EVALUATION OF DIFFERENT SUBSTRATES AND QUEEN CUP MATERIALS ON THE ARTIFICIAL REARING OF HONEY BEE QUEEN (*Apis mellifera* Linnaeus) USING GRAFTING METHOD

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Entomology

By

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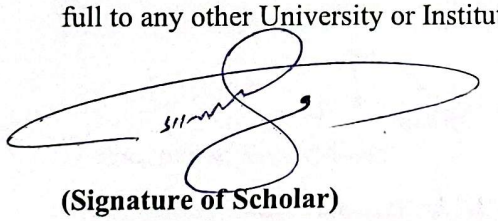
DECLARATION

I, hereby declared that the presented work in the thesis entitled **"Evaluation of Different Substrates and Queen Cup Materials on The Artificial Rearing of Honey Bee Queen (*Apis mellifera* Linnaeus) Using Grafting Method"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.) in Agriculture (Entomology)** is outcome of research work carried out by me under the supervision of **Dr. Ankush M. Raut**, working as Associate Professor, in the Department of Entomology, School of Agriculture of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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CERTIFICATE I

This is to certify that the work reported in the Ph. D. thesis entitled **"Evaluation of Different Substrates and Queen Cup Materials on The Artificial Rearing of Honey Bee Queen (*Apis mellifera* Linnaeus) Using Grafting Method"** submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.) in Agriculture (Entomology)**, is a research work carried out by **Mohammed Ahmed Mogbel Abdullah Al Shogari, 12109776** is bonafide record of hisher original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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CERTIFICATE II

This is to certify that the thesis **"Evaluation of Different Substrates and Queen Cup Materials on The Artificial Rearing of Honey Bee Queen (*Apis mellifera* Linnaeus) Using Grafting Method"** submitted by **Mohammed Ahmed Mogbel Abdullah Al Shogari (Registration No. 12109776)** to the Lovely Professional University, Phagwara in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY (Ph.D.)** in the discipline of **AGRICULTURE (ENTOMOLOGY)** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an external examiner.

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Place: LPU, Punjab

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LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviated Form	Full Form	Abbreviated Form	Full Form
%	Percentage	SE	Standard error
ANOVA	Analysis of Variance	NS	Non-significant
Spp.	Species Pluralis	NE	No emerged
etc.	Et cetera	N	Indicates North Latitude
Fig.	Figure	E	Indicates East Longitude
gm	Gram	B	Emerging Capped Brood Comb
mg	Milligram	HP	Honey And Pollen Comb
mm	Millimeter	GF	Grafting Frames
kg	Kilogram	F	Feeder Frame
cm^2	Square Centimeter	®	Registered Trademark
µL	Microliter	S	Substrate
<	Less than symbol	C	Cup Materials
>	Greater than symbol	i.e.	id est
±	Plus-Minus Sign		
°C	Degree Celsius		
SPSS	Statistical Package for the Social Sciences		
DMRT	Duncan's Multiple Range Test		
'**'	Significant level at 0.01		
'*'	Significant level at 0.05		
Df	Degrees of Freedom		
P_{value}	Probability Value		

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Title	**:**	**Evaluation of different substrates and queen cup materials on the artificial rearing of honey bee queen (*Apis mellifera* Linnaeus) using grafting method**
Name of Student	**:**	**Mohammed Ahmed Mogbel Abdullah Al Shogari**
Registration No.	**:**	**12109776**
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Designation	**:**	**Associate Professor, Department of Entomology**
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ABSTRACT

Honey bees contribute significantly to the pollination of several crops and the maintenance of biodiversity. The bee colony's strength and productivity are largely based on the bees' floral resources, prevailing environmental conditions, and the age and genetic characteristics of the queen. Artificial queen rearing is the first choice for beekeepers to produce high-quality and healthy queens, essential for sustaining colony strength and productivity. Therefore, this study aims to investigate the potential effects of seasons, substrates, and queen cup materials on larvae acceptance rates, queen emergence rates, and morphometric traits of queens. The experiments were carried out during the Spring and Autumn seasons, at the Apiculture Area, Department of Entomology, School of Agriculture, Lovely Professional University, Phagwara, Punjab, India. The suitable age larvae (12-24 hours old) for grafting were taken from the chosen mother colonies. Three queen cell builder colonies were used for artificial queen rearing. Six treatments were evaluated in total, comprising five different substrate types (priming media) and one control treatment (dry grafting without substrate). Paraffin wax, beeswax, and plastic queen cups were used in this study. The Doolittle grafting method was used for queen rearing in both seasons (Spring and Autumn). The data were analyzed utilizing a two-way analysis of variance and a t-test using SPSS software (version 22). Duncan’s Multiple Range Test

(DMRT) was utilized to compare and rank the groups. Our findings revealed that the highest mean larval acceptance (50.00 %), (33.33 %) and queen emergence rates (43.33 %), (33.33 %) were significantly achieved in mixture solutions and apple juice, respectively, used as the priming media in bee wax cups and plastic cup materials during the Spring season. Morphometric traits were significantly greater in beewax cups compared to plastic cups during the Spring season. During the Autumn season, overall larval acceptance and queen emergence rates were lower compared to Spring. The control group (dry grafting) showed the highest mean larval acceptance (43.33 %) and queen emergence rates (26.67 %) in bee wax cups and plastic cup materials. Morphometric traits were also reduced in Autumn compared to Spring. Comparative impact of grafting folds indicated that the first grafting fold (04/10/2024) had better larval acceptance and queen emergence rates (15.83 %, 14.167 %), respectively, during Autumn, while the second grafting fold (27/03/2024) performed better in queen emergence rates (24.17 %) during Spring. Comparative impact of grafting bar level indicated that a lower grafting bar level showed significantly higher larval acceptance (36.67%) and queen emergence (30.00%) in Autumn. In contrast, there were no significant differences between the grafting bar levels during the Spring season. Further research is recommended to examine the specific type of substrates, such as apple juice and properties of beeswax used in the preparation of artificial queen cups, as these factors may influence larval acceptance, the queen emergence rates, and morphometric traits.

Keywords: *Apis mellifera*, seasons, substrates, cup materials, queen rearing, grafting method, morphometric traits.

Chapter 1
Introduction

CHAPTER - 1

INTRODUCTION

Honeybees are significant in agricultural production systems (Sharma et al., 2020; Khalifa et al., 2021). Honey bees directly impact 1/3 of the global food crops, contributing significantly to the pollination of several crops and the maintenance of biodiversity (Papa et al., 2022). Beekeeping is essential to exist plans for sustainable agriculture and integrated rural development programmes (Abrol, 2013). It offers rural populations nutritional, economic, and ecological security, particularly in developing nations like India (Chanthayod et al., 2017; Amulen et al., 2019; Abrol, 2023). Moreover, it is an extra revenue-producing activity (Prodanović et al., 2024). The South and Southeast Asian areas are rich in honey bee species and genetic diversity (Anandaraj, 2017).

Honey bees are directly advantageous to humans by producing many crucial bee products, including royal jelly, honey, propolis, beeswax, pollen, and bee venom etc. (Pasupuleti et al., 2017; Durazzo et al., 2021) and indirectly help in sustaining biological variety via hybrid vigor through cross-pollination (Khalifa et al., 2021; Johannsmeier and Mostert, 2001). Honeybees play an important role in controlled and natural ecosystems by pollinating flowering plants (Bixby et al., 2020). Therefore, they greatly aid food crop production: nearly a third of food crop species worldwide increase their yield due to animal pollination, particularly by bees (Khalifa et al., 2021; Klein et al., 2007).

A perfect community of diligent workers in the honeybee colony plays an important role in promoting development of colony and ultimately enhancing overall productivity (Mattiello et al. 2022). The queen bee quality directly influences the disease resistance, vigor colony, and overall productivity, making the selection and rearing of queens a critical aspect of successful beekeeping (Hatjina et al., 2014; Ozbakir 2021; Yu et al., 2022).

The bee colony's strength and productivity are largely based on the bees' floral resources, prevailing environmental conditions, and the age and genetic characteristics of the queen (Mahbobi et al. 2012; Ozbakir 2021). Therefore, queen rearing is the first choice

for beekeepers to produce high-quality and healthy queens, essential for sustaining colony strength and productivity. (Guda et al., 2024; Zhong et al., 2024). The most crucial member of a honeybee colony is the honey bee queen, as she is solely responsible for laying both fertilized and unfertilized eggs, thereby ensuring the continuity of overlapping generations, (Winston, 1991; Delaney et al., 2011) and she also secretes pheromones that suppress the development of ovaries in worker bees and prevent them from rearing new queens (Abou-Shaara et al., 2021; Carroll et al., 2023).

The queens that have high-quality traits can be raised from superior-performing colonies to enhance the desirable hereditary traits provided via the mother colonies, as the queen serves as the primary custodian of the colony's genetic characteristics (Yu et al., 2023). However, genetics influence many behavioral traits of the colonies like resistance to diseases, defensiveness and their enemies, swarming and absconding tendencies, degree of gentleness, rate of population growth, industriousness, ability to collect honey and pollen, and many other qualities (Yu et al., 2022; Ozbakir 2021; Hatjina et al., 2014). In other words, the great performance of a colony of a honeybee is decided by the quality of the queen, which may be measured through various traits, like ovarioles number, weight, fecundity rate (eggs number laid per day), size of the spermatheca, and the quality of brood produced (Kovacic & Puškadija, 2016; Amiri et al., 2017; Facchini et al., 2021; Arslan and Arslan, 2024). For any activities made on colony growth and for economically successful beekeeping use of standard and good quality queens is a must (Buchler et al., 2013).

Honeybee queen rearing typically occurs during periods of supersedure, swarming, or when the existing queen has been mistakenly killed or lost (Büchler et al., 2024; Holmes et al., 2023; Seeley, 2014). Therefore, adult honey bee workers raise fresh queens from worker larvae that are below 24 h. old (Gąbka et al., 2011; Rehman et al., 2024). However, one cannot depend on queens raised throughout the swarming season because, first, it requires constant vigilance and, second, the colonies reared from such queens may have more swarming instinct, which is generally discouraged; it also limits the selection of queens from a good colony (Sharma, 2019; Kamboj et al., 2023). In swarming and supersedure impulse, no beekeepers can know exactly which colony is going to swarm or

supersedure and if it is known, it is only probable that it may not be in the colonies, which beekeepers desire to increase (Suryanarayana et al., 1988).

In particular queen cells, honey bee workers rear queens by building up a queen cup after the queen has placed the egg within (Abbasi et al., 2015). The larvae that are feeding on royal jelly via the nursing bees develop as queens and come out after ten to eleven days (Woodward, 2007; Remolina and Hughes, 2008; Kamakura, 2011; Alhosin, 2023). The royal jelly is the nutritive material for larvae of drones and workers during the first to third day and is utilized as a sustenance for queen bees during their larval stages (Khan et al., 2021; Ghosh et al., 2024). Queen bee raising is the most crucial beekeeping process for rapidly reproducing honeybee colonies (Yu et al., 2022). Each year before the honey flow season, old queens are substituted to raise honey production (Khan and Ghramh 2022). New queens are also introduced into colonies in the event of sudden loss due to transportation, colony manipulation, or attacks by honeybee diseases or enemies (Amiri et al., 2017; Sharma et al., 2020). The artificial queen raising practice has completely changed the industry of beekeeping due to, in association with the replacement of natural queen (supersedure), enabling the timely addition of a young, fresh, and mated queen to a colony, thereby minimizing the period during which egg-laying and new bee production are interrupted (Contreras-Martinez et al., 2017; Smilga-Spalvina et al., 2024). On the other hand, when a queen is naturally superseded via the workers, it typically takes around twenty-five to thirty days for a fresh queen to be successfully mated, developed, and mature enough to start egg-laying (Holmes et al., 2023). Ensuring the availability of young, mated queens for timely requeening necessitates their artificial rearing under controlled conditions (Contreras-Martinez et al., 2017; Büchler et al., 2024). Recently mated queens from selected races contribute to preserving the genetic diversity of the colony, enhancing productivity and overall health, and are generally less likely to initiate swarming compared to older queens (Yu et al., 2022; Adgaba et al., 2019; Guzman-Novoa, 2007).

A honeybee colony may create a new queen without human involvement as long as fertilized eggs are available (Buchler et al., 2013). In industrial apiaries, replacing aging queen bees becomes essential, as their egg-laying capacity declines over time, potentially

affecting colony productivity (Dhaliwal et al., 2017). The queen typically lays approximately 50% of her lifetime eggs during her first year, 30% in the second year, and 10% in the third year, with the remaining 10% distributed over the subsequent two years. (Goodman 2008). There are various conventional methods like the Alley method, Miller method, Smith method, Hopkin method, and some modern methods like the Doolittle method, and Karl jenter method of mass queen bee raising (Sharma, 2019). The most common method for large-scale queen raising followed by commercial queen breeders is the Doolittle or Larvae Grafting method (Arslan et al., 2021; Gatoria et al., 2004). The reactions of colonies towards diverse queen-raising procedures are influenced by the changes in environmental, behavioral, and biological factors (Büchler et al. 2024; Crailsheim et al., 2013; Nuru, 2012).

The important factors impacting the quality of the queen include raising duration and grafting techniques (Rafique et al., 2019). Honey bee queen rearing is best performed during periods of favorable climatic conditions, particularly when temperatures are warm, drone populations are ample, and the availability of high-quality nectar and pollen is abundant, thereby ensuring optimal colony productivity and successful mating outcomes (Guda et al. 2024). Environmental conditions such as relative humidity, temperature, and source of pollen were found to be crucial factors impacting the quality and acceptance of artificially raised queens (Adgaba et al., 2019; Tlak and Mutinelli, 2024).

Queen quality may be improved through management methods. Grafting with several procedures such as wet or dry grafting was investigated for a high rate of queen emergence and acceptance by utilizing different substrates (priming media) (Adgaba et al., 2019; Kamel et al., 2013; Buchler et al., 2013). The crucial step in queen rearing involves transferring a young larva, aged between 12 to 24 hours, from the worker cells into the specially prepared queen cell cups (Dhaliwal et al., 2017). Cell priming refers to the practice of placing a drop of priming media into a queen cell cup prior to grafting a larva. This technique facilitates the grafting process, reduces the risk of larval dehydration and damage, enhances larval acceptance, and contributes to improved queen quality (Khan et al., 2021; Sharma et al., 2020; Rafique et al. 2019; Contreras-Martinez et al., 2017).

The European honeybee (*Apis mellifera* L.) serves as the foundation of the thriving apiculture industry globally (Papa et al., 2022). Since it was first introduced in India, extensive need-based research has been conducted across various domains, including colony management, bee forage resources, behavioral studies, and disease management in honey bees (Sharma et al., 2020; Thomas et al., 2002). In India, beekeeping and stock development have remained ignored (Singh et al., 2007). However, there are opportunities for the enhancement of several traits of the honey bee by utilizing the selection of superior bee stock (Maucourt et al., 2020; Maucourt et al., 2021; Morfin et al., 2023). Different *A. mellifera* L. strains have been introduced in India (Sharma, 2019). Only *A. mellifera ligustica* queen bees survived and were mostly established in India (Atwal and Sharma, 1970).

Globally, beekeepers have conducted practical assessments using various substrates and queen cell cup materials. However, there is a notable scarcity of studies investigating the impact of substrates and queen cup materials on acceptance rates of larvae, emergence rates of queen, and morphometric traits of queens. Additionally, there are inconsistencies in the results, especially in the impact of the substrates on the grafted larval acceptance and queen emergence rates. To date, research has primarily focused on substrates such as royal jelly, distilled water, and their combinations with other compounds, as well as queen cup materials including beeswax derived from old brood combs, fresh beeswax, and various wax mixtures (Ebadi & Gary 1980; Macicka 1985; Contreras-Martinez et al., 2017; Dhaliwal et al. 2017; Rafique et al. 2019; Sharma et al., 2020; Khan et al., 2021; Lashari et al. 2022; Ustadi et al. 2022; Kamboj et al., 2023). A research gap remains in standardizing the optimal season for queen rearing. However, a lack of consensus and standardized guidelines persists in identifying the optimal season that yields the best results consistently across diverse geographic regions and climatic conditions. Therefore, this study aims to investigate the potential effects of seasons, substrates, and queen cup materials on larvae acceptance rates, queen emergence rates, and morphometric traits of queens.

Taking into account the importance of artificial queen rearing on the larval acceptance rates, queen emergence rates, and morphometric traits of queens, the current study aims to investigate of "Evaluation of different substrates and queen cup materials on the artificial rearing of honey bee queen (*A. mellifera*) using grafting method", and divide this goal into sub-objectives as follows:

1- To standardize optimum season for *A. mellifera* queen rearing.
2- Effect of different substrates on *A. mellifera* queen rearing.
3- Effect of queen cell cup materials on the acceptance of grafted larvae and *A. mellifera* queen rearing.

Chapter 2
Review of literature

CHAPTER - 2

REVIEW OF LITERATURE

Colonies exhibiting good performance and high quality are selected for queen rearing, as they serve as a genetic bank within the colony, playing a vital role in preserving desirable traits (Kamboj et al., 2023). A queen with undesirable traits can be replaced with another queen with desirable traits by raising honey bee queens, thus improving the characteristics of the honey bee colony (Holmes et al., 2023; Amiri et al., 2017). The Doolittle grafting method is the most commonly employed technique by commercial queen breeders for rearing (Doolittle, 1889 and Contreras-Martinez et al., 2017). The grafted larva age is considered a biological factor, cell cups of the queen as a nutritional factor, external factors, and mechanical factor such as climate, food supply, rearing conditions, and weather that impact in queen quality (Rehman et al., 2024; Ustadi et al., 2022; Cengiz et al., 2019; Weiss 1983). Artificial queen rearing is a crucial beekeeping procedure, particularly for commercial beekeepers, as it enables the regular requeening of honey bee colonies to maintain colony productivity, health, and genetic quality (Ahmat et al., 2024 and Simeunovic et al., 2014).

The literature relating to different factors and elements of the current research entitled "Evaluation of different substrates and queen cup materials on the artificial rearing of honey bee queen (*A. mellifera*) using grafting method" is provided under the following heads:

2.1. Selection of honeybee colonies for queen bee rearing:

Selection of honeybee colonies is a critical factor in the queen rearing success, as it includes the biological and economic characteristics of bee colonies, including stores of honey, stores of pollen, and brood area, all of which help us to know the strength of the hive (Sharma, 2019).

