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USE OF POLLEN PROTEINS AS A NUTRITIONAL FEEDS THAT ALLEVIATES THE IMMUNITY OF HONEY BEE

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A B S T R A C T

Introduction- Honey bee (*Apis mellifera)* is the significant pollinator of all the plant species. The main dietary source of honey bee are pollen that provides proteins, amino acids and lipids which are essential to adult bee for its physiological development. In addition, pollens have some nutritive impact on health of honey bees. Insecticides and pathogens like bacteria, viruses and protozoans only leads to colony collapse disorder.

Objective- This study has been conducted to emphasize more on the nutritional pollen protein (sunflower, *Helianthus annuus* and soyabean, *Glycine max*) uptake of honey bee and to understand the dynamics of nutrition and immunity relationship.

Methods- In this study, protein extracted from the pollens of sunflower and soyabean and directly feed or exposed to hemolymph of honey bee for determining its immunity. First of all, pollen protein was analyzed by using FPLC and then analyzed or quantified its immunity against honey bees using standard immunocompetence assays i.e. total hemocyte count and assessed regularly to study the growth kinetics of hemocytes and also observed its effect in the form of forward (shape and size) and side scatter (granularity of the cell) through flow cytometer.

Results-The results of this study showed that pollen ofsoyabean showed higher amount of protein as compared to sunflower. In addition, there is enormous increased in hemocytes count after infected with bacteria i.e. *bacillus subtilis* as compared to hemocytes control. In continuation of this study, pollen proteins of sunflower and soyabean showed declined in both forward and side scatter in case of bees which is infected with *bacillus subtilis*.

Conclusion- Herein, the current study helped us to illustrate the nutritional feed of pollen protein i.e. sunflower and soyabean that will be helpful for eliciting the immunity of honey bee.

INTRODUCTION

Apis mellifera (honey bee) was introduced in India during late 70's or early 80's, first into Punjab, Haryana and Himachal Pradesh and later into South Indian states. These bees are native to Africa, Middle East Asia and Europe. Unlike *A. cerana these bees are larger in size,* builds larger multiple combs having greater honey storage capacity than its Indian counterpart. Fecundity, brood rearing and colony build up are enriched in this species [1-3]. After independence, Khadi and Village industries commission (KVIC), Govt. took up beekeeping as one of its important venture. States like Jammu and Kashmir, Karnataka, Uttar Pradesh, and Himachal Pradesh established Department of beekeeping under their ministry of Agriculture. Further considering the importance of applied and basic research in apiculture, KVIC established Central Bee Research and Training Institute (CBRTI) at Pune in 1962. Presently, it is estimated that India has 14 lakh hive bee colonies (6.73 lakh of *Apis mellifera* and 7.30 of *Apis cerena)*. On the basis of FAO statistics on honey production, India ranks no. 5 in the world.

Generally, honey bee foragers collect nectar from plants to produce honey, which is the main source of carbohydrate nutrition for the colony.These carbohydrates are required to both the stages of honey bee i.e. larva and adult stage for normal growth and development and also generate energy for muscle activity and are absolutely essential for fueling flight muscles [1, 2].The elevated intake of carbohydrates in the form of honey is correlated with proper wax gland function and secretion. In addition, the carbohydrates are used as fuel to thermoregulate the brood nest and as flight fuel for foragers [1-4].

Pollen is an indispensable source of lipids for honey bees. Lipids and fatty acids are the building blocks of phospholipids which are a major component of cell membranes. Lipids are the precursors of important hormones that are involved in the molting process during insect's life cycle and serve as a primary energy store for insects in their fat bodies. These fatty acids also are used for derivation of different defensive secretions in some insects. The loss and depletion of fatty acids is also linked to the desiccation of many insects [3, 4]**.** Fatty acid (FA) content of pollen ranges between 1% and 20%, meets essential polyunsaturated fatty acids (PUFA) in their diet, and linolenic acids usually satisfy their nutritional

