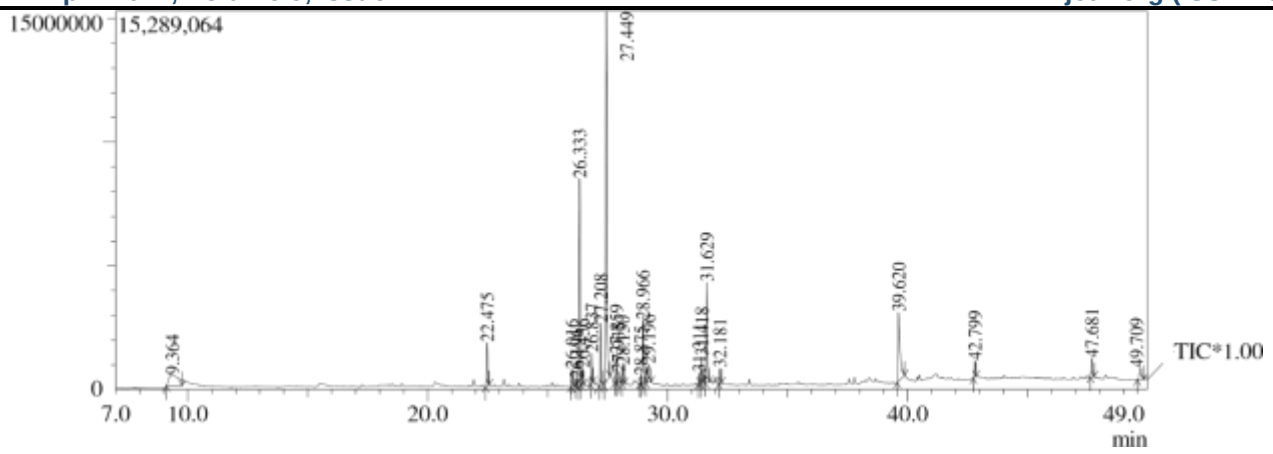
21	42.799	1703292	1.00	673575	1.42	Squalene	69.05
22	47.681	3141439	1.85	767605	1.62	Vitamin E	165.10
23	49.709	3665176	2.16	482455	1.02	CAMPESTEROL	55.05
		170009137	100.00	47511242	100.00		

Figure 1. Developmental stages of Psyche**. A**, Early instar of L. nina**. B**, Later instar of L. nina.

C, Pre-pupatory pause of L. nina. **D**, Pupa of L. nina**. E, F**, Adult L. nina on C. hirsuta.

Retention time

Figure 2. GCMS chromatograph of methanolic leaf extracts of C. hirsuta



Retention time

Figure 2. GCMS chromatograph of methanolic leaf extracts of C. hirsuta

DISCUSSION

One of the mechanisms that evolved in plants for dealing with herbivorous insects are the chemical defense, in which secondary metabolites plays an important role. In the order Brassicales, glucosinolates serves as a defensive secondary metabolite. They are sulfur containing compounds. A naturally occurring enzyme called myrosinase have the ability to hydrolyze these glucosinolates. These enzymes are stored in specialized plant cells. Glucosinolates and myrosinase are in a co-occur state. However, they are compartmentalized (Rask et al. 2000).

By the chewing activity of insects, the plant tissues are damaged. This results in the release of glucosinolate from the vacuole and comes in contact with myrosinase. The myrosinase activity on glucosinolate results in the release of glucose and sulfate and also several toxic and pungent products such as isothiocyanates, nitriles and oxazolidinethiones (Bones and Rossiter, 2006). The chemical product formed by the reaction is directly depend on the chemical structure of the side chain, the concentration of ferrous ions and the presence epithiospecifier protein (ESP) are also an important factor for contributing the final outcome of glucosinolate-myrosinase reaction (Burrow et al. 2006). Glucosinolate serves as the classical case of token stimuli in insect plant interactions (Schoonhoven et al., 2005). Their occurrence is restricted to certain plant taxa, and chemoreception of such compounds allow unambiguous recognition of species host plant. There are three physiological strategies exist in specialist insects to adapt to their host plants containing toxic compounds. They are enzymatic detoxification, excretion, and sequestration (Mainguet et al. 2000).

Of the hydrolytic products, only isothiocyanates have toxic activity (Halkier and Gershenzon, 2006). Certain insect herbivores show counter adaptation against these toxic compounds. The counter mechanism involves the avoidance of isothiocyanate production by altering normal metabolism of glucosinolate. The larvae of Pieris rapae redirects the normal hydrolytic reaction catalyzed by myrosinase (Wittstock et al. 2004). The redirection process results in the formation of nitriles instead of toxic isothiocyanates. This redirection process is due to a protein called Nitrile Specifier Protein (NSP). The NSP based mechanism has been considered as a key innovation in the Pieridae clade (Wheat et al. 2007). This NSP based diversification to cope with glucosinolate within Pieridae family supports the coevolutionary scenario of insect-plant interaction (Ehrlich and Raven, 1964).

During enzymatic hydrolysis of glucosinolate, the glucoside entity will undergo rearrangement to form either isothiocyanate or nitriles, while the R group of glucosinolates remain unchanged (Bones and Rossiter, 1996). Benzyl nitrile was one of the compounds present in methanolic extracts of C. *hirsuta* leaves. It is the hydrolytic product of benzyl glucosinolate. This indicates that the leaves of C. *hirsuta* contain benzyl glucosinolate.

The present study focuses on why the caterpillar of Psyche survive and completes their life cycle on C. *hirsuta*. The result revealed that, when the larvae of psyche eat the leaves of C. *hirsuta* plant, the benzyl glucosinolates present in the leaves was hydrolyzed into benzyl nitrile due to the combined action of plant myrosinase and larval NSP. Thus, the present study provide evidence to support the hypothesis of coevolution between the butterflies and their host plants mediated by chemical defense.

CONCLUSION

The present study was an attempt to analyze the compound that was responsible for the survival of Psyche's caterpillar on C. *hirsuta*. The GCMS analysis of methanolic extract of C. *hirsuta* leaves reveals the presence of a compound called benzyl nitrile, which was the hydrolytic product of benzyl glucosinolate. The NSP system present in the gut of Psyche's larvae enables the hydrolysis of glucosinolate into non-toxic nitriles instead of forming isothiocyanates, which are toxic to insects. This indicates that, NSP system and the glucosinolate are responsible for the survival of Psyche's caterpillar on C. *hirsuta*. The survival of

caterpillar on C. *hirsuta* suggests their coevolution.

In the present study, the phytochemical constituents present in the methanolic extracts of the plant C. *hirsuta* is analyzed through GCMS. The analysis reveals the presence of 23 compounds. It could be concluded that C. *hirsuta* contains various bioactive compounds. These bioactive compounds can be utilized for developing beneficial drug to cure various human diseases and disorders. However, further studies are with this plant required to sequestrate, characterize and interpret the structure bioactive compounds for industrial drug formulation.

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