Multilevel selection, involving the evaluation of both colonies and patrilines, can significantly enhance artificial selection for hygienic behavior in honeybees. Colonies

headed by hygienic patrilineal queens exhibit nearly twice the level of hygienic behavior compared to those headed by non-hygienic patrilineal queens, thereby significantly improving honeybee breeding programs (Pérez-Sato et al., 2009).

Evaluating colony performance based on factors such as swarming tendency, gentleness, colony strength, honey production, racial characteristics, extent of capped brood, cubital index (Ci), the existence of *Nosema* spp. spores, and hygienic behavior serve as an effective tool for ranking and selecting colonies in each queen-rearing apiary (Gregorc and Lokar, 2010).

Swarming was once considered a desirable trait in honey bee colonies several decades ago; however, in modern apiculture, it is generally regarded as an undesirable characteristic due to its negative impact on colony productivity and management efficiency (Gregorc, 2005). Evaluating the strength of colonies biannually provides an opportunity to rank colonies based on their economic potential, aiding in the selection of superior strains for beekeeping procedures (Pechhacker et al., 1991). The brood production and colony productivity are directly influenced by the quality and age of the queen bee (Tarpy, 2000). Evaluating the strength of colonies based on the brood number of combs is an effective method to identify and select the productive colonies (Gregorc & Lokar, 2010).

Standardized methods for estimating colony strength parameters in *Apis mellifera* L. are valuable tools for evaluating colony performance. These methods generally fall into two primary categories: the objective mode, that involves experiential measurements, including area (cm^2) or weight (mg, g, or kg), and the subjective mode, which depends on visual assessments made through one or more inspectors. Additionally, a 3rd emerging mode, computer-assisted digital image analysis, is gaining prominence for its potential to enhance the accuracy and consistency of colony strength evaluations (Delaplane et al. 2013).

X-ray computed tomography (CT) provides one of the most empirically precise, comprehensive, and non-invasive techniques for monitoring the honey bees' colony strength and other social insects, offering detailed internal insights without disrupting

colony structure (Greco, 2010). Although X-ray computed tomography (CT) represents the gold standard for non-invasive monitoring of colony strength, its high technical demands and cost limit its accessibility for most honey bee researchers (Delaplane et al., 2013).

Genetic improvement in honey bee colonies can be achieved by simultaneously selecting for productivity, health, and hardiness using a selection index within a selection program, focusing on traits such as honey production, hygienic behavior, *Varroa destructor* infestation level, winter weight loss, and spring development (Maucourt et al., 2020).

Bee breeding primarily focuses on enhancing traits of apicultural significance that directly influence the beekeeping industry, including reducing defensive behavior, increasing honey yield, and minimizing swarming tendencies (Büchler et al., 2013; Uzunov et al., 2017; Kovačić et al., 2020).

Genotype-environment interactions significantly affect bee behavior, productivity, and survivability, leading to the recommendation of using locally adapted bees, a practice increasingly recognized as crucial for enhancing bee health and colony survival, especially in light of recent concerns about honey bee health and colony losses (Costa et al., 2012; Büchler et al., 2014; Hatjina et al., 2014; Uzunov et al., 2014).

Long-term breeding and selection practices significantly influence bee behavior, particularly traits such as calmness on the comb, defensiveness, and the tendency to swarm (Kovačić et al., 2020).

A high-quality queen can be raised from superior-performing colonies to enhance desirable genetic traits passed on by the mother colony, as the queen serves as the principal custodian of the colony's genetic characteristics (Sharma et al., 2020; Kumar and Mall, 2018). Sharma et al. (2020) reported significant differences among colony parameters, viz. strength of colony (frames numbers with bees), honey stores (g), pollen area (cm^2), brood area (cm^2), and prolificness (eggs laid numbers/day).

The queen bee quality directly influences the benefits derived from a colony, as her progeny determines key traits such as temperament, productivity, and overall behavior, which permit beekeepers to strategically manage and enhance colony performance (Kumar, 2018). The quality and development rate of queens are influenced by a different of environmental factors, including temperature, nutrition, humidity, and the availability of flora (Mahbobi et al., 2012).

Honeybee colonies can exhibit varying behavioral capabilities in performing essential tasks such as queen rearing, royal jelly secretion, comb construction, hygienic behavior, and the production of wax, honey, and propolis (Akongte et al., 2023). The expression of certain behavioral patterns in honeybee colonies is believed to be regulated by a combination of instinctual responses and cognitive processes (Gallo and Chittka, 2018). Furthermore, bees' capability to understand past experiences enhances their movement skills (Abramson et al., 2016; Mirwan et al., 2015) and anticipate the outcomes of their behaviors, thereby improving task performance and adaptability (Webb, 2004). Colonies with perfect wax production and comb-construction capabilities are better equipped to store greater quantities of honey and pollen under favorable conditions, such as the presence of a queen, robust brood rearing, active nectar flow, and temperatures above 15 °C, while also facilitating efficient communication within the colony (Bogdanov, 2016).

Therefore, to optimize hive product yields and meet various beekeeper objectives, selecting and breeding colonies with high productive potential is essential, as factors like queen cell acceptance rate influence royal jelly secretion and colony management affects overall product output (Akongte et al., 2023).

Khan and Ghramh (2022) reported that the acceptance of queen cells was considerably higher in hygienic colonies; however, non-hygienic colonies condition was performed impact on larval acceptance rate according to Akongte et al. (2023). Additionally, Akongte et al. (2023) found that royal jelly production and the dead brood removal percentage varied among colonies, indicating distinct behavioral and physiological differences across honeybee populations.

Based on the previous studies mentioned above, it can be concluded that the selection of high-performing and superior-quality colonies plays a crucial role in queen rearing, as such colonies serve as a genetic bank, preserving and transmitting desirable traits essential for colony improvement.

2.2. Grafting method in queen bee rearing and other methods:

Queen rearing is a very important factor to improve and increase honey bee products, as the study of the behavior and nature of honey bee breeding motivated beekeepers to develop queen rearing techniques to produce high quality queens (Wakjira et al., 2019).

Queen-rearing techniques have been improved to enable apiarists to reproduce high-quality colonies and to substitute undesirable or aged queens within colonies, thereby enhancing colony performance and sustainability (Joseph Latshaw, 2011). Artificial queen rearing enables beekeepers and researchers to economically select desirable genetic stock while also providing a valuable tool for studying honeybee genetics and behavior (Hamdan, K. 2010).

However, environmental factors such as the relative humidity, availability of flora, and temperature were identified as key factors influencing both the larval acceptance rate and queen quality raised artificially (Adgaba et al., 2019; Büchler et al., 2013).

To ensure better growth of the colonies throughout the year and maximize the monetary outputs, the beekeepers must rear and provide high-quality queens before the onset of honey flow season (Kamboj et al., 2023).

Quinby (1854) described a method for requeening queenless colonies through inserting a comb piece containing small larvae between the hives' combs. However, he did not advocate this method for regular queen production, as he believed that queens produced through this method were of inferior quality compared to those reared under the natural swarming impulse. Variations in the queen cells' positioning on the comb, typically along the lower edge during swarming or on the sides during supersedure, lead to differences in the climatic conditions, particularly temperature, which can influence the metabolic

processes and developmental outcomes of the queen larvae (Lukoschus, 1955 and Lukoschus, 1956).

Queen bees reared using the grafting method (Doolittle method) exhibit superior traits, such as greater sealed queen cell lengths, higher emergence weights, larger spermatheca diameters, and increased spermatozoid counts, compared to those reared using the natural queen cell method (Kumar, 2018; Abbasi et al., 2015; Dodologlu et al., 2004).

Miller (2006), in "Fifty Years Among the Bees," describes the Miller method for raising queen cells, which involves cutting wax foundation sheets into a V-shape, placing them in mother colonies for comb raising, extracting the combs after queen egg-laying, leaving 24 eggs, removing excess eggs, and transferring the marked frames to strong queen-right cell builder colonies for queen cell development.

Hamdan (2011) described the Hopkins method as a queen rearing technique that involves removing a frame containing newly hatched larvae or eggs from a selected breeder queen and placing it in a queenless cell builder colony. Unlike standard placement, the frame is laid horizontally above the brood nest. In response to queenlessness, nurse bees are stimulated to feed the larvae generously with royal jelly and initiate queen development. This method is suitable for producing a limited number of queens. Although it is possible to rear 20 to 30 or more queens from a single frame, limiting the number to around 20 is recommended to ensure high-quality queen development.

The Doolittle grafting method is the most commonly employed technique by commercial queen breeders for rearing (Rehman et al., 2024). It involves transferring a young larva (< 24 hours old) into artificial queen cell cups (Vung et al., 2018). This method requires a high level of skill, as it demands accurate identification of the tiny larvae and careful handling during the grafting process to ensure successful queen development and avoid rejection by worker bees (Dhaliwal et al., 2017; Wakjira et al., 2019).

However, novel techniques such as the Karl Jenter and Cup kit queen rearing apparatus eliminate the need for wax cell cup fabrication and grafting, reducing the risk of larval injury and simplifying the transfer process to cell builder colonies (Dhaliwal et al., 2019).

Using the Karl Jenter and Cupkit systems in queen bee rearing facilitates the larvae transfer without displacement or injury, as the larvae remain in the same plastic queen cell cups in which they hatch. This approach significantly simplifies and expedites the queen rearing process while minimizing the risk of damage to the delicate young larvae (Gatoria et al., 2004).

Numerous queen-rearing techniques have been developed to enable beekeepers to propagate superior genetic stock, replace suboptimal or old queens, and establish new colonies, supporting sustainable and productive apiculture (Kumar, 2018).

Queen bees reared through the grafting method generally exhibit superior performance compared to those produced from natural queen cells, due to controlled selection of larval age, genetic lineage, and optimal rearing conditions (Doolittle, 1915; Abrol et al., 2005; Abbasi et al., 2015).

Kamboj et al., (2023) deployed Doolittle, Miller, Smith, and swarming instinct methods to mass rear and study the quality traits of *A. mellifera* queens. The study showed that the Doolittle method yielded the highest larval acceptance rate, development of the largest queen cells, as well as the earliest egg laying. Researcher recorded that queens from the Doolittle method also had the highest mean body weight (201.75 mg) and the highest sealed queen cells number and neonate queens per colony. The study identified the Miller method as the next best alternative. Therefore, the researcher recommended the Doolittle method as the most suitable approach for the mass rearing high-quality queens in commercial beekeeping operations.

Based on the previous findings, it can be concluded that queen rearing by the artificial grafting technique has a notable positive impact on honey yield and development of brood, while also contributing to reduced mite infestation, lower swarming tendency, and absence

of absconding behavior compared to queens produced by natural methods. Therefore, the artificial grafting technique is recommended for beekeepers seeking to achieve optimal colony performance and productivity (Abbasi et al. 2015).

2.3. Effect of larvae age on queen bee rearing:

The artificial queen-rearing methods used in beekeeping have a positive influence on the queens' development for desirable traits, thereby enhancing colony performance, productivity, and overall apiary management (Rehman et al., 2024).

The honeybee queen quality is determined through various factors, such as the grafted larvae source, the availability of nutritional resources within the starter and finisher colonies, the larvae age, the young nurse bees' population, and the presence or absence of existing honey bee queens in the colony (Mahbobi et al., 2012 and Morse 1994).

The queen cells' acceptance rate and morphological characteristics in honeybees are significantly affected by the larval age (Emsen et al., 2003; Lashari, 2025). The optimal age is between one and two days, when larvae are still actively feeding and have not yet started to pupate, resulting in higher acceptance rates and morphological characteristics (Mahbobi et al., 2012; Ozbakir 2021; Vung et al., 2018; Ustadi et al., 2022; Rehman et al., 2024). These findings are corroborated by Zhong et al. (2024), who reported that queens raised with one-day-old larvae exhibited significantly superior morphological and physiological parameters compared to those reared from two-day-old and three-day-old larvae.

A recent study by Okuyan and Akyol (2018) demonstrated that queen bees can be reared from larvae up to 3 days old using the grafting method. However, subsequent research by Zhong et al. (2024) indicated that queen quality declines with increasing larval age, which is attributed to the reduced duration of the larval stage and limited exposure to royal jelly, essential for optimal queen development. Furthermore, older larvae spend a shorter duration in the larval stage and consume less royal jelly, which negatively impacts their development and results in queens of lower quality (Yi et al., 2020; Zhong et al. 2024).

The grafted larval age significantly influences the development of honey bee queens, affecting cephalic volatiles, hind leg size, and ovariole number (Dedej et al., 1998). Younger larvae grafted for queen rearing tend to develop more queen-like morphological features, while juvenile hormone (JH) application during larval stages enhances physiological pathways related to ovary development and metabolism (De Souza et al., 2019). Each day increase in grafted brood age decreases body weight, size of spermatheca, the number of ovarioles, and the spermatozoa number in inseminated queens (Woyke, 1971). Nurse bee feeding behavior plays a crucial role in controlling the growth of larvae and the number of ovarioles through food delivery manipulation at specific larval stages. Body mass is highly responsive to feeding through the first to fourth instar, while the number ovarioles is highly affected through the fifth instar (Wang et al., 2014). These results underscore the intricate interplay among larval age, nutrition, and hormonal factors in honey bee caste determination and reproductive development.

Research indicates that the age of grafted larvae significantly affects the quality and characteristics of queen honey bees. Queens reared from younger larvae (1-day-old) are generally heavier and larger than those from older larvae (2-day-old or 3-day-old) (Vung et al., 2018; Mahbobi et al., 2012; Ozbakir, 2021). These queens also exhibit superior reproductive potential, including earlier egg-laying, more stored spermatozoa, and longer lifespans (Vung et al., 2018). Morphological advantages of queens from younger larvae include larger spermathecae and higher ovariole numbers (Njeru et al. 2017; Ozbakir, 2021). The weight at emergence is positively correlated with various post-emergence weights and spermatheca size (Kahya et al., 2008). Additionally, queens from younger larvae positively influence colony growth, including increased worker and drone brood production (Vung et al., 2018). These findings suggest that using the youngest possible larvae for queen rearing can improve overall queen quality and colony fitness.

Based on the previous findings, it can be concluded that the larval age at grafting plays a critical role in producing a high-quality, and healthy queen, whereas does not affect the larval acceptance rate. Therefore, before the queen rearing artificially should be selected as the suitable age of larvae for grafting.

2.4. Effect of season on queen bee rearing:

The honey bee occupies a highly ecologically and economically significant position among the species of bee within the genus of *Apis* (Aktürk et al., 2023). Honey bees get their essential nutrients from two primary floral resources: pollen, which provides proteins, lipids, vitamins, and minerals; and nectar, which serves as their main source of carbohydrates (Vaudo et al., 2015; Nicolson, 2011; Roulston and Cane, 2000).

The season factor plays an important role in artificial queen-rearing, the success of the larval acceptance rate, and the production of healthy queens (Al-Fattah et al., 2021; Jagdale et al., 2021; Khan et al., 2021; Shakeel et al., 2020; Taha and Al-Kahtani, 2020; Helaly 2018; Brar et al., 2018; Cengiz et al., 2009; Şahinler and Kaftanoğlu 2005).

Honey bee colonies follow an annual cycle, their feeding dependent on the seasonally available flowering plants. In autumn, the production of brood decreases as the colony prepares for overwintering, whereas in spring, the development of colonies occurs through active brood rearing. However, the specifics of this cycle can vary depending on the geographical location of the colonies, particularly in overwintering activities (DeGrandi-Hoffman et al., 2021).

Climate change poses a significant threat to honeybee populations, which are crucial pollinators for agricultural ecosystems and global food security (Le Conte & Navajas, 2008; Reddy et al., 2012; Mahankuda and Tiwari, 2024). Honeybees are responsible for pollinating approximately 62-73% of cultivated crops worldwide (Reddy et al., 2012; Fikadu, 2019). Climate change may also lead to mismatches between bee activity and floral resources, impacting both plant pollination and bee food availability (Reddy et al., 2012).

Variations in patterns of temperature resulting from climate change can hinder the honey bee's ability to regulate the internal temperature of the hive, potentially affecting brood development and overall health of colony (Varshneya et al., 2007; Mishra et al., 2023).

Honey bee colonies exhibit seasonal variations in brood rearing and foraging activities (Chaand et al., 2017). In temperate regions during winter, unfavorable environmental conditions restrict foraging, compelling colonies to depend on honey reserves accumulated during the spring and summer months for survival (Villagomez, 2021).

To conserve resources, honey bee colonies typically stop brood raising in most of the winter (Nürnberger et al., 2018). However, by late winter, they begin actively raising the colony temperature and initiate brood production in preparation for spring, ensuring a sufficient workforce is available once foraging resumes (Seeley, 1995).

After the winter season, when food reserves in honey bee colonies are significantly depleted, bees become highly dependent on spring-blooming plants for nourishment (Villagomez, 2021). In contrast, many of these early-flowering plants depend on honey bees as well as other pollinators for successful pollination, creating a mutually beneficial relationship critical to plant and honey bee colony reproduction (Khalifa et al., 2021).

Environmental conditions play an important role in the success of queen rearing. Key factors include regulating temperature and humidity, the vitality and safety of queen cells, and the availability of food resources, such as natural nectar flow or supplemental feeding, to support the nurse colony (Taha 2014; Büchler et al., 2024).

In tropical and subtropical regions, where bee flora is continuously available, honeybees forage for nectar and pollen year-round (Taha, 2014). However, their foraging activity, particularly for pollen, is significantly affected by prevailing weather conditions and the availability of floral resources (Neupane & Thapa, 2005).

The influence of seasonal pollen nutrients and corresponding bee responses in honey bee colonies can be attributed to seasonal variations in the nutritional composition of pollen, as well as genetic factors linked to the specific queen lines utilized (DeGrandi-Hoffman et al., 2021). These combined factors play a crucial role in determining colony health, productivity, and brood development throughout the year.