need. All of the essential minerals i.e. sodium, potassium, calcium, magnesium, chlorine, phosphorus, iron, copper, iodine, manganese, cobalt, zinc, and nickel are present in pollen. Addition of pollen ash (1 %) to synthetic honey bee diet for increased its brood rearing but exceeding the ash content by more than 2% did not seem to be advantageous [5, 6]. Pollen contains all the essential amino acids i.e. arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine including B-complex vitamins (thiamine, riboflavin, pyridoxin, pantothenic acid, niacin, folic acid and biotin) [3-8]. These vitamins are crucial for hypopharyngeal gland development e.g. Pantothenic acid is now known to play a critical role in queen/worker differentiation; Riboflavin, nicotinic acid and pyridoxine have also been correlated to brood rearing etc. [3-8] In this study, our group focused on pollen protein extracted from sunflower and soyabean and tried to use as nutritional feed that alleviates the immunity of honey bee.

MATERIALS AND METHODS

Surveillance

Survey was carried out in Apiary, VSBT (18°10'24"N 74°36'36"E), Baramati in the month of September 2015 and January-March 2016pertaining to collect data relating beehives, environment information (flowering and climatic conditions), number of living and healthy colonies and estimate the prevalence of certain disease.

Sample collection

One frame of brood and 50 foragers adult honey bees were collected from 10 colonies from Apiary,VSBT, Baramati. Pollen samples of Sunflower and Soyabeanwere collected from Nakshatra Udyan, VSBT, Baramati. Bee pollen was also collected from Apiary, VSBT, Baramati.

Morphometric analysis

Measurements of forewing and head with its width and length, femur and tibia length, inner forewing length and width of foragers in order to detect size differences between colonies or developmental stages were done.

Extraction of protein from pollen

Extraction of protein from collected pollen was performed by using Tris HCl(pH 7.2) and acetone.The pollen samples were weighed and crushed in mortar and pestle using liquid nitrogen (-196ºC).The pollen powder was incubated in Tris HCl (pH 7.2) for 10 minutes, followed by centrifugation at 6000 rpm for 10 minutes at 4 ^{0}C. Acetone was added in the collected supernatant and incubated for 20 minutes. Again, centrifuging at 6000 rpm at 4 ^{0}C for 10 minutes.Pellet settled at the bottom and the supernatant was discarded and PBS added.The pellet was filter sterilized in autoclaved membrane filters (0.22µm) and stored at 4^{0}C [9, 10].

Analysis of protein through Fast Protein Liquid Chromatography (FPLC)

Separation of pollen proteins was carried out using size exclusion chromatography under AKTA FPLC (GE Health care) protein purification system. For this purpose, Sephacryl 100 HR resins (GE Health care) were used.$XKC_{16/70}$ (GE Health care) was used as column which was filled with 120 ml of resins.Column was equilibrated with PBS buffer (pH 7.2). Flow rate was adjusted at 0.5ml/min andpollen extract (2ml) was injected into the previously equilibrated column.Protein elution profile was monitored in UV range at 215nm, 254nm and 280nm.Total duration of chromatography run was upto 180ml.Fractions were collected manually in 50ml falcon tubes.

Collection of Hemolymph

Hemolymph was collected from forager adult bee by severing the abdomen and collecting samples from the proximal abdominal opening (2nd and 3rd tergites). Five microliters of hemolymph was collected from the resulting bubble of hemolymph, transferred to a vial containing 95 ml PBS (pH 7.2) and frozen at 4^{0}C to disrupt hemocytes for analysis [11].One microliter of hemolymph was collected from the vial which contained 99 µl of PBS (pH 7.2) and used for total hemocyte count. Any fluid which appeared yellow or brown was avoided as this was likely not hemolymph but gastric fluid.

In vitro hemocyte Cell Cultureand viability assay

In this assay, Grace's insect medium (5 ml) were added into sterile tissue culture flask ($T_{12.5}$). 10µl of hemocytes were transferred into tissue culture flask. Cultures were maintained at 32^{0}C with 5 % CO_2 in Grace's insect media supplemented with 10% FBS and Gentamicin (50µg/ml). Afterwards, hemocyte cell suspension was taken for viability assay.A clean hemocytometer slide was taken and the overslip was placed onto it. The counting chamber of the hemocytometer was located with cell suspension mixed with trypan blue 1:1 ratio. After 1-2 min, the slide was placed on stage of microscope, and focused with a 10X objective to observe counting grid. The total number of cells including colorless and bluish colored stained cells was counted.