The rearing season and queens' origin play a crucial role in determining several quality parameters of queen bees, including the pre-oviposition period, the volume and length of queen cells, the queen weight at emergence, acceptance, and spermatozoa stored number in the spermatheca (Guda et al. 2023; Canverdi et al., 2023; Koç & Karacaoğlu, 2011; Al-ghzawi & Zaitoun, 2008). Honey bee queens can be effectively raised from late March to late April, with the highest quality queens (Kumar et al., 2013; Shafey et al., 2021). During this optimal period, queens exhibit higher acceptance rates of grafted larvae, shorter pre-oviposition periods, and greater mating success (Koç & Karacaoğlu, 2004; Hussain et al., 2020). These findings indicate that both larval acceptance and queen emergence are strongly affected by the rearing season.

The acceptance rate of virgin queens by experimental honey bee colonies is significantly higher during the spring season, particularly when nectar flow is abundant (Moretto et al., 2004; Al-ghzawi & Zaitoun, 2008; Khattaby et al., 2018). This period provides optimal environmental and nutritional conditions for successful queen rearing. Therefore, spring is considered the most favorable time for producing high-quality virgin queens. (Al-ghzawi & Zaitoun 2008 Brar et al., 2018; Amiri et al., 2017; Hussain et al., 2020; DeGrandi-Hoffman et al., 2021; Ozbakir, 2021; Shafey et al., 2021; Chatha et al., 2022; Zhang, 2022; Aktürk et al., 2023; Canverdi et al., 2023; Holmes et al.2023; Büchler et al., 2024; Rehman et al., 2024).

Moreover, it can be concluded that the season factor plays an important role in artificial honey bee queen rearing and producing healthy queens. In addition, the climate factors, the nectar and pollen availability, and the presence of enemies of the honey bee play a very important in the limiting of foraging activity and honey bees' productivity. The colonies must be provided with pollen substitutes during periods of scarcity to maintain colony strength. Therefore, all the findings that are mentioned above highlight the critical importance of understanding and managing the season factor to reduce the influence of seasonal dearth periods on the colonies of the honey bee.

2.5. Effect of substrates (priming media) on queen bee rearing:

Queen rearing of honey bees is a crucial method in beekeeping that ensures high-quality production queens, which are essential for the bee colonies' health and productivity (Yu et al., 2022). The queen-rearing success is largely dependent upon the priming media or substrates utilized during the queen-rearing method (Khan et al. 2021).

Bee colonies are more resilient and productive overall when they have high-quality queens (Holmes et al., 2023). The process involves rearing queen larvae in controlled conditions, where the selection of priming media can significantly affect outcomes as that can either keep the appropriate humidity levels or act as nourishment for the larvae (Emsen et al., 2003; Contreras-Martinez et al., 2017; Rafique et al., 2019).

The priming media offers the necessary nutrients and generates a medium that is ideal for the development of queen larvae (Sharma, 2019). The effects of different substrates on queen rearing have been investigated, including sugar coconut water, syrup, honey solution, royal jelly, apple nectar, and artificial substrates (Ebadi and Gary, 1980; Contreras-Martinez et al., 2017; Rafique et al., 2019; Sharma et al., 2020; Khan et al., 2021; Kamboj et al., 2023). Adgaba et al., (2019) reported that honey bee colonies reared notably more queens in wet grafting conditions and queenless colonies, compared to those in dry grafting and queenright conditions. This suggests that both the presence of a queen and the grafting technique play a critical role in the success of queen rearing. Contreras-Martinez et al., (2017) demonstrated that using liquid substrates enhances acceptance rates of larvae and reared queens' quality. Among the tested substrates, apple nectar yielded the highest larval acceptance rate. Their findings suggest that liquid acidic substrates, particularly apple nectar, can significantly improve grafted larval acceptance and may serve as an effective medium to increase queen honey bee production during artificial rearing. Apple nectar may have exhibited a highly sustained influence compared to other priming media due to its superior hygroscopic features, attributed to its high sugar content and natural constituents like pectin found in the fruit pulp and cell walls (Berk, 2016). Additionally,

this effect might be due to the acidic nature of the substrates, which may enhance larval acceptance and queen quality during rearing (Contreras-Martinez et al., 2017).

Similarly, Khan et al. (2021), Sharma et al., (2020), and Rafique et al. (2019) found that using royal jelly as a priming media exhibits better morphological traits, and results in more healthy and strong queens compared to artificial substitutes. Researchers have investigated the remarkable physiological properties of royal jelly, highlighting its composition, which includes amino acids, proteins, lipids, vitamins, and sugars (Fratini et al., 2016; Maleszka, 2018; Ahmad et al., 2020).

Larval acceptance rates are a critical evaluation in the queen rearing of honey bees, where the choice of priming media can either increase or decrease larvae acceptance by worker bees. The queen bee quality, as indicated by her size, weight, and reproductive capacity, is influenced by the priming media used during rearing (Khan et al., 2021; Adgaba et al., 2019; Gene et al., 2005). In addition, Ebadi & Gary (1980) demonstrated that substrates of royal jelly with 10% honey yielded the highest larval acceptance rate during queen rearing, whereas the lowest acceptance was observed when royal jelly was mixed with 10% bee-stored pollen.

In conclusion, the selection of priming medium is an important factor in the success and quality of honey bee queen rearing. High-quality queens are pivotal for the health and productivity of bee colonies, and choosing suitable priming media can significantly influence larval acceptance rates and the morphometric traits of queens. Research has demonstrated that priming media such as apple nectar and royal jelly provide better results due to their nutrient content and advantageous properties, such as hygroscopicity and physiological activity. Therefore, improving priming media is critical for enhancing queen-rearing techniques and ultimately increasing colony resilience and productivity.

2.6. Effect of queen cell cup materials on queen bee rearing:

The queen cup materials are a critical factor affecting the success of queen bee rearing (Abrol et al., 2005). Different queen cup materials can influence acceptance rates of larvae, the queen's development, and the quality and health of the queens (Ustadi et al., 2022).

This literature review surveys different studies that have investigated the effect of queen cell cup materials on queen bee rearing. Currently, some research studies the effect of queen cup materials using materials with different components and proportions to determine the extent to which they affect the larval acceptance rate and queen emergence (Ebadi & Gary, 1980; Sharma et al., 2020; Lashari et al., 2022; Ustadi et al., 2022). Thus, it becomes crucial to determine whether differences occur in the grafted larval acceptance and the queen's emergence in various queen cup materials.

Several studies have conducted direct comparisons between different cup materials. Lashari et al., (2022) reported that using uncapping bee wax for preparing the queen cell cups can significantly increase the larval acceptance rates during queen bee rearing, but that using fresh comb bee wax does not significantly affect larval acceptance, while using the brown plastic queen cups decreases the larval acceptance. In contrast, Ebadi & Gary (1980); Sharma et al., (2020) recorded that the use of old brood comb beeswax as cup materials provided the highest larval acceptance and emergence of queen rate, while the lowest in the wax of artificial queen cups during the time that queen pheromone was existent and that pure paraffin wax cups were completely rejected by the bees according to Ebadi & Gary (1980), whereas in plastic cups according to Sharma et al., (2020). Additionally, Ustadi et al., (2022) demonstrated that the use of 50% *Apis cerana* wax + 50% *Apis dorsata* wax results in the highest acceptance rate, while the lowest in 100% *Apis cerana* wax and 100% *Apis dorsata* wax. Natural bee wax cups are traditionally used in queen rearing due to their similarity to the properties of natural bee wax. The bees' familiarity with the texture and scent of wax is thought to contribute to this preference.

Additionally, plastic cups are commonly used in commercial queen rearing due to their durability and ease of handling. Dhaliwal et al., (2017) found that plastic cups provide the maximum emergence rate of the queen by using the Doolittle method. However, some studies have shown that larval acceptance rates can be lower in plastic cups compared to wax cups (Ebadi & Gary 1980; Rafique et al., 2019; Sharma et al., 2020; Lashari et al., 2022). Therefore, the absence of a natural wax scent might be a factor in lower acceptance rates. Despite this, plastic cups are favored for their cost-effectiveness and reusability.

In conclusion, the choice of queen cup materials significantly impacts the queen rearing success. While natural wax cups are often preferred for their high larval acceptance rates and natural feel, plastic cups offer practical advantages in terms of durability and ease of handling. Ongoing researches continue to improve queen rearing practices by identifying queen cell cup materials that enhance both larval acceptance and developmental success. Overall, beekeepers must consider the specific needs of their colonies and the practical aspects of their queen rearing procedures when selecting queen cell cup materials. Future research should focus on further optimizing these materials to enhance larval acceptance and developmental success.

2.7. Effect of supplementary feeding on queen bee rearing:

Beekeeping is facing recurrent annual colony losses, largely due to the combined effects of multiple stressors, including pathogens and pesticide exposure (Smith et al., 2013). However, malnutrition has emerged as a growing concern, exacerbating the impacts of these other stressors through synergistic interactions (López-Uribe et al., 2020). Poor nutrition in colonies of honey bee is associated with several sub-lethal effects, including compromised immune function and heightened vulnerability to diseases and agrochemical exposure (Di Pasquale et al., 2013; DeGrandi-Hoffman and Chen, 2015; Gong and Diao, 2017; Koch et al., 2017; Tritschler et al., 2017; Dolezal and Toth, 2018; Vodovnik et al., 2021).

Nectar provides the primary source of carbohydrates for honey bees, whereas pollen is their exclusive source of lipids, proteins, and essential micronutrients (Brodschneider ang Crailsheim, 2010; Noordyke and Ellis, 2021).

However, a diverse range of floral sources is essential to fulfill the nutritional requirements of honey bees, as the nutrient composition of pollen varies significantly depending on the plant species (Roulston et al., 2000; Roulston and Cane, 2000; Ricigliano et al., 2022).

The queen bee rearing success is intricately tied to the nutritional status of the colony, with supplementary feeding playing an important role in enhancing a productivity and

quality of queen bees (Shadmehr., et al 2023). This literature review explores the impact of various supplementary feeding methods on queen bee rearing, drawing on recent and relevant studies.

However, supplementary feeding provides essential nutrients that may be lacking in the bees' natural foraging environment, especially during periods of dearth (Noordyke and Ellis, 2021; Lamontagne-Drolet et al., 2019; Mortensen et al., 2019; Mattila and Otis, 2006). This practice ensures that the colony has sufficient resources to rear high-quality queens. The nutrition of larvae, particularly those destined to become queens, is crucial for the overall health and development of the colony (Standifer et al., 1980; Taha, 2015).

Researchers have identified several types of supplementary feeds that benefit queen rearing, including protein supplements, sugar syrups, and pollen substitutes (Sultana et al., 2024; McMenamin et al., 2023; Khan and Ghramh, 2022; Ullah et al., 2021; Kumar et al., 2021; Dolasevic et al. 2020; Wijayati et al., 2019; Cengiz et al. 2019; Stevanovic et al., 2018; Puškadija et al., 2017; Mahfouz, 2016; Amro et al., 2016; Abd El-Wahab et al., 2016; Pande et al., 2015; Gemeda, 2014). Protein supplementation, commonly administered as pollen patties, is essential as it provides the amino acids necessary for larval development (Ullah et al., 2021; Jeannerod et al., 2022; Ricigliano et al., 2022). Studies have shown that colonies receiving protein supplements produce more robust queens with better reproductive capabilities (Sultana et al., 2024).

Sugar syrups are another common form of supplementary feeding (Pande et al., 2015). They provide the necessary carbohydrates for energy, enabling worker bees to continue their foraging and brood-rearing activities even when natural nectar sources are scarce (Thompson and Drew, 2022). This continuous supply of energy is crucial for maintaining the colony's strength and health during the queen rearing process (El Ghouizi et al., 2023).

The quality of the queens reared under supplementary feeding diets has been a focal point of several studies. For instance, Cengiz et al., (2019) demonstrated that colonies supplemented with protein had queens with significantly higher weights and better-developed ovaries compared to those that did not receive supplements. Similarly, research

by Sultana et al., (2024) found that supplementary feeding improved the overall health and longevity of queens, which are critical factors for colony productivity.

Additionally, the period of supplementary feeding is also playing a critical role. Feeding during the early spring can stimulate brood rearing and prepare the colony for the queen rearing season, whereas late-season feeding can help ensure that colonies have enough resources to support queen production during periods when natural forage is limited (Mattila and Otis, 2006).

In conclusion, supplementary feeding is a crucial practice in queen bee rearing, significantly impacting the quality and productivity of reared queens. By providing essential nutrients, beekeepers can enhance the health and reproductive capabilities of queens, thereby improving overall colony performance. Future research should continue to improve feeding practices, focusing on optimizing the composition and timing of supplements to maximize their benefits while minimizing potential disadvantages.

Chapter 3
Materials And Methods

CHAPTER - 3

MATERIALS AND METHODS

To achieve the objectives of the study, quantitative method was used, which described the subject phenomenon of the study and analyzed the data, to reveal significant differences among the different substrates, queen cell cup materials, and different seasons and the effects that occur.

3.1. Study area:

The experiments were carried out in between than February to April 2024 (Spring season) and in between than September to November 2024 (Autumn season) at the Apiculture Area, Department of Entomology, School of Agriculture, Lovely Professional University, located in Phagwara, Punjab, India (N 31° 13' 4" – E 75° 46' 10"). The experimental site is characterized by a semiarid climate under the Köppen climate classification, with an average annual rainfall of 816 mm and a temperature of 23.2 °C.

Fig. 3.1: Satellite image of study area and location

3.2. Effect of the season on the queen bee rearing:

The first experiment was conducted in the Spring season (between February and April 2024), while the second experiment was conducted in the Autumn season (between September and November 2024). The objective was to standardize the optimum season for

A. mellifera queen rearing. All treatments were applied consistently across both seasons, and the collected data were analyzed comparatively to assess seasonal variations.

3.3. Preparation and Selection of breeder (mother) colony:

Among the top chosen strong colonies, the one colony with gentleness, high reproduction, and excellent brood raising capacity was used as the breeder colony (Fig. 3.2). The suitable age larvae (12-24 hours old) for grafting were taken from chosen mother (breeder) colony (Fig. 3.3) (Ustadi et al., 2022). The colony was periodically maintained through the provision of sugar solution, pollen supplements, and by introducing capped hygienic worker brood from other colonies within the apiary to support colony strength and development (Gatoria et al., 2000).

Fig. 3.2: Breeder (mother) colony

Fig. 3.3: Suitable age larvae (12-24 hours old)

3.4. Preparation of queen cell builder colonies:

Three queen cell builder colonies maintained in Langstroth beehives were prepared via dequeening a honey bee colony (Fig. 3.4). Each colony was standardized to contain four frames of emerging capped brood (Fig. 3.5), two frames with honey and pollen (Fig. 3.6), three grafting frames (Fig. 3.7), and one feeder frame (Fig. 3.8). The adult bee population in each colony occupied both sides of six frames, indicating that all colonies were strong and healthy (Delaplane et al., 2013). The comb arrangement of the cell builder colony was described below as per Gatoria et al., 2004 and McKinley, 1963 with some modifications as F-HP-B-GF-B-GF-B-GF-B-HP (Alshogari and Raut, 2024 patent).

Where,

B - Emerging capped brood comb (Fig. 3.5).

HP- Honey and pollen comb (Fig. 3.6).

GF- Grafting frames (Fig. 3.7).

F- Feeder frame (Fig. 3.8).

Fig. 3.4: Three Langstroth beehives brood chambers

Fig. 3.5: Four frames of emerging capped brood

Fig. 3.6: Two frames with honey and pollen

Fig. 3.7: Three grafting frames

Fig. 3.8: One feeder frame

These conditions were preserved throughout the experiment by regularly transferring frames from strong, healthy colonies to ensure uniform colony strength. In addition, every colony received supplementary feeding consisting of 0.5 liter of 50 % sugar syrup (1 sugar: 1 water) (Fig. 3.9) and a protein supplement 50 grams (Fig. 3.10) consisting of low-fat

soybean flour 70%, pollen 20%, and honey 10% (Fig. 3.11) where that amount of honey we pour on the ingredients until get a cohesive patty. The protein supplement and the syrup were given weekly to each cell builder colony.

Fig. 3.9: Sugar syrup

Fig. 3.10: Protein supplement

Fig. 3.11: Honey, Pollen, Low-fat soybean flour.

3.5. Preparation of priming media (substrates):

Before grafting the larvae, approximately (5 µL) of the priming media was carefully dispensed into the base of each queen cup using a micropipette (Fig. 3.12). Six treatments were evaluated in total, comprising five different substrate types (priming media) and one control treatment (dry grafting without substrate). The treatments are apple juice (B Natural®, India) (S_1), commercial royal jelly (enriched with honey) (S_2), sugar syrup (1 sugar : 1 distilled water) (S_3), honey solution (1 honey : 1 distilled water) (S_4), mixture solution (apple juice, commercial royal jelly (enriched with honey), sugar syrup, honey solution) (1:1:1:1) (S_5), and control (dry grafting without substrate) (S_6) (Fig. 3.13). The royal jelly utilized in the study was commercial (enriched with honey) and was used at 100% undiluted. The sugar and honey were taken from the apiary, and the apple juice from a grocery store.