Establishment of primary cell culture of hemocytes

Adult bees were washed with sterile PBS (pH 7.2). Afterwards, bees were sterilized by giving two washes of disinfectant like 0.525 % Sodium hypochlorite and 70% ethanol (2 washes) followed by several rinses of sterile PBS. Finally, bees were rinsed with PBS (pH 7.2) containingantibiotic and antimitotic solution (2%) twice.Hemolymph was collected by severing abdomens (2nd and 3rd tergites) and used for establishing hemocytes cell culture.

Eliciting bacterial infection in Honey bee

Bacillus Subtilis was grown in Nutrient brothto 0.5 Absorbance at 550 nm. 1ml of culture was centrifuged and resuspended in 1X PBS (pH 7.2). Culture was injected into the honey bee and incubated for 5 h in an incubator. Thereafter, hemolymph was extracted from the dorsal part of honey bee. Trypan blue was added to the collected hemolymph in 1:1 ratio. Cell count was done using hemocytometer.

Flow cytometric analysis

In this study, hemolymph samples of honey bees (infected and non-infected) were exposed to proteins of pollen (sunflower and soyabean). Incubate haemolymph samples along with pollen protein samples for 2 h at 37 °C in carbon dioxide incubator. After incubation, add FACS lysing solution and incubated for 10 minutes. Afterwards, centrifuge the protein

sample at 2300 rpm for 7 min at 4 °C and washed with phosphate buffered saline (PBS) and then analyzed through flow cytometer (FACS Calibur) for estimating forward (shape and size) and side scatter (granularity of the cell) gating applied for data acquisition of 10000 events of cell populations representing different phenotypes analyzed using cell quest software [12, 13].

RESULTS

Surveillance

Selected site of apiary is totally clean and free from dry leaves. The recommended methods are used to control swarming, colonies management, mass queen rearing, stopping laying workers, robbing and migration. The humidity of the hives to ensure proper incubation of eggs. When diagnosed with certain disease, preventive measures is to be taken. Honey bee colonies in the hives will be periodically maintained hygienically as shown in **Table 1**.

Table 1 Surveillance Inspection chart of the Apiary site

Date of inspection	7/9/2015	12/1/2016	15/3/2016
Total number of colonies	20	8	4
Cleanliness	Ok	Ok	Ok
Steadiness on comb	Fast	Slow	Slow
Attempts on desertion	No	Yes	Yes
Disease and pests	No	Wax moth	No
Pollen stores	Yes	No	No
Honey and bee pollen stores	Yes	No	No
Floral condition (pollen and nectar)	Sunflower, Soyabean,	No	No
Drones breeding stage	No	No	No
Number and stage of queen cells	New queen cells	Old queen cells	No
Temper	Aggressive	Dull	Dull
Feed supplements	No	Sugar feeding	Pollen Supplements

When bee pasturage is available bee feed on nectar and pollen that they have collected. While, during dearth period 50% sugar syrup and artificial pollen diet also provided in order to maintain healthy colonies.

Morphometric analysis

Morphometric analysis of those worker bees collected from hive shows no significant differencesintheir body parts.However, the dead bees collected from nearby regions of hive box indicate the severe extent of infectionsince bees exhibited shorter length and width of femur, tibia, forewing and head as well as compared to bees fed during flowering days (soyabean, Sunflower). It has been observed that bees were weak and therefore, exhibited organs of reduced size due to malnutrition during dearth period (**Table 2**).