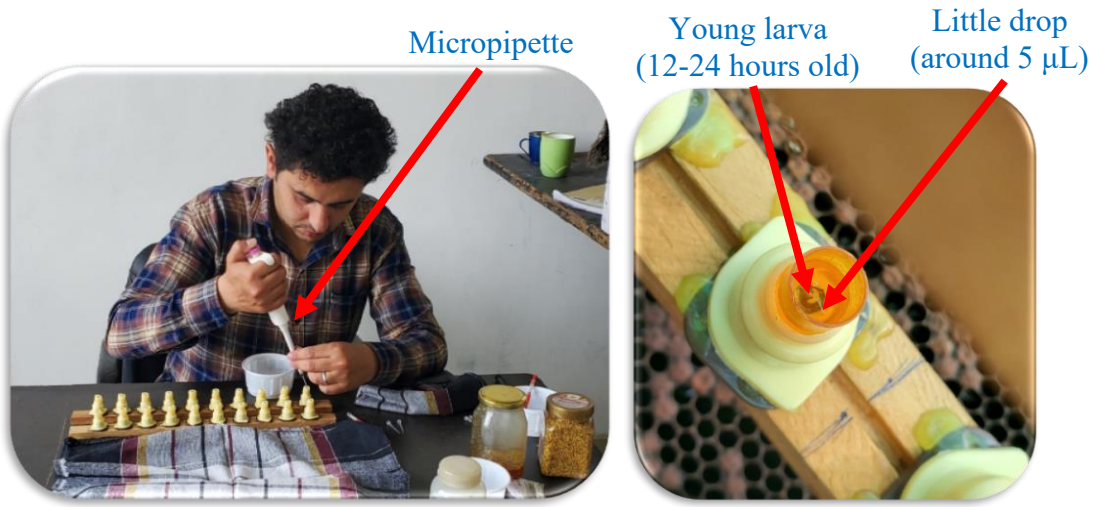


Fig. 3.12: Priming media placed into the base of each queen cup using a micropipette

Fig. 3.13: Different substrates (priming media)

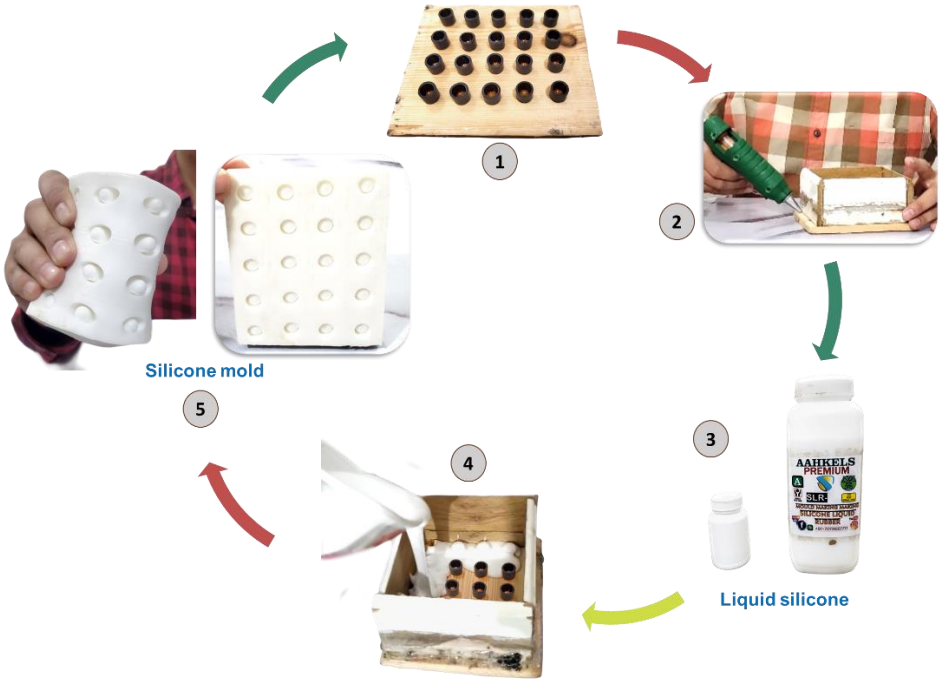
3.6. Preparation of queen cell cup materials:

Paraffin wax, bee wax, and plastic queen cups were used in this study (9 mm diameter and 10 mm height) (Fig. 3.14). Light-colored beeswax and paraffin wax were utilized to construct artificial queen cups (Fig. 3.14 A, B). Additionally, brown-colored plastic queen cups of the same diameter were purchased from an online market (Fig. 3.14 C). The silicone mold method was used, as a silicone mold was made by using liquid silicon to prepare bee wax and paraffin wax cups (Fig. 3.15 A, B). The cups were carefully removed from the silicone mold by gently twisting them using the thumb and index finger (Fig. 3.15 B, C). These cups were installed on a cell cup holder. The cell cup holder gets pushed on the cell bar holder. The cell bar holder was installed onto a wooden cell bar using nails onto a

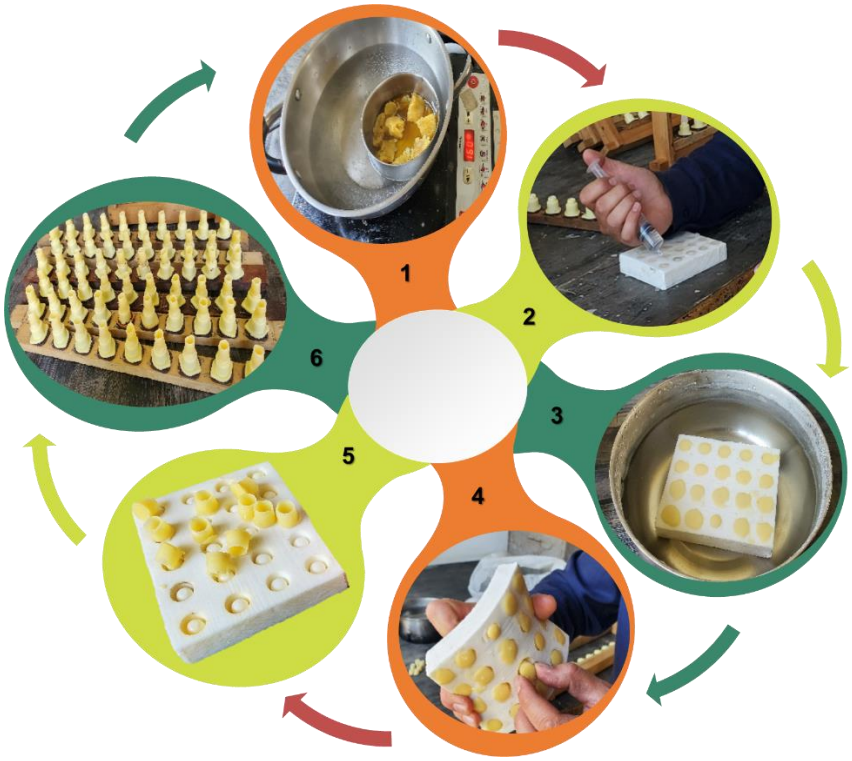
grafting frame. Each frame contained two removable wooden cell bars, with ten queen cell cups affixed to each bar (Fig. 3.16).

A. Bee wax B. Paraffin wax C. Plastic queen cups

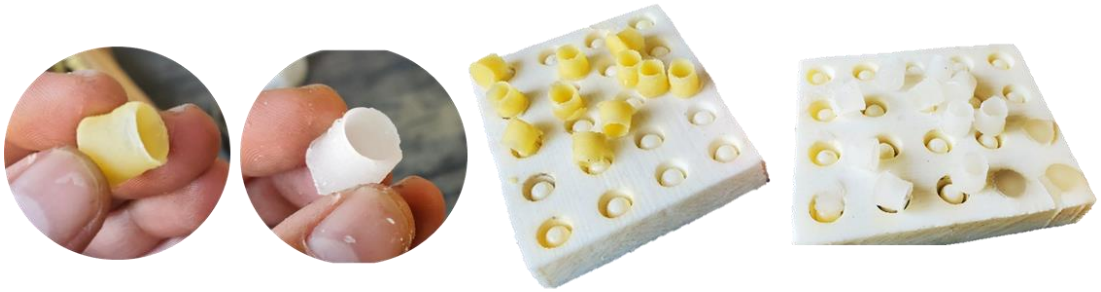
Fig. 3.14: Queen cup materials



A. Preparation of silicone mold using liquid silicone



B. Preparation of bee wax and paraffin wax queen cups using a silicone mold



C. Bee wax and paraffin wax queen cups ready for use

Fig. 3.15: Preparation of queen cell cups for artificial queen rearing using a silicone mold

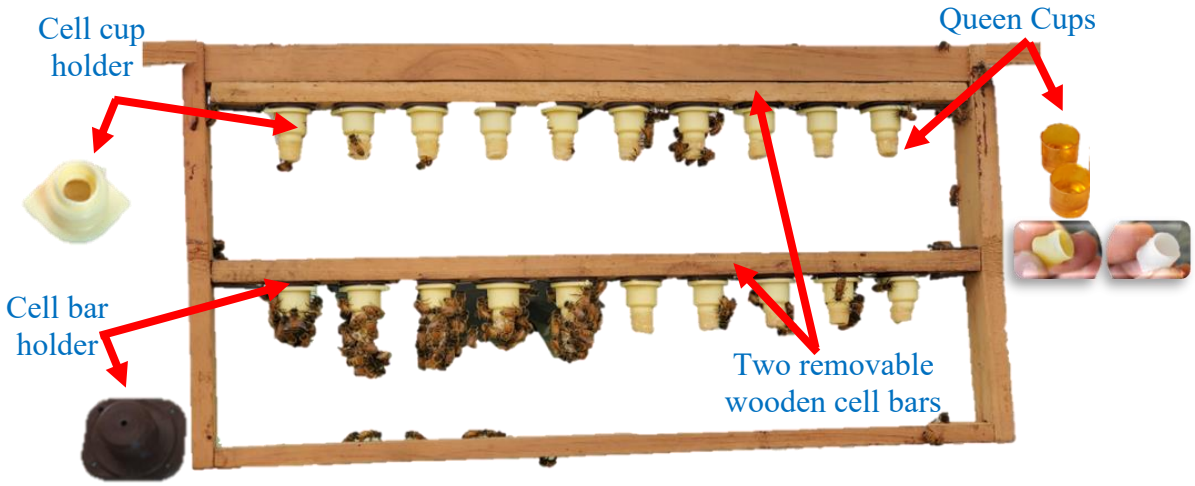


Fig. 3.16: Install the queen cups on the bars of the grafting frames

3.7. Grafting method:

Before one day of priming with the substrates, three grafting frames that have the queen cups were inserted into queenless cell builder colony for polishing (Fig. 3.17) (Khan et al., 2021; Ustadi et al., 2022; Büchler et al., 2024). After priming with the substrates (excluding the control treatment), each queen cup was grafted with a <24-hour-old larva collected from the comb of a breeder (mother) colony utilizing a plastic grafting tool. The larvae were transferred along with a small amount of royal jelly from the comb (Fig. 3.18). The same source colony was utilized for larval collection during each grafting session to ensure uniformity and minimize genetic and developmental variability. As groups of ten queen cups (replications) were utilized for each treatment during each grafting session, 60 queen cups were employed across all treatments (Fig. 3.19). These 60 queen cups were fixed onto three grafting frame bars and inserted into one cell builder colony. Grafting was performed in each of the three cell builder colonies, with 60 larvae grafted per colony, resulting in 180 grafted larvae (Fig. 3.19). Three queen cell cup materials were used in the experimental grafting (bee wax, paraffin wax, and plastic queen cups) (Fig. 3.20). Larval acceptance or rejection was verified and recorded three days after grafting (Fig. 3.21 A) (Ma et al., 2022; Khan et al., 2021), whereas the emerged queens number was recorded eleven to twelve days, after grafting (Fig. 3.21 C). The larvae acceptance and queen emergence percentage

of the grafted larvae were calculated with the data that were collected as per the following formula (Sharma et al., 2020):

$$Percentage\ of\ acceptance = \frac{Number\ of\ larvae\ accepted}{\text{Total number of larvae grafted}} \times 100$$

$$Percentage\ of\ emergence = \frac{Number\ of\ queens\ emerged}{\text{Total number of larvae grafted}} \times 100$$

Before polishing After polishing

Fig. 3.17: Queen cups polishing

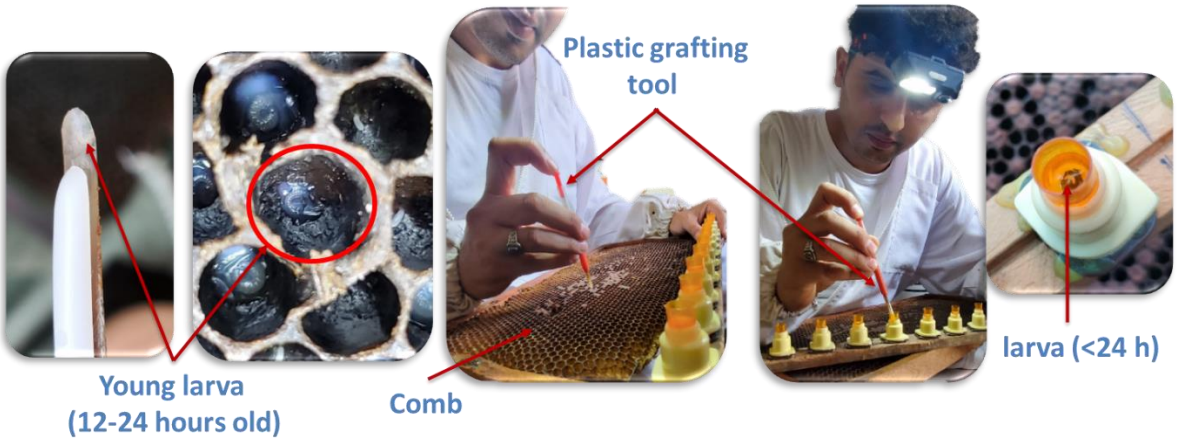


Fig. 3.18: Doolittle grafting method

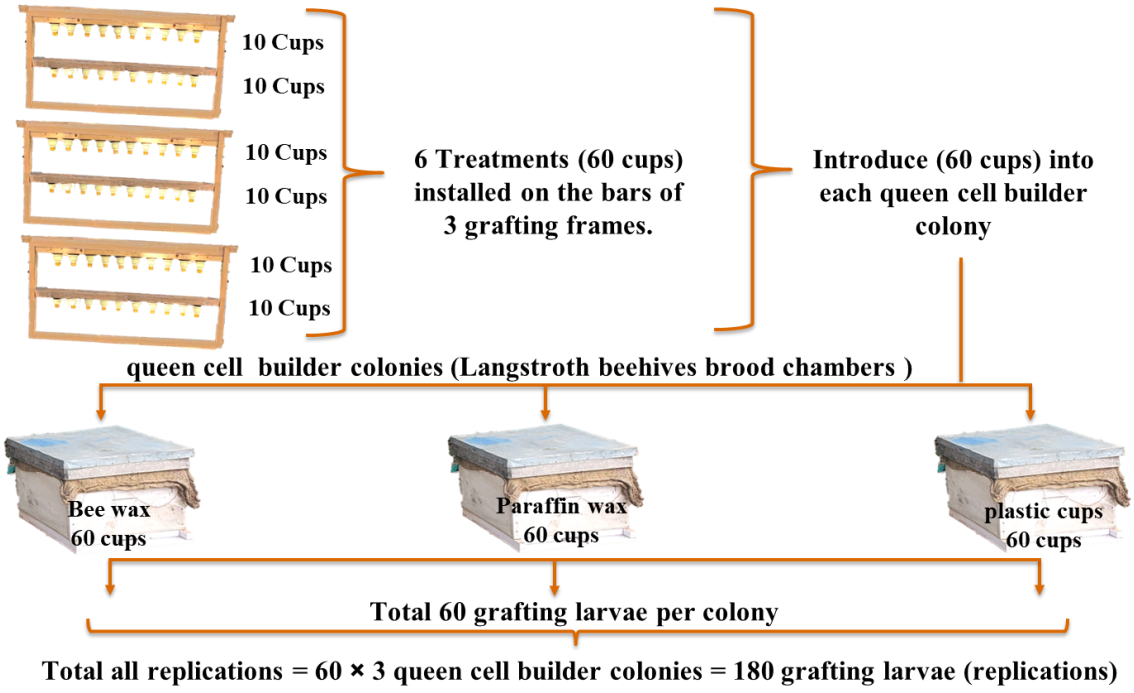


Fig. 3.19: Systematic representation of artificial queen rearing by grafting methods

Fig. 3.20: Queen cell cup materials

Fig. 3.21: Acceptance of larvae and emerged queens

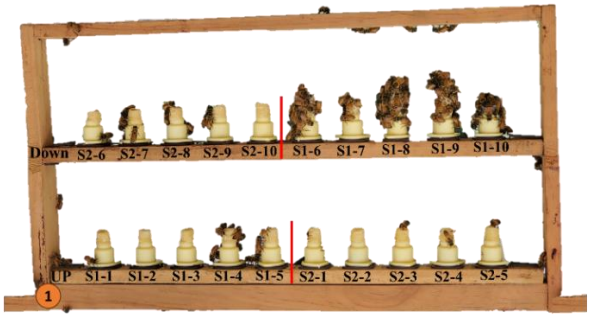
3.8. Effect of grafting folds on acceptance and emergence rate of the *A. mellifera* L. queens:

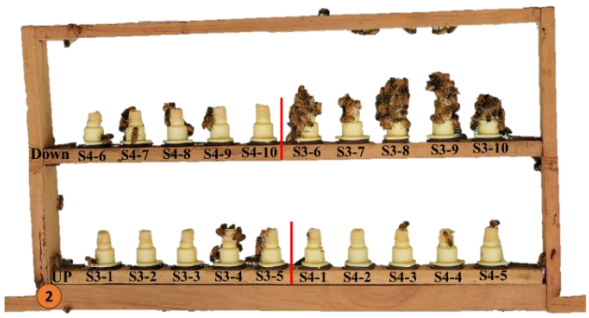
Grafting was carried out at three different folds during two separate seasons (Spring and Autumn 2024). Grafting fold was divided into three parts: (First grafting on 24/03/2024), (Second grafting on 27/03/2024), and (Third grafting on 25/04/2024) in the Spring season, whereas in the Autumn season was divided into three parts also: (First grafting on 04/10/2024), (Second grafting on 07/10/2024), and (Third grafting on 11/10/2024).

3.9. Effect of grafting bar level on acceptance and emergence rate of the *A. mellifera* L. queens:

The grafting frame was divided into two levels (Up and Down). The up-level bar and the down-level bar were split into two parts. Ten replications of each substrate were used five in each level (Fig. 3.22), (Fig. 3.23).

For example, in the case of apple juice (S_1) and commercial royal jelly enriched with honey (S_2), the up-level bar consisted of two parts "(S_{1-1}, S_{1-2}, S_{1-3}, S_{1-4}, S_{1-5}, and S_{2-1}, S_{2-2}, S_{2-3}, S_{2-4}, S_{2-5})" in the case of beeswax (Fig. 3.22-1), whereas in the plastic cups "(S_{1-6}, S_{1-7}, S_{1-8}, S_{1-9}, S_{1-10}, and S_{2-6}, S_{2-7}, S_{2-8}, S_{2-9}, S_{2-10})" (Fig. 3.23-1),. The down-level bar consisted of two parts "(S_{1-6}, S_{1-7}, S_{1-8}, S_{1-9}, S_{1-10}, and S_{2-6}, S_{2-7}, S_{2-8}, S_{2-9}, S_{2-10})" in the case of beeswax (Fig. 3.22-1), whereas in the plastic cups "(S_{1-1}, S_{1-2}, S_{1-3}, S_{1-4}, S_{1-5}, and S_{2-1}, S_{2-2}, S_{2-3}, S_{2-4}, S_{2-5}) (Fig. 3.23-1)".





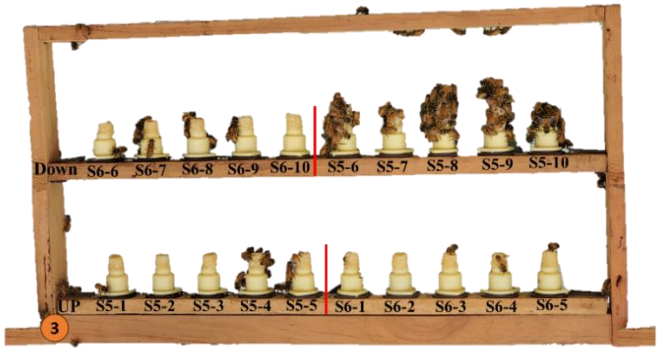


Fig. 3.22: Grafting frames in beeswax cups

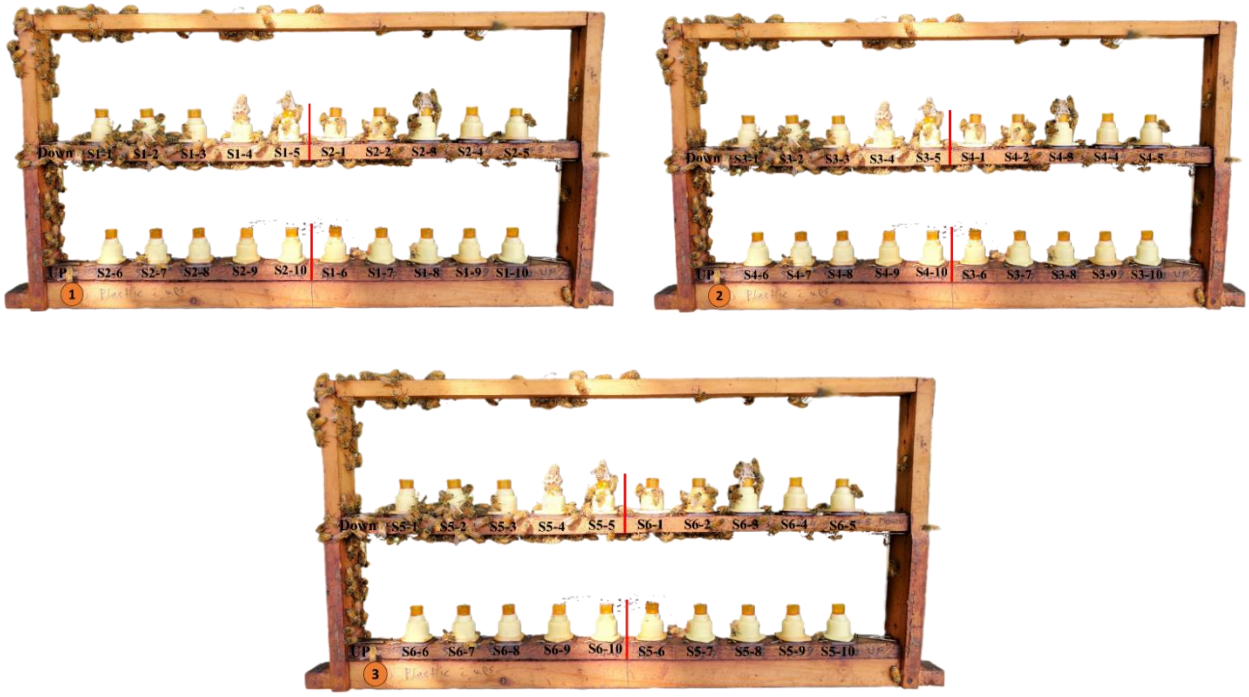
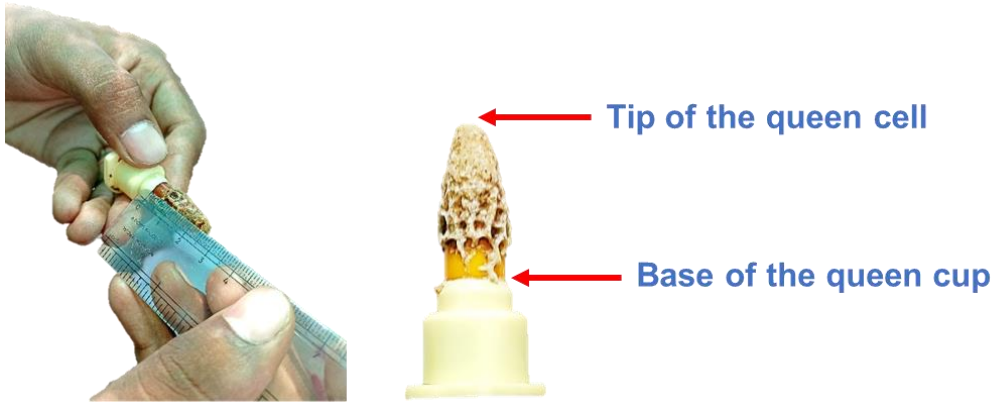


Fig. 3.23: Grafting frames in plastic cups

3.10. Morphometric measures of virgin queens:

Measuring was carried out immediately after the newly emerged queen bees, then the morphometric data were collected and recorded the length of sealed queen cell, the length of the virgin queen, and weight of virgin queen at emergence (Fig. 3.24 A, B, C) (Mattiello et al., 2022). The length of sealed queen cells was recorded by measuring the distance from the base of the queen cup to the tip of the queen cell (Fig. 3.24 A). The length of the virgin

queen was recorded by measuring the distance from the front of the head (excluding antennae) to the end of the abdomen (excluding the sting) using the ruler or digital vernier calliper (Mitutyo®) (Fig. 3.24 B, C). The weight of virgin queen at emergence was recorded using a weighing balance (Wensar®) with a maximum capacity of 1000g (Fig. 3.24 D). All the measurements were taken in millimeters and milligrams. The morphological images were captured using 200x lens (APL-MS002BK) fixed on the phone camera (Samsung Galaxy Note 10+). The samples were preserved in ethanol 70% and submitted to the Entomology lab, LPU.



A. Measure the length of the sealed queen cells

B. Measure the length of the virgin queen

C. Digital vernier calliper

D. Measure the weight of the virgin queen

Fig. 3.24: Morphometric measures of the virgin queens

3.11. Statistical analysis:

The data were analyzed utilizing a two-way analysis of variance to evaluate the effects of queen cup materials and different substrates. A significance level of 0.05 was applied for all statistical tests, and Duncan's Multiple Range Test (DMRT) was utilized to compare and rank the groups. The effects of substrates on acceptance rate and emergence rate were performed using ANOVA using SPSS software (version 22). The morphometric data within the queen cup materials (bee wax cups and plastic cups) were performed the t-test however, paraffin wax cups not considered due to non-preference by the bees.

Chapter 4
Results and Discussion

CHAPTER - 4

RESULTS AND DISCUSSION

Experimental findings and their interpretations on the topic **"Evaluation of Different Substrates and Queen Cup Materials on the Artificial Rearing of Honey Bee Queen (*Apis mellifera* Linnaeus) Using Grafting Method"** are given in this chapter concerning main and interaction effects. This chapter shows and discusses the impact of seasons, substrates, and queen cup materials on larvae acceptance rates, queen emergence rates, and morphometric traits of queens. Furthermore, a deliberate effort has been made to apply pertinent scientific arguments from the literature to show how the experimental results correspond to the associated cause and effect.

4.1. Effect of substrates and queen cup materials on artificial queen rearing during Spring season 2024:

4.1.1. Larval acceptance rates of queen *A. mellifera* L. during Spring season (2024):

The acceptance rate of queen larvae is an initial indicator of queen-rearing success. The data was recorded 72 hours after grafting, where the larval acceptance rates in different substrates and cup materials were recorded as statistically highest (90%) in mixture solution as priming media and plastic cups as queen cup materials compared to the various substrates. However, the statistically highest larval acceptance rates were observed in the treatment mixture solution (60%) followed by sugar syrup (60%), apple juice (50%), commercial royal jelly (enriched with honey) (50%), honey solution (10%), and control (dry grafting) (10%) respectively in bee wax cups. The statistical highest larval acceptance rates were observed in the plastic cups in the mixture solution (90%), followed by apple juice (50%), control (dry grafting) (50%), honey solution (40%), sugar syrup (30%), and commercial royal jelly (enriched with honey) (0%), respectively. However, the paraffin wax cups were not accepted at all (Table 4.1, Fig. 4.1, 4.2, 4.3).

Moreover, our findings revealed that the highest mean larval acceptance rates were significantly achieved in mixture solutions (50.00 %) and apple juice (33.33 %) used as the

priming media in bee wax cups and plastic cup materials. In contrast, no larvae were accepted in paraffin wax queen cups.

Therefore, the apple juice as a sole priming media will enhance the larval acceptance rates and finally, the queen emergence because of the acidic nature of the priming media (Contreras-Martinez et al., 2017). This demonstrates that substrates, such as the mixture solution and apple juice used as priming media and bees wax and plastic cups used as queen cups, contribute to increasing the larval acceptance rates and thus improving the artificial queen rearing success. Using different substrates for grafting larvae plays an important role in enhancing larval acceptance rates, as they influence the suitability of the grafting environment (Kamboj et al., 2023; Khan et al., 2021; Sharma et al., 2020; Rafique et al., 2019; Adgaba et al., 2019). This might be due to their nutrient content and advantageous properties, such as hygroscopicity and physiological activity.

Additionally, queen cup materials are also a critically important factor for artificial queen rearing. However, bee wax and plastic cups had a significantly higher mean acceptance rate of queen larvae observed in our research (40.00 %) and (43.33 %), respectively. Furthermore, no significant differences were found in beeswax and plastic queen cups in the mean larval acceptance rates. Therefore, the bee wax and plastic cups functioned equally well in the mixture solution and apple juice. A similar result was observed that the maximum acceptance rate of queen larvae obtained from bee wax cups (Ebadi & Gary 1980; Rafique et al., 2019; Sharma et al., 2020; Lashari et al., 2022; Kamboj et al., 2023). The research findings align with the observations reported by Dhaliwal et al. (2017), who recorded highest larval acceptance rates in plastic queen cups when employing the Doolittle grafting method. None of the larvae were accepted in paraffin wax queen cups in the different substrates were used. The possible clarification for this finding may be due to the smell of paraffin wax and its artificial properties. The findings were consistent with the research conducted by Ebadi & Gary in 1980.

Table 4.1: Effect of different substrates and queen cup materials on larval acceptance rates of queen *A. mellifera* L. during Spring season in 2024

Cup materials / **Substrates**	**Acceptance rates (%)**			**Mean**
	Beewax cups	Paraffin wax cups	Plastic cups	
Apple juice	50.0	0.0	50.0	33.33[ab]
Commercial royal jelly (enriched with honey)	50.0	0.0	0.0	16.66[b]
Sugar syrup	60.0	0.0	30.0	30.00[b]
Honey solution	10.0	0.0	40.0	16.66[b]
Mixture solution	60.0	0.0	90.0	50.00[a]
Control (dry grafting)	10.0	0.0	50.0	20.00[b]
Mean	40.00[a]	0.00[b]	43.33[a]	
Factors	**F_{value}**	**P_{value}**	**Df**	
Cup materials (C)	25.691	0.000**	2	
Substrates (S)	3.698	0.003**	5	
C x S	3.404	0.000**	10	

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test. Number of replications 10 in each substrate.

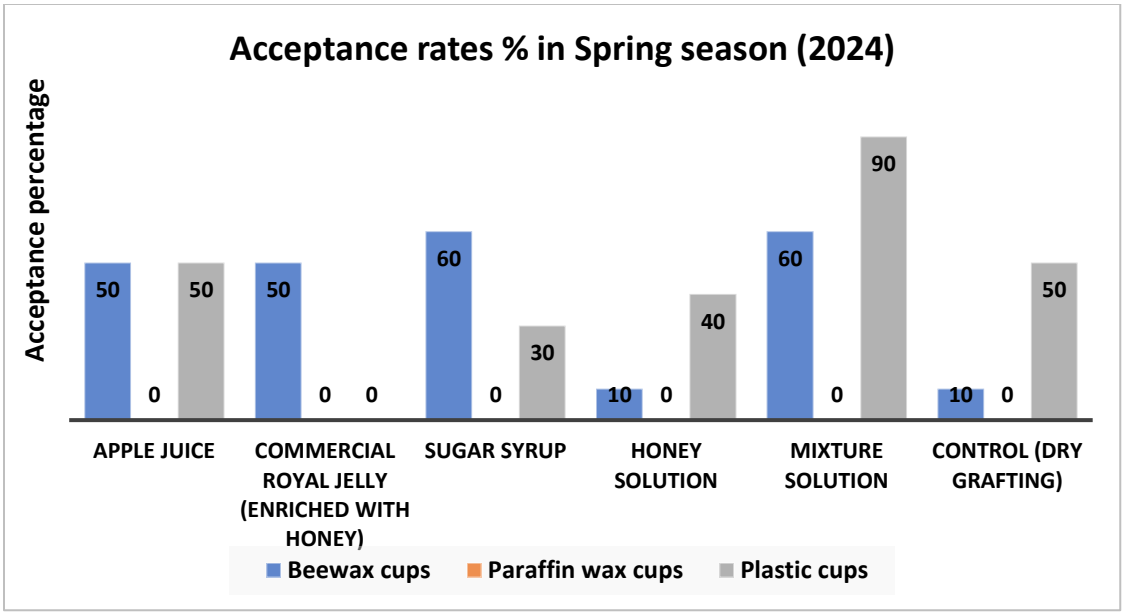


Fig. 4.1: Acceptance rates of queen larvae *A. mellifera* L. during Spring season (2024)

Fig. 4.2: Acceptance rates of queen larvae *A. mellifera* L during Spring season 2024 in Bee wax cups

Fig. 4.3: Acceptance rates of queen larvae *A. mellifera* L during Spring season 2024 in Plastic cups

4.1.2. Queen emergence rates of *A. mellifera* L. during Spring season (2024):

The emergence rate of the queens is a crucial indicator of their development and success of queen rearing. The data was recorded eleven to twelve days after grafting, where the emergence rate of queen in different substrates and queen cup materials was recorded as statistically highest (90%) in mixture solution as priming media and plastic cups as queen cup materials compared to the various substrates. However, the statistically maximum queen emergence rates were recorded in the apple juice (50%), followed by mixture solution (40%), commercial royal jelly (enriched with honey) (40%), sugar syrup (40%), control (dry grafting) (10%), and honey solution (0%) respectively in bee wax cups. The statistical highest emergence rates of queen were observed in plastic cups in the mixture solution (90%) followed by apple juice (50%), control (dry grafting) (50%), sugar syrup (30%), honey solution (30%), and commercial royal jelly (enriched with honey) (0%) respectively. However, the paraffin wax cups have no emerged at all (Table 4.2, Fig. 4.4, 4.5, 4.6).

Furthermore, our findings revealed that the highest mean emergence rate of the queens was significantly achieved in mixture solutions (43.33 %) and apple juice (33.33 %) used as the priming media in plastic cups. In contrast, no queens emerged in paraffin wax queen cups. On the other hand, significant differences were found between the beeswax and plastic cups in queen emergence rates, with the highest mean emergence rate of queens in plastic cups (41.66 %). The findings were consistent with the study carried out by Dhaliwal et al., in 2017. None of the queens emerged in paraffin wax queen cups in the different substrates that were used. The findings were consistent with the research conducted by Ebadi & Gary in 1980. Several studies have explored the impact of different queen cup materials and their compositions on the acceptance of larvae and the queen emergence rates. Researchers have investigated how varying components and proportions influence these parameters (Ebadi & Gary, 1980; Sharma et al., 2020; Lashari et al., 2022; Ustadi et al., 2022). The findings suggest that both the choice of substrate and queen cup material can play a crucial role in successful queen bee rearing.

Table 4.2: Effect of different substrates and queen cup materials on the queen emergence rates of *A. mellifera* L. after successful grafting during Spring season in 2024

Cup materials / **Substrates**	**Emergence rates (%)**			**Mean**
	Beewax cups	Paraffin wax cups	Plastic cups	
Apple juice	50.0	0.0	50.0	33.33^{ab}
Commercial royal jelly (enriched with honey)	40.0	0.0	0.0	13.33^{bc}
Sugar syrup	40.0	0.0	30.0	23.33^{bc}
Honey solution	0.0	0.0	30.0	10.00^{c}
Mixture solution	40.0	0.0	90.0	43.33^{a}
Control (dry grafting)	10.0	0.0	50.0	20.00^{bc}
Mean	30.0^{a}	0.00^{b}	41.66^{a}	
Factors	**F_{value}**	**P_{value}**	**Df**	

Cup materials (C)	21.296	0.000**	2	
Substrates (S)	3.628	0.004**	5	
C x S	3.167	0.001**	10	

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test. Number of replications 10 in each substrate.