Table 2 Morphometric analysis of worker bees *(Apis mellifera)*

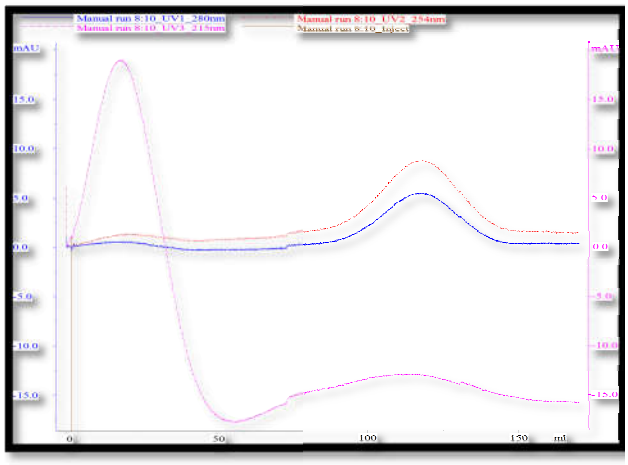
Morphometric analysis (mm)	Soyabean	Infected Bees	Sunflower
Inner Forewing	4.32	4.1	4.3
Maximum length of inner forewing	9.01	8.2	9
Head width	3.86	3.2	3.85
Head length	4.01	3.8	4
Femur Length	3.5	3	3.5
Tibia length	3	2.7	2.96
Forewing length	9.4	8.6	9.37
Forewing width	2.8	2.4	2.8

Quality analysis of pollen in terms of protein content

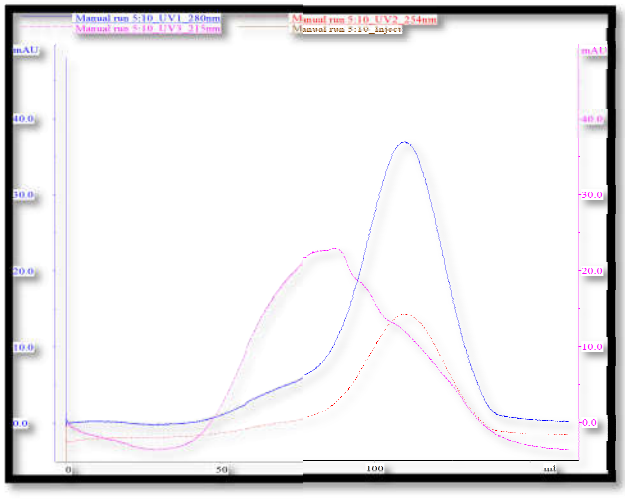
Pollen from different flowers are collected and analyzed further for their protein content. Inorder to carry out analysis, protein from different pollens such as Soyabean (9.8 mg/ml) and Sunflower (7.04 mg/ml) are carried out using Lowry's method.

FPLC

AKTA FPLC system was used to analyze the mixture of proteins of Sunflower and soyabean protein as shown in **Fig.1**. Collectively, FPLC analysis suggests the higher protein content of soyabean pollen as compared to sunflower.



A Sunflower



B Soyabean

Fig.1 Peak obtained by FPLC of pollen protein of Sunflower and Soyabean

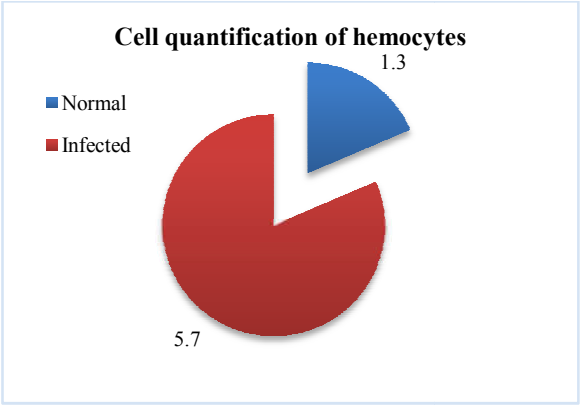
Hemocyte count and eliciting bacterial infection in honey bee

There is significant increase in total hemocyte count of cellswhich is infected with *bacillus subtilis* as shown in **Fig.2**. Due to increase in hemocyte count that results in phagocytosis of the invading foreign material (innate immunity).