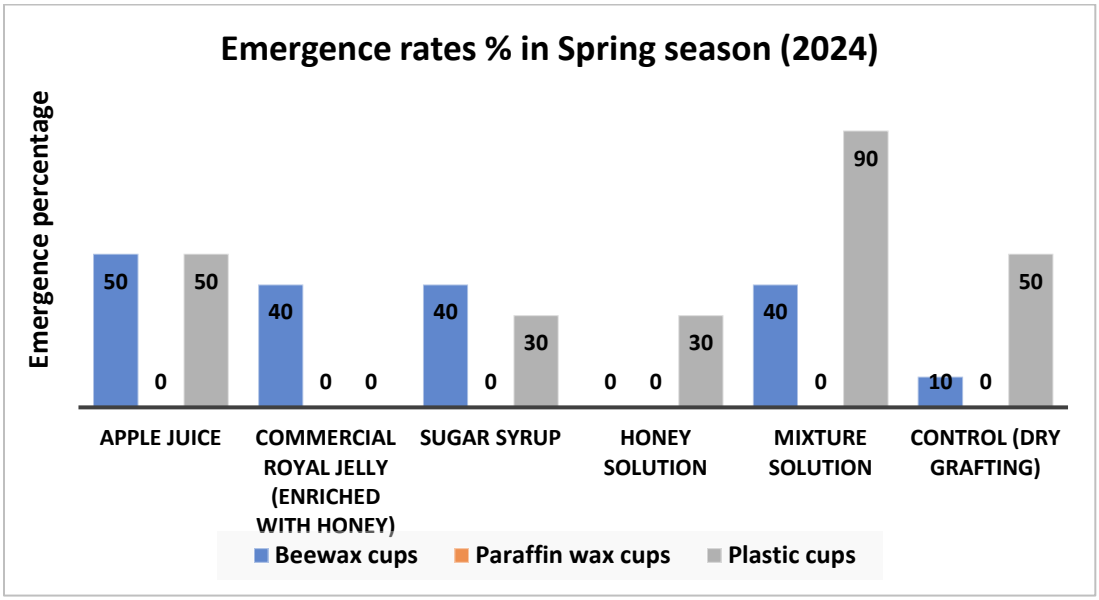


Fig. 4.4: Emergence rates of queen *A. mellifera* L. during Spring season (2024)

Fig. 4.5: Emergence rates of queen *A. mellifera* L during Spring season 2024 in Bee wax cups

Fig. 4.6: Emergence rates of queen *A. mellifera* L during Spring season 2024 in Plastic cups

4.2. Morphometric measures of artificial queen rearing during Spring season (2024):

The morphometric traits of the length of sealed queen cell, the length of the virgin queen, and virgin queen's weight at emergence are important morphometric criteria for quality queen selection (Alqarni et al., 2013 Masry et al., 2015; Metorima et al., 2015; Mattiello et al., 2022).

4.2.1. Morphometric measures of length of sealed queen cells during Spring season (2024):

The length of the sealed queen cells is one of the critical morphometric traits to determine the health and quality of queens. The data was recorded before the queen bee's emergence, where the length of sealed queen cells in different substrates and queen cup materials was reported as statistically highest (29.20 mm) in apple juice as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum length of sealed queen cells was recorded in the apple juice (29.20 mm), followed by commercial royal jelly (enriched with honey) (29 mm), sugar syrup (27.33 mm), mixture solution (26.75 mm), control (dry grafting) (26.50 mm), and honey solution (no emerged) respectively in bee wax cups. In the plastic cups, the statistically maximum length of sealed queen cells was recorded in the control (dry grafting) (20.60 mm), followed by the apple juice (20 mm), honey solution (19.75 mm), sugar syrup (19.67 mm), mixture solution (19.00 mm), and commercial royal jelly (enriched with honey) (no emerged) respectively. However, the paraffin wax cups were not accepted at all (Table 4.3, Fig. 4.7).

In other words, our findings demonstrated that the longest length of the sealed queen cells was significantly achieved in apple juice (29.20 mm) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. The reason for this observation might be attributed to the odor of paraffin wax and its synthetic characteristics. However, highly significant differences were recorded among the substrates, between the cup materials, and between the substrates and queen cup materials. This might be due to the acidic nature of the substrates (Contreras-Martinez et al., 2017), and the similar properties of bee wax to natural bee wax, which is made from honeybee comb.

Sealed queen cells length may be varied due to the artificial diet provided to the cell builder colony, which workers influence the wax production in the hive. Along with primary feed, i.e., royal jelly, which affects the larvae's development, nutritional requirement, and genetic variation. The morphometric variation in the queen's body parts

may significantly vary due to the availability of pollen quality, as it affects the hypopharyngeal gland in nurse bees, which are crucial for royal jelly production (Di Pasquale et al., 2016; Corby-Harris & Snyder, 2018; Abdel-Rahman et al., 2021).

Table 4.3: Effect of different substrates and queen cup materials on the morphometric traits of length of sealed queen cells during Spring season in 2024

Cup materials / **Substrates**	**Length of the sealed queen cells (mm± SE)**			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	29.20± 1.32	20.00± 0.32	6.782	0.000**
Commercial royal jelly (enriched with honey)	29.00 ± 1.22	NE	23.678	0.000**
Sugar syrup	27.33 ± 0.99	19.67 ± 0.67	6.429	0.000**
Honey solution	NE	19.75 ± 0.95	20.867	0.000**
Mixture solution	26.75 ± 0.85	19.00 ± 0.35	7.43	0.002**
Control (dry grafting)	26.50 ± 1.50	20.60 ± 0.60	3.652	0.119^{NS}
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	93.45		0.000**	
Cup materials (C)	178.13		0.000**	
S x C	159.32		0.000**	

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

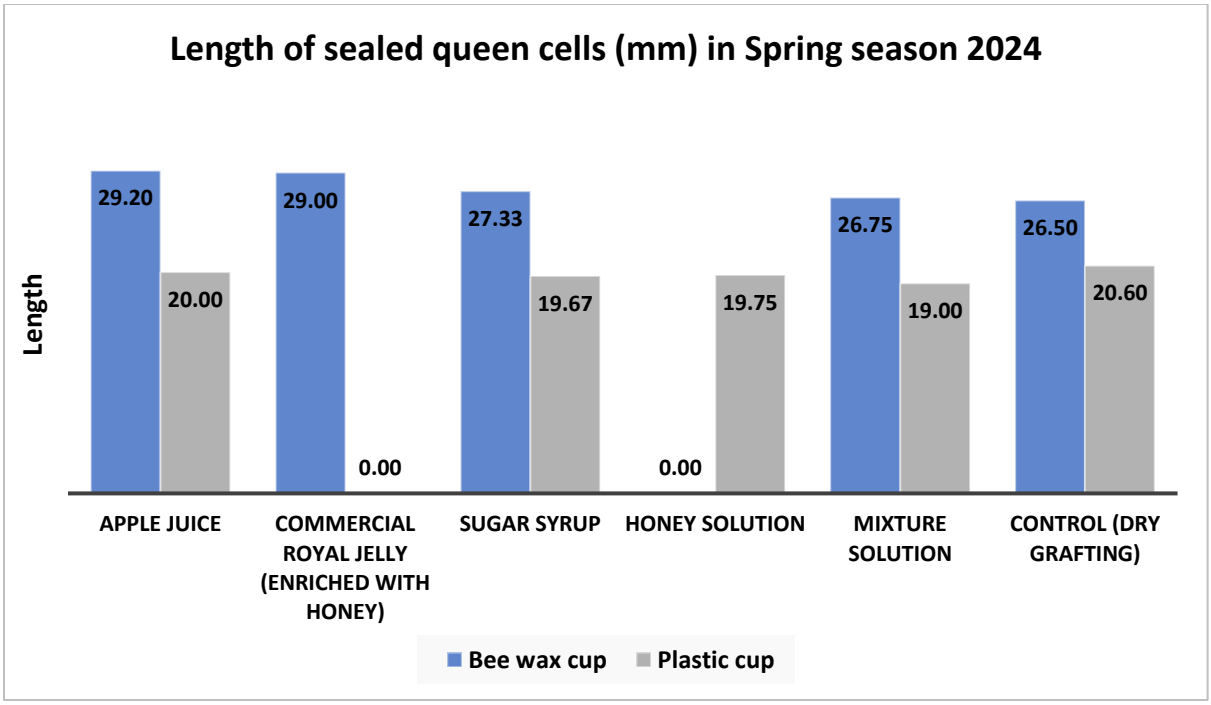


Fig. 4.7: Morphometric measures of length of sealed queen cells during Spring season 2024

4.2.2. Morphometric measures of length of virgin queen during Spring season 2024:

The length of the virgin queen is one of the critical morphometric traits to determine the health and quality of queens. The data was recorded after the queen bee's emergence, where the length of the virgin queen in different substrates and queen cup materials was recorded as statistically highest (18.95 mm) in apple juice as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum length of the virgin queen was recorded in apple juice (18.95 mm), followed by commercial royal jelly (enriched with honey) (18.83 mm), mixture solution (18.63 mm), control (dry grafting) (18.50 mm), sugar syrup (18.48 mm), and honey solution (no emerged) respectively in bee wax cups. In the plastic cups, the statistically maximum length of the virgin queen was recorded in the apple juice (17.45 mm) followed by mixture solution (17.38 mm), control (dry grafting) (17.09 mm), sugar syrup (16.18 mm), honey solution (16.05 mm), and commercial royal jelly (enriched with honey) (no emerged) respectively. However, the paraffin wax cups were not accepted at all (Table 4.4, Fig. 4.8).

Moreover, our findings revealed that the highest length of the virgin queen was significantly achieved in apple juice (18.95 mm) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. However, highly significant differences were recorded among the substrates, between the cup materials, and between the substrates and queen cup materials. Therefore, apple juice as the substrate affected the morphometric traits of queens because of the acidic nature of the substrate (Contreras-Martinez et al., 2017).

In addition, beeswax cups, as queen cup materials, performed significantly better than plastic cups due to their properties, which are similar to natural bee wax, which is made from honeybee comb. Similar findings are reported in the previous study (Rafique et al., 2019; Sharma et al., 2020; Lashari et al., 2022; Kamboj et al., 2023; Abou-Shaara et al., 2024).

Table 4.4: Effect of different substrates and queen cup materials on the morphometric traits of length of the virgin queen during Spring season in 2024

Cup materials / **Substrates**	**Length of the virgin queen (mm± SE)**			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	18.95 ± 0.12	17.45 ± 0.87	1.72	0.161^{NS}
Commercial royal jelly (enriched with honey)	18.83 ± 0.53	NE	35.23	0.000**
Sugar syrup	18.48 ± 0.47	16.18 ± 0.33	4.01	0.016*
Honey solution	NE	16.05 ± 1.13	14.23	0.000**
Mixture solution	18.63 ± 0.82	17.38 ± 1.14	1.09	0.338^{NS}
Control (dry grafting)	18.50 ± 0.05	17.09 ± 0.08	14.95	0.004**
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	132.59		0.000**	
Cup materials (C)	20.25		0.000**	

S x C	187.36	0.000**

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

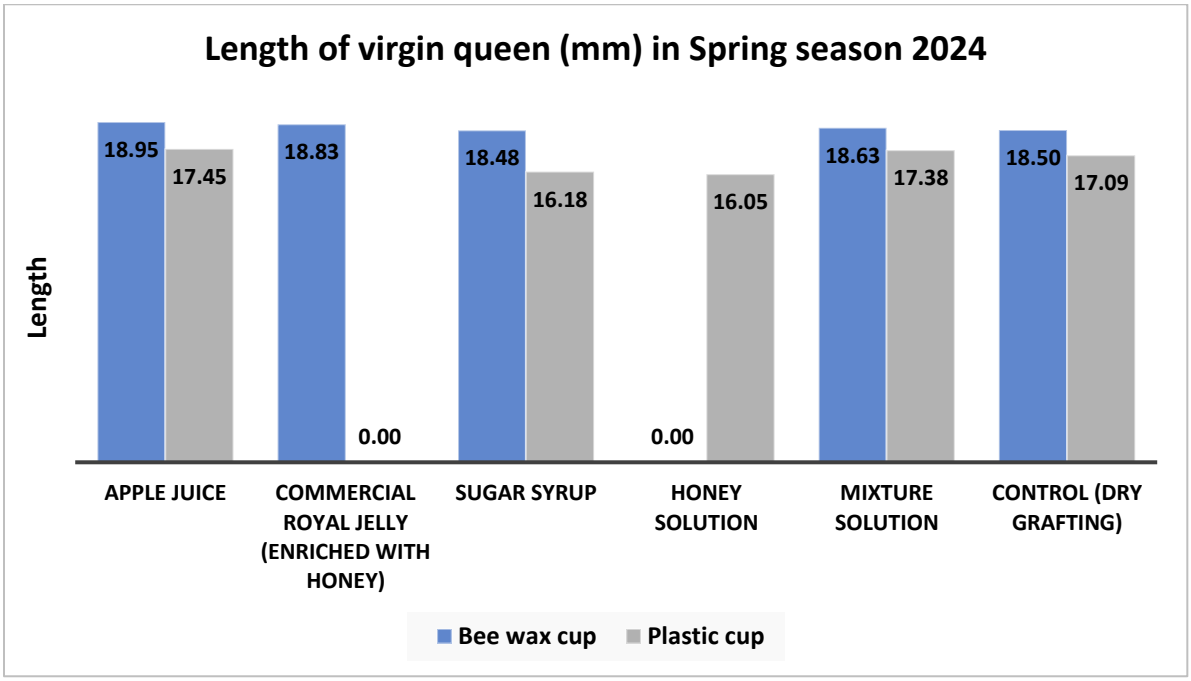


Fig. 4.8: Morphometric measures of length of the virgin queen during Spring season 2024

4.2.3. Morphometric measures of virgin queen's weight at emergence during Spring season 2024:

The virgin queen's weight at emergence plays a critical role in determining morphometric traits that determine the health and quality of queens. It is also a vital physiological characteristic for the queen's growth. The data was recorded immediately after the queen bee's emergence, where the virgin queen's weight at emergence in different substrates and queen cup materials was recorded as statistically highest (258 mg) in apple juice as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum virgin queen's weight at emergence was recorded in the apple juice (258 mg), followed by commercial royal jelly (enriched with honey) (255 mg), sugar syrup (232.5 mg), control (dry grafting) (220 mg), mixture solution (215 mg), and honey solution (not accepted) respectively in bee wax cups. In the plastic

cups, the statistically maximum virgin queen's weight at emergence was recorded in the apple juice (256 mg), followed by mixture solution (212.22 mg), sugar syrup (206.67 mg), control (dry grafting) (194 mg), honey solution (190 mg), and commercial royal jelly (enriched with honey) (not accepted) respectively. However, the paraffin wax cups were not accepted at all (Table 4.5, Fig. 4.9).

Furthermore, our findings revealed that the highest virgin queen's weight at emergence was significantly achieved in apple juice (258 mg) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. However, highly significant differences were reported among the substrates and between the substrates and queen cup materials, while no significant differences were found between the cup materials. Apple juice was noted to have a significant impact due to the acidic nature of the substrate (Contreras-Martinez et al., 2017).

The heavier weights of the virgin queen may vary due to the influence of the availability of honeybee floral resources, temperature, and relative humidity (Rehman et al., 2024; Zhang, 2022; Chatha et al., 2022; Abd Al-Fattah et al., 2021; Nürnberger et al., 2018; Doeke et al., 2015). A queen weight greater than 290 mg is considered a criterion for queen quality, and similar findings were reported in previous research (Akyol et al., 2008; Kahya et al., 2008; De Souza et al., 2013; Arslan et al., 2021; Kamboj et al., 2023; Arslan and Arslan, 2024). The weights of virgin queen at the emergence were categorized by quality into three quality groups i.e. light (<190 mg), medium (190–200 mg), and heavy (>210 mg) (Akyol et al., 2008; Hatjina et al., 2014; Arslan et al., 2021; Arslan and Arslan, 2024). The heavier queens are more successful than the lighter-weight queens when introduced into the hives to achieve a high level of social status (Tarpy and Mayer, 2009; De Souza et al., 2013). One study reported 79.23% success for heavy queens compared to lower rates for medium and light queens (Masry et al., 2015). Newly emerged honeybee queens experience gradual weight loss, losing 1-2 mg per day due to water loss (Harano et al., 2007; Skowronek et al., 2004), as the added weight quantitatively reduces the number of flights (Hayworth et al., 2009).

Table 4.5: Effect of different substrates and queen cup materials on the morphometric traits of virgin queen's weight at emergence during Spring season in 2024

Cup materials / **Substrates**	**Virgin queen's weight at emergence (mg± SE)**			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	258.0 ± 22.89	256.0 ± 18.60	0.68	0.948^{NS}
Commercial royal jelly (enriched with honey)	255.0 ± 36.63	NE	6.962	0.000**
Sugar syrup	232.5 ± 16.52	206.67 ± 17.64	1.069	0.337^{NS}
Honey solution	0.00 ± 0.00	190.0 ± 5.77	32.909	0.000**
Mixture solution	215.0 ± 25.33	212.22 ± 13.41	0.097	0.927^{NS}
Control (dry grafting)	220.0 ± 20.00	194.0 ± 14.35	1.056	0.395^{NS}
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	19.07		0.000**	
Cup materials (C)	2.58		0.118^{NS}	
S x C	22.99		0.000**	

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

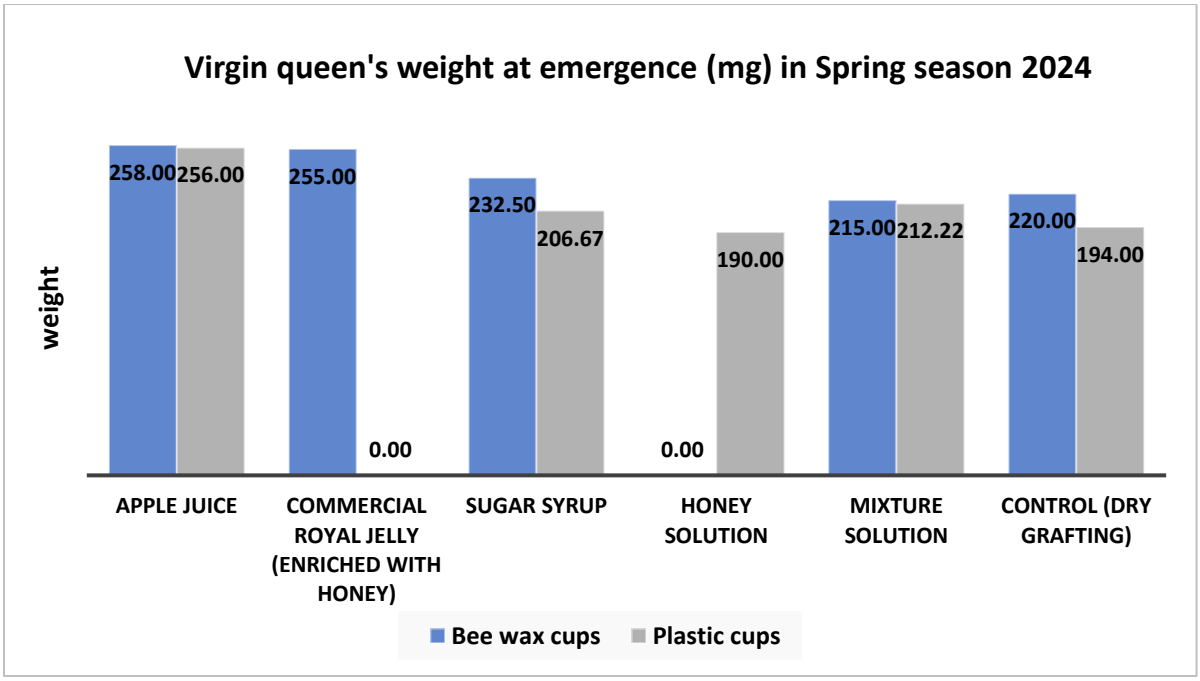


Fig. 4.9: Morphometric measures of virgin queen's weight at emergence during Spring season 2024

4.3. Effect of substrates and queen cup materials on artificial queen rearing during Autumn season 2024:

4.3.1. Larval acceptance rates of queen *A. mellifera* L. during Autumn season (2024):

The acceptance rate of queen larvae is an initial indicator of queen-rearing success. The data was recorded 72 hours after grafting, where the larval acceptance rates in different substrates and queen cup materials were recorded as statistically highest (80%) in control (dry grafting) as priming media and plastic cups as queen cup materials compared to the various substrates. However, the statistically highest larval acceptance rates were observed in the control (dry grafting) (50%), followed by commercial royal jelly (enriched with honey) (30%), apple juice (20%), mixture solution (20%), sugar syrup (20%), and honey solution (0%) respectively in bee wax cups. The statistical highest larval acceptance rates were observed in the plastic cups in the control (dry grafting) (80%), followed by apple juice (30%), mixture solution (30), commercial royal jelly (enriched with honey) (20%), sugar syrup (20%), and honey solution (10%) respectively. However, the paraffin wax cups were not accepted at all (Table 4.6, Fig. 4.10, 4.11, 4.12).

Moreover, our findings revealed that the highest mean larval acceptance rates were significantly achieved in control (dry grafting) (43.33 %) used as the priming media in bee wax cups and plastic cup materials. In contrast, no larvae were accepted in paraffin wax queen cups. However, plastic cups had a significantly higher mean acceptance rate of queen larvae observed in our research (31.67 %). Furthermore, significant differences were found between the beeswax and plastic cups in the mean larval acceptance rates (23.33 %), (31.67 %), respectively.

The findings of this research align with the observations reported by Dhaliwal et al. (2017), who recorded the highest larval acceptance rates in plastic queen cups when employing the Doolittle grafting method. None of the larvae were accepted in paraffin wax queen cups in the different substrates were used. The findings were consistent with the research conducted by Ebadi & Gary in 1980.

Table 4.6: Effect of different substrates and queen cup materials on larval acceptance rates of queen larvae *A. mellifera* L. during Autumn season in 2024

Cup materials / **Substrates**	**Acceptance rates (%)**			**Mean**
	Beewax cups	Paraffin wax cups	Plastic cups	
Apple juice	20.0	0.0	30.0	16.67^{b}
Commercial royal jelly (enriched with honey)	30.0	0.0	20.0	16.67^{b}
Sugar syrup	20.0	0.0	20.0	13.33^{b}
Honey solution	0.0	0.0	10.0	3.33^{b}
Mixture solution	20.0	0.0	30.0	16.67^{b}
Control (dry grafting)	50.0	0.0	80.0	43.33^{a}
Mean	23.33^{a}	0.00^{b}	31.67^{a}	
Factors	**F_{value}**	**P_{value}**	**Df**	
Cup materials (C)	13.279	0.000**	2	

Substrates (S)	4.353	0.001**	5	
C x S	1.451	0.163^{NS}	10	

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test. Number of replications 10 in each substrate.

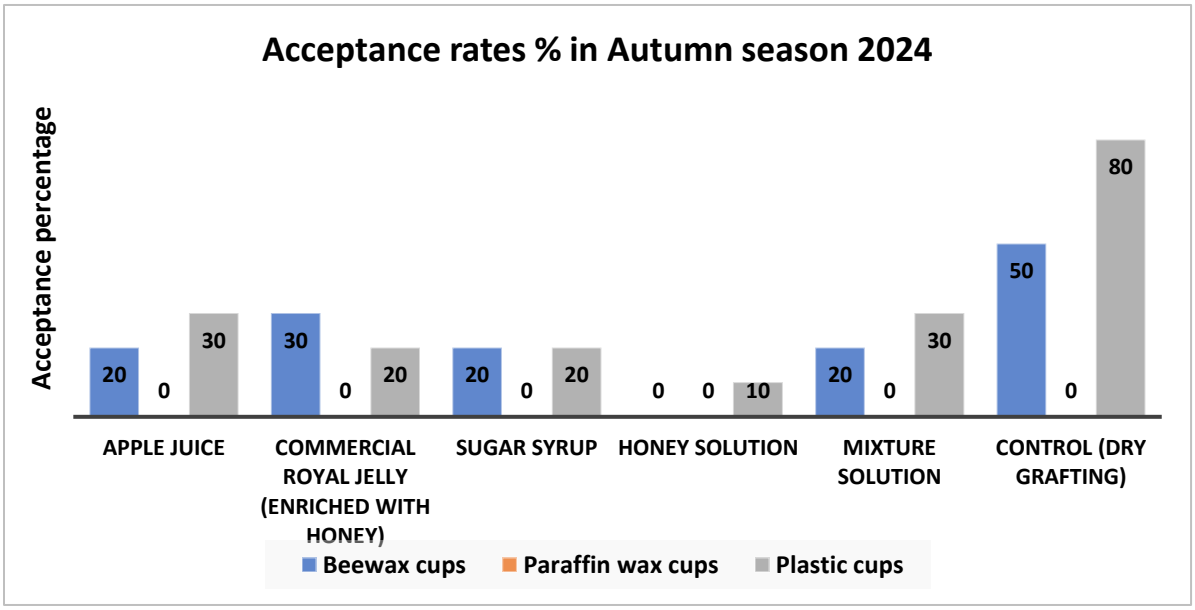


Fig. 4.10: Acceptance rates of queen larvae *A. mellifera* L. during Autumn season (2024)

Fig. 4.11: Acceptance rates of queen larvae *A. mellifera* L during Autumn season 2024 in Bee wax cups

Fig. 4.12: Acceptance rates of queen larvae *A. mellifera* L during Autumn season 2024 in Plastic cups

4.3.2. Queen emergence rates of *A. mellifera* L. during Autumn season (2024):

The emergence rate of the queens is a crucial indicator of their development and success of queen rearing. The data was recorded eleven to twelve days after grafting, where the emergence rate of queen in different substrates and queen cup materials was recorded as statistically highest (40%) in control (dry grafting) as priming media and bee wax cups and plastic cups as queen cup materials compared to the various substrates. However, the statistically maximum queen emergence rate was recorded in the control (dry grafting) (40%) followed by commercial royal jelly (enriched with honey) (30%), apple juice (20%), mixture solution (20%), sugar syrup (20%), honey solution (0%) respectively in bee wax cups. The statistical highest emergence of queen rates was observed in plastic cups in the control (dry grafting) (40%) followed by mixture solution (30%), apple juice (20%), commercial royal jelly (enriched with honey) (20%), sugar syrup (20%), and honey solution (0%) respectively. However, the paraffin wax cups were not accepted at all (Table 4.7, Fig. 4.13, 4.14, 4.15).

Furthermore, our findings revealed that the highest mean emergence rate of the queens was significantly achieved in control (dry grafting) (26.67 %) used as the priming media in bee wax and plastic cups. In contrast, no queens emerged in paraffin wax queen cups. On the other hand, no significant differences were recorded in the mean queen emergence rates between the bee wax and plastic cups. None of the queens emerged in paraffin wax queen cups in the different substrates that were used. The findings were consistent with the research conducted by Ebadi & Gary in 1980. In addition, highly significant differences were observed among the queen cup materials, while there were no significant differences among the substrates and between the queen cup materials and the substrates in the emergence rate of queens.

Table 4.7: Effect of different substrates and queen cup materials on the queen emergence rates of *A. mellifera* L. after successful grafting during Autumn season in 2024

Cup materials / **Substrates**	**Emergence rates (%)**			**Mean**
	Beewax cups	Paraffin wax cups	Plastic cups	
Apple juice	20.0	0.0	20.0	13.33^{ab}
Commercial royal jelly (enriched with honey)	30.0	0.0	20.0	16.67^{ab}
Sugar syrup	20.0	0.0	20.0	13.33^{ab}
Honey solution	0.0	0.0	0.0	0.00^{b}
Mixture solution	20.0	0.0	30.0	16.67^{ab}
Control (dry grafting)	40.0	0.0	40.0	26.67^{a}
Mean	21.67^{a}	0.00^{b}	21.67^{a}	
Factors	**F_{value}**	**P_{value}**	**Df**	
Cup materials (C)	8.224	0.000**	2	
Substrates (S)	1.947	0.090^{NS}	5	
C x S	0.574	0.833^{NS}	10	

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test. Number of replications 10 in each substrate.

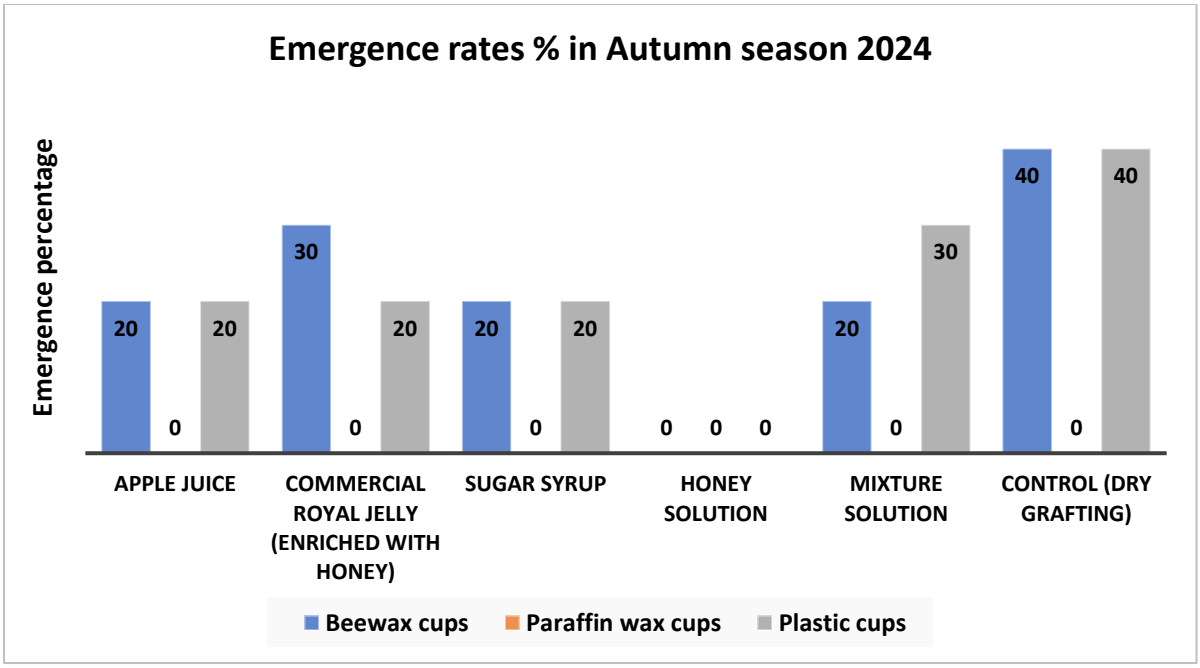


Fig. 4.13: Emergence rates of queen *A. mellifera* L. during Autumn season (2024)

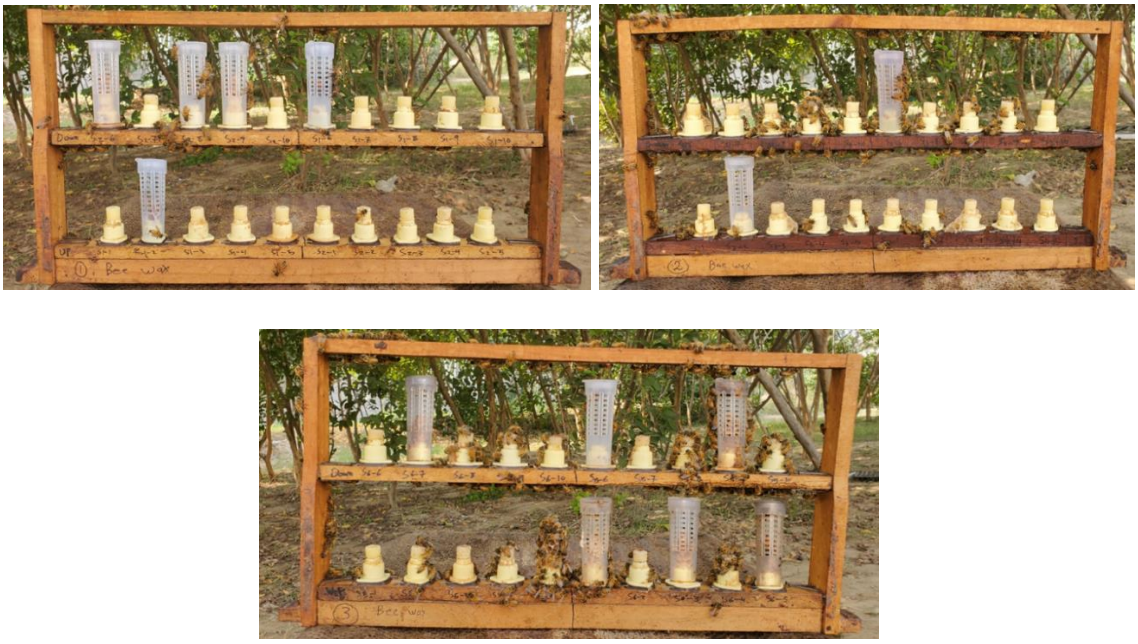


Fig. 4.14: Emergence rates of queen *A. mellifera* L during Autumn season 2024 in Bee wax cups

Fig. 4.15: Emergence rates of queen *A. mellifera* L during Autumn season 2024 in Plastic cups

4.4. Morphometric measures of artificial queen rearing during Autumn season (2024):

The morphometric traits of the length of sealed queen cell, the length of virgin queen, and the virgin queen's weight at emergence are important morphometric criteria for quality queen selection (Alqarni et al., 2013; Masry et al., 2015; Metorima et al., 2015; Mattiello et al., 2022).

4.4.1. Morphometric measures of length of sealed queen cells during Autumn season (2024):

The length of the sealed queen cells is one of the critical morphometric traits to determine the health and quality of queens. The data was recorded before the queen bee's emergence, where the length of sealed queen cells in different substrates and queen cup materials was recorded as statistically highest (30.44 mm) in apple juice as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum length of sealed queen cells was recorded in the apple juice (30.44

mm), followed by sugar syrup (30.32 mm), mixture solution (30.03 mm), commercial royal jelly (enriched with honey) (28.13 mm), control (dry grafting) (28.13 mm), and honey solution (no emerged) respectively in bee wax cups. In the plastic cups, the statistically maximum length of sealed queen cells was recorded in the control (dry grafting) (19.41 mm), followed by the mixture solution (19.16 mm), sugar syrup (17.66 mm), apple juice (16.75 mm), commercial royal jelly (enriched with honey) (16.56 mm), and honey solution (no emerged) respectively. However, the paraffin wax cups were not accepted at all (Table 4.8, Fig. 4.16).

In other words, our findings demonstrated that the longest length of the sealed queen cells was significantly achieved in apple juice (30.44 mm) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. In contrast, no queens emerged when using the honey solution at all. However, highly significant differences were reported among the substrates, between the cup materials, and between the substrates and queen cup materials. This might be due to the acidic nature of the substrates (Contreras-Martinez et al., 2017), and the similar properties of bee wax to natural bee wax, which is made from honeybee comb.

Sealed queen cell length may be varied due to the artificial diet provided to the cell builder colony, which workers influence the wax production in the hive. Along with primary feed, i.e., royal jelly, which affects the larvae's development, nutritional requirement, and genetic variation. The morphometric variation in the queen's body parts may significantly vary due to the availability of pollen quality, as it affects the hypopharyngeal gland in nurse bees, which are crucial for royal jelly production (Di Pasquale et al., 2016; Corby-Harris & Snyder, 2018; Abdel-Rahman et al., 2021).