Flow cytometric analysis

The effect of pollen protein extracted from sunflower and soyabean which is exposed to hemolymphas shown in **Fig.3**. The results of this study showed that there is enhancement in forward and side scatter of this protein content as compared to control. In addition, soyabean and sunflower protein showed

more declined in forward and side scatter in case of infected bees as shown in **Fig.3.**



Days	Cells/ml
0	0.7 x10^6
1	0.9 x10^6
3	1.5 x10^6
5	2.35x10^6
8	5.35x10^6
12	5.48x10^6

Fig.2 Hemocytes. A) Cell quantification. B) Growth curve of Hemocyte culture *in vitro.*

disorder. In this study, immunocompetence assay revealed that their immunity is based on the pollen diet and nectar they feed on and the risk of pathogens that they encounter. The risk of disease is more during dearth period when bee pasturage is negligible [14, 15]. The immune system becomes weak and their body becomes more prone to invading pathogenic effect. This data was recorded during surveillance of Apiary site in VSBT and data collected for morphometric analysis and it was based on the flowering conditions and during dearth period. Normally, worker bees fed on pollen diet and maintained their health [16].

In this study, pollen protein was extracted from soyabean and sunflower. Out of two, higher protein concentration is observed in soyabean as compared to sunflower. As per the results of FPLC, analysis of pollen protein appeared at 215nm, 254nm and280nm in soyabean and sunflower. Hence, peptides are present in pollen and small molecular weight proteins are also present in less concentration.In case of Sunflower, Initially at 50ml volume of mobile phase, no peak is visible which suggests absence of proteins in the respective sample. Further, peak appeared at 280 nm indicating proteins is observed at 100-150 ml volume of mobile phase.

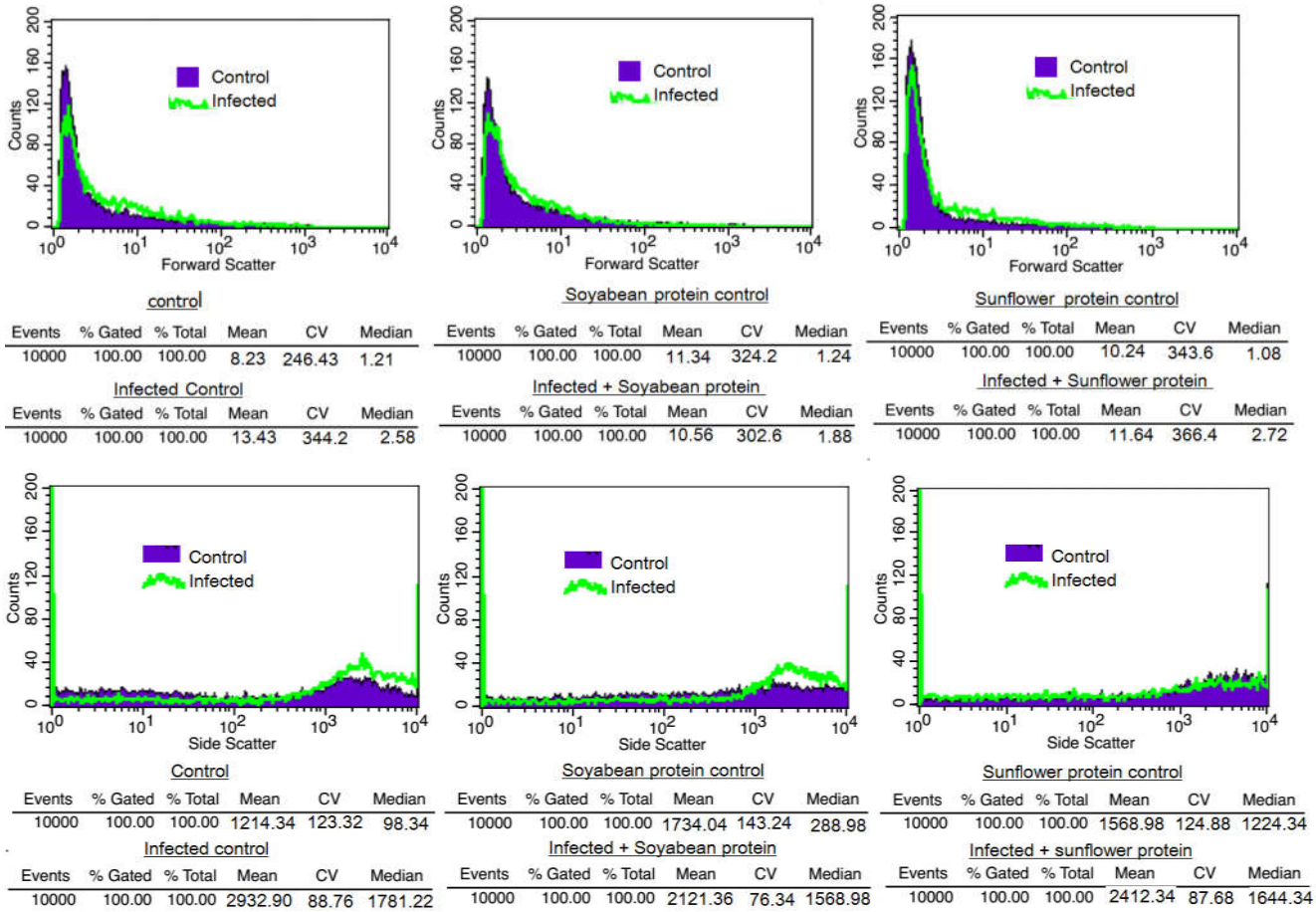


Fig.3 Flow cytometric analysis of infected (bacillus subtilis) and non-infected protein extracted from soyabean and sunflower pollen exposed to hemolymph of honey bee and determined forward (shape and size) and side scatter (granularity). Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software.

DISCUSSION

Honey bees are significant pollinators and frequently encountered with an infection that results in colony collapse

While peak at 254 nm at same elution volume indicates presence of small peptides whereas in soyabean, peak starts appearing at 50 ml volume of mobile phase, which suggests high concentration of proteins in the initial volume of elution

itself. Further, peak appeared at 280nm, peptide portion of the sample appeared at 60-130 ml volume of mobile phase. While peak at 215 nm at same elution volume indicates presence of small peptides.

In addition, bacterial infection elicited immune response in honey bee when bacterial suspension was injected into the dorsal part of honey bee for determining its immunity. Cellular response was triggered and hence, hemocyte count increased. In other words, hemocytes, blood cells circulating in the hemolymph of honey bee mediates the cellular response. Prohemocytes can differentiate into three primary kinds of hemocytes: Crystal cells, plasmatocytes, and lamellocytes [17-19].Antimicrobial peptides are produced especially in the fat body which is a major immune-responsive tissue. It is a large biosynthetic organ located inside the open circulatory system. Defensin 1 and Defensin 2 are produced in hypopharyngeal gland and fat body cells and hemocytes of honey bee. The production of such antimicrobial peptides become second line of defense in immunity of honey bee [17-19].

In another study, pollen protein of soyabean and sunflower is exposed to hemolymph and showed variability in forward and side scatter which is determined through flow cytometer. The results of these studies clearly indicates that soyabean pollen protein showed higher in forward and side scatter as compared to sunflower. In other words, soyabean proteins when exposed to hemolymph, there is enhancement in shape, size and granularity of protein as compared to control. Similarly, when hemolymph of infected bees were collected and exposed to these pollen proteins, the results showed more declined in shape and size including granularity of pollen. This inhibition is due to the presence of antimicrobial peptides that are present in pollen proteins i.e. defensins [17-19]. Overall, this test is relatively inexpensive and required small quantities of inexpensive chemicals and kept only few hundred bees. In addition, identification of these hemocytes that are present in a fluid containing proteins of soyabean and sunflower which is measured through light scatter properties as it has been shown that there is a direct relationship between forward scattered light and cell volume and this has become common practice in flow cytometry.

CONCLUSION

Overall the data represents that when pollen proteins exposed to hemolymph content of honey bees is a useful, rapid, practical and accurate method for determining its immunity in the form of forward (shape and size) and side scatter (granularity) quantitatively using flow cytometry. In other words, these proteins extracted from pollens of soyabean and sunflowers that are responsible for enhancing its immunity related to honey bees.

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