Table 4.8: Effect of different substrates and queen cup materials on the morphometric traits of length of sealed queen cell during Autumn season in 2024

Cup materials / Substrates	Length of the sealed queen cells (mm± SE)			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	30.44±0.56	16.75±0.15	23.350	0.002**
Commercial royal jelly (enriched with honey)	28.13±0.63	16.56±0.17	17.79	0.002**
Sugar syrup	30.32±0.67	17.66±0.94	10.944	0.008**
Honey solution	NE	NE	NE	NE
Mixture solution	30.03±0.70	19.16±0.422	13.30	0.009**
Control (dry grafting)	28.13±0.60	19.41±0.49	11.165	0.000**
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	338.164		0.000**	
Cup materials (C)	546.222		0.000**	
S x C	21.433		0.000**	

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

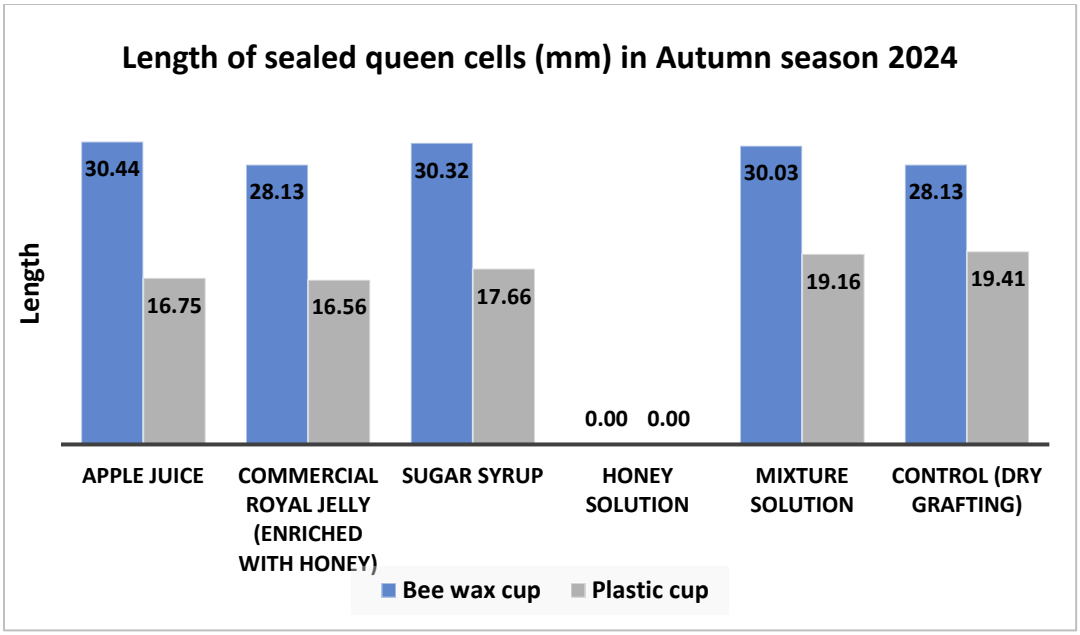


Fig. 4.16: Morphometric measures of length of sealed queen cells during Autumn season (2024)

4.4.2. Morphometric measures of length of virgin queen during Autumn season (2024):

The length of the virgin queen is one of the critical morphometric traits to determine the health and quality of queens. The data was recorded after the queen bee's emergence, where the length of virgin queen in different substrates and queen cup materials was recorded as statistically highest (18.88 mm) in sugar syrup as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum length of virgin queen was recorded in sugar syrup (18.88 mm), followed by apple juice (18.45 mm), commercial royal jelly (enriched with honey) (17.73 mm), mixture solution (17.65 mm), control (dry grafting) (17.63 mm), and honey solution (no emerged) respectively in bee wax cups. In the plastic cups, the statistically maximum length of the virgin queen was recorded in the mixture solution (17.78 mm), followed by control (dry grafting) (17.41 mm), commercial royal jelly (enriched with honey) (17.16 mm), sugar syrup (17.16 mm), apple juice (16.63 mm), and honey solution (no emerged) respectively. However, the paraffin wax cups were not accepted at all (Table 4.9, Fig. 4.17).

Moreover, our findings revealed that the highest length of the virgin queen was significantly achieved in the sugar syrup (18.88 mm) and apple juice (18.45 mm) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. In contrast, no queens emerged when using the honey solution at all. However, there were highly significant differences among the substrates and between the substrates and queen cup materials, while there were significant differences between the cup materials. Therefore, apple juice as the substrate affected the morphometric traits of queens because of the acidic nature of the substrate (Contreras-Martinez et al., 2017).

In addition, beeswax cups, as queen cup materials, performed significantly better than plastic cups due to their properties, which are similar to natural bee wax, which is made from honeybee comb. Similar findings are reported in the previous study (Rafique et al., 2019; Sharma et al., 2020; Lashari et al., 2022; Kamboj et al. 2023; Abou-Shaara et al., 2024).

Table 4.9: Effect of different substrates and queen cup materials on the morphometric traits of length of virgin queen during Autumn season in 2024

Cup materials / **Substrates**	**Length of virgin queen (mm± SE)**			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	18.45±0.97	16.63±0.42	1.714	0.229^{NS}
Commercial royal jelly (enriched with honey)	17.73±0.50	17.16±0.07	1.130	0.372^{NS}
Sugar syrup	18.88±1.02	17.16±0.13	1.664	0.238^{NS}
Honey solution	NE	NE	NE	NE
Mixture solution	17.65±0.02	17.78±0.22	0.551	0.636^{NS}
Control (dry grafting)	17.63±0.19	17.41±0.38	0.531	0.614^{NS}
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	391.802		0.000**	

Cup materials (C)	5.451	0.031*
S x C	1.149	0.000**

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

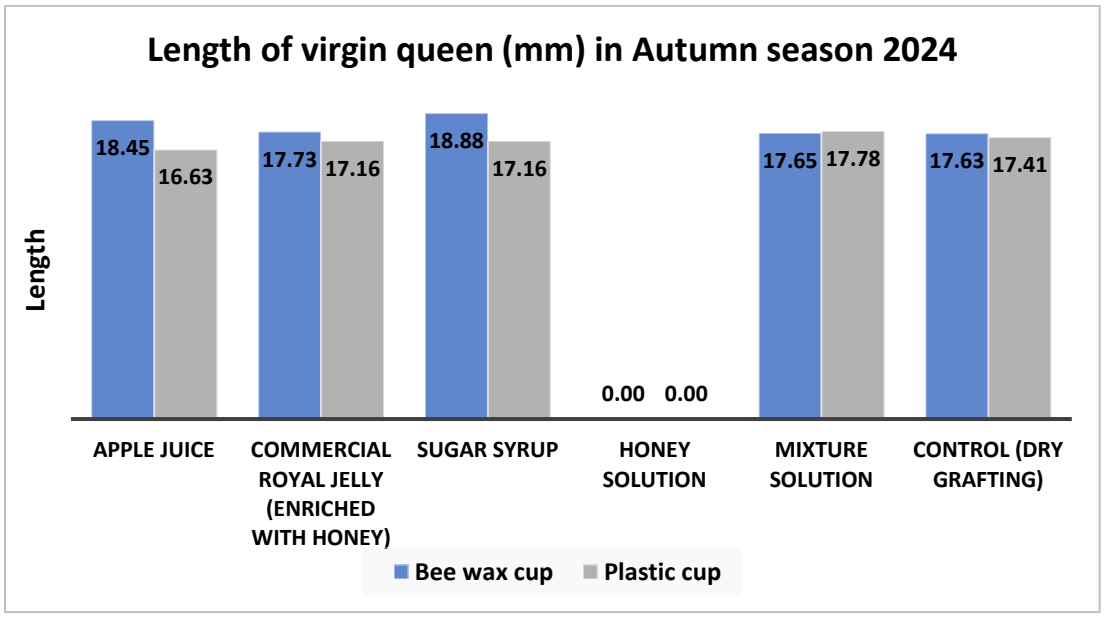


Fig. 4.17: Morphometric measures of length of the virgin queen during Autumn season (2024)

4.4.3. Morphometric measures of virgin queen's weight at emergence during Autumn season (2024):

The virgin queen's weight at emergence plays a critical role in determining morphometric traits that determine the health and quality of queens. It is also a vital physiological characteristic for the queen's growth. The data was recorded immediately after the queen bee's emergence, where the virgin queen's weight at emergence in different substrates and queen cup materials was recorded as statistically highest (175 mg) in apple juice and sugar syrup as priming media and bee wax cups as queen cup materials compared to the various substrates. However, the statistically maximum virgin queen's weight at emergence was recorded in the apple juice (175 mg), followed by sugar syrup (175 mg), mixture solution (155 mg), control (dry grafting) (135 mg), commercial royal jelly (enriched with honey) (123.3 mg), and honey solution (no emerged) respectively in bee

wax cups. In the plastic cups, the statistically maximum virgin queen's weight at emergence was recorded in the apple juice (145 mg), followed by sugar syrup (125 mg), commercial royal jelly (enriched with honey) (115 mg), control (dry grafting) (107.5 mg), mixture solution (103 mm), and honey solution (no emerged) respectively. However, the paraffin wax cups were not accepted at all (Table 4.10, Fig. 4.18).

Furthermore, our findings revealed that the highest virgin queen's weight at emergence was significantly achieved in apple juice (175.0 mg) and sugar syrup (175.0 mg) used as the priming media in bee wax cups, while no queens emerged in paraffin wax queen cups. In contrast, no queens emerged when using the honey solution at all. However, highly significant differences were found among the substrates and between the cup materials, while no significant differences were observed between the substrates and queen cup materials. Apple juice was noted to have a significant impact due to the acidic nature of the substrate (Contreras-Martinez et al., 2017).

Table 4.10: Effect of different substrates and queen cup materials on the morphometric traits of virgin queen's weight at emergence during Autumn season in 2024

Cup materials / **Substrates**	**Virgin queen's weight at emergence (mg± SE)**			
	Bee wax cups	Plastic cups	t test	P_{value}
Apple juice	175.0±15.0	145.0±15.0	1.414	0.293^{NS}
Commercial royal jelly (enriched with honey)	123.3±14.53	115.0±5.0	0.542	0.633^{NS}
Sugar syrup	175.0±25.0	125.0±5.0	1.961	0.189^{NS}
Honey solution	NE	NE	NE	NE
Mixture solution	155.0±5.0	103.0±6.67	6.200	0.009**
Control (dry grafting)	135.0±6.45	107.5±8.54	2.569	0.045*
Factors	**F_{value}**		**P_{value}**	
Substrates (S)	28.785		0.000**	

Cup materials (C)	10.170	0.005**
S x C	0.673	0.649^{NS}

'**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant, "NE" no emerged.

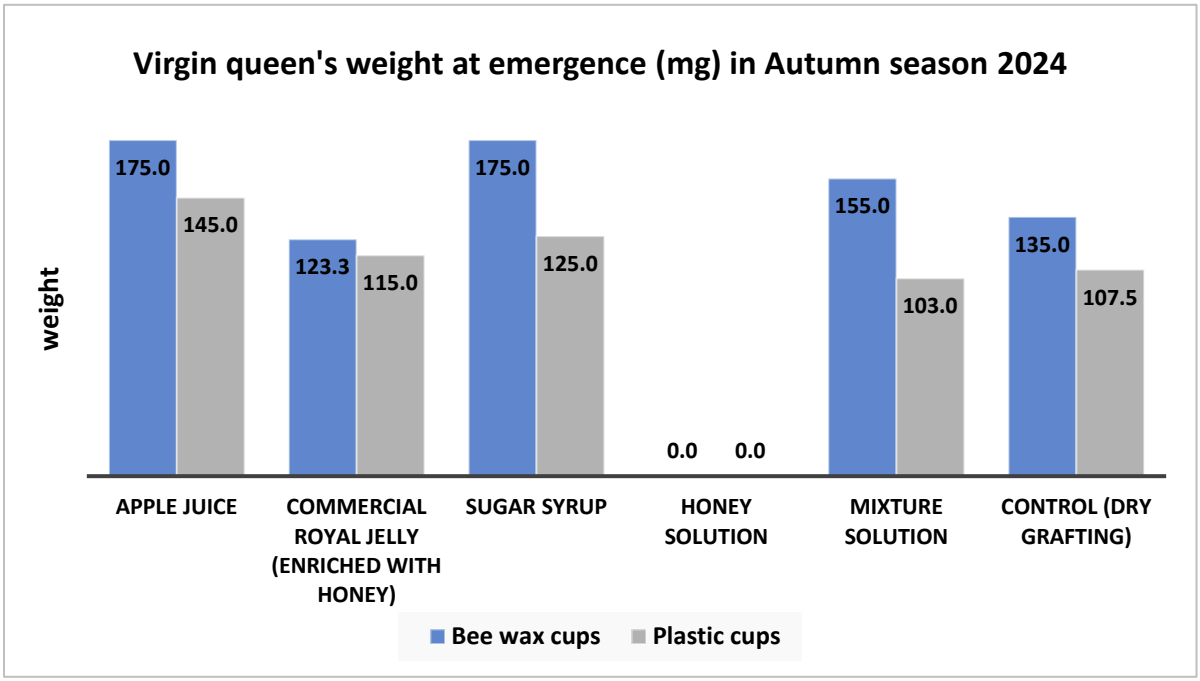


Fig. 4.18: Morphometric measures of virgin queen's weight at emergence during Autumn Season (2024)

4.5. Comparative impact of seasons, substrates, and queen cup materials on larvae acceptance, queen emergence rates, and morphometric traits of *A. mellifera* L. queens:

The larval acceptance, queen emergence rates, and morphometric measures of *A. mellifera* L. queens were significantly higher in the Spring season compared to the Autumn season (Fig. 4.19). These observations are closely consistent with the findings made by (Al-ghzawi & Zaitoun 2008; Amiri et al., 2017; DeGrandi-Hoffman et al., 2021; Shafey et al., 2021; Ozbakir, 2021; Chatha et al., 2022; Zhang, 2022; Aktürk et al., 2023; Canverdi et al., 2023; Holmes et al.2023; Büchler et al., 2024; Rehman et al., 2024). However, significant differences were recorded between the Spring and Autumn seasons (Table

4.11), (Fig. 4.19). The possible reasons for the present findings may be because of the optimal temperature and relative humidity ranges, favorable seasonal conditions, peak forage seasons, and floral abundance resources. Therefore, the warmer months typically provide more favorable foraging opportunities, resulting in increased larval feeding and enhanced queen development (DeGrandi-Hoffman et al., 2021).

The larval acceptance and queen emergence rates showed no significant difference between bee wax and plastic cups (Table 4.11), (Fig. 4.20). However, the morphometric measures of queens were significantly higher in bee wax cups compared to plastic cups (Table 4.11). Similar results were observed in the high morphometric traits of queens achieved from bee wax cups (Rafique et al., 2019, Kamboj et al., 2023). The reason for this might be due to their similar properties to natural bee wax, which is made from honeybee comb. The queen emergence and larval acceptance rates were significantly higher in the mixture solution when used as a primer (Fig. 4.21). The morphometric measures of queens were significantly higher in the apple juice because of the acidic nature of the substrate (Table 4.11), (Fig. 4.21) and the present result was supported by Contreras-Martinez et al., 2017.

There were no significant differences found in the seasons and queen cup materials. Highly significant differences were reported in the seasons and substrates in the larval acceptance rates and the length of the virgin queen (Table 4.11). Highly significant differences were recorded in the queen cup materials and the substrates in morphometric measures of *A. mellifera* L. queens. Significant differences were found in the queen cup materials and the substrates in the larval acceptance rates (Table 4.11). No significant differences were reported in the queen cup materials and the substrates in the queen emergence rates. However, no significant differences were recorded among the seasons, queen cup materials, and substrates in the larval acceptance, queen emergence rates, and the length of the sealed queen cells. Highly significant differences were observed among the seasons, queen cup materials, and substrates in the length and weight of the virgin queen (Table 4.11).

Seasonal influences play an important role in honey bee success (*Apis mellifera*) queen rearing, affecting the quality and morphometric traits of the queens (Chatha et al., 2022; Zhang, 2022). Previous research has shown that the queen rearing success is highly dependent on optimal temperature and relative humidity ranges, floral abundance, and colony strength (Chatha et al., 2022). Additionally, colonies under favorable seasonal conditions are likely to produce queens with more desirable morphometric traits, including larger body size and heavier weight, which are associated with reproductive health and colony performance (Amiri et al., 2017; Aktürk et al., 2023). However, research indicates that queen rearing by grafting gives better results during peak forage seasons than during periods when floral resources are scarce (Rehman et al., 2024).

Moreover, queens reared during seasons rich in forage resources show higher morphometric traits, which leads to their reproductive success and longevity in the colonies (Ozbakir, 2021; Shafey et al., 2021; Holmes et al.2023; Canverdi et al., 2023; Büchler et al., 2024). The substrates in queen cups are crucial for development of queen larvae and the queen rearing success. The different grafting techniques, such as dry and wet grafting, affect the larval acceptance, queen emergence rates, and morphometric traits of the queens (Gatoria et al., 2004; Büchler et al., 2013; Kamel et al., 2013; Adgaba et al., 2019; Kamboj et al., 2023). Additionally, the queen cup materials play a very important role in artificial queen rearing (Dhaliwal et al. 2017; Rafique et al. 2019; Sharma et al., 2020; Khan et al., 2021; Lashari et al. 2022).

Table 4.11: Comparative impact of seasons, substrates, and queen cup materials on larval acceptance, queen emergence rates, and morphometric traits of *A. mellifera* L. queens

Parameters / Factors	Acceptance rates (%)	Emergence rates (%)	Length of sealed queen cells (mm)	Length of virgin queen (mm)	Weight of virgin queen (mg)
Seasons					
Spring	41.70^{a}	35.80^{a}	20.26^{a}	14.65^{a}	189.46^{a}
Autumn	27.50^{b}	21.70^{b}	15.84^{b}	14.48^{a}	113.174^{b}
$F_{value\ (1,207)}$	6.277	6.543	$_{(1,44)}$ 6.987	$_{(1,29)}$ 0.069	$_{(1,30)}$ 54.435
P_{value}	0.013*	0.011*	0.011*	0.795^{NS}	0.000**
Cup materials					
Bee wax cups	31.70^{a}	25.80^{a}	21.62^{a}	15.15^{a}	167.154^{a}
Plastic cups	37.50^{a}	31.70^{a}	14.48^{b}	14.08^{b}	135.481^{b}
$F_{value\ (1,207)}$	1.064	1.109	$_{(1,44)}$ 18.243	$_{(1,29)}$ 15.89	$_{(1,30)}$ 9.616
P_{value}	0.303^{NS}	0.293^{NS}	0.000**	0.000**	0.004**
Substrates					
Apple juice	37.50^{ab}	35.00^{ab}	20.53^{a}	17.93^{a}	228.00^{a}
Commercial royal jelly (enriched with honey)	25.00^{bc}	22.50^{bc}	17.28^{a}	16.00^{b}	156.66^{c}
Sugar syrup	32.50^{abc}	27.50^{ab}	20.08^{a}	17.61^{a}	192.00^{b}

Honey solution	15.00[c]	7.50[c]	5.17[b]	8.02[c]	95.00[d]
Mixture solution	50.00[a]	45.00[a]	21.62[a]	17.88[a]	171.81[bc]
Control (dry grafting)	47.50[a]	35.00[ab]	23.60[a]	17.70[a]	152.00[c]
$F_{value\ (5,207)}$	3.723	3.627	$_{(5,44)}$ 10.846	$_{(5,29)}$ 202.12	$_{(5,30)}$ 16.972
P_{value}	0.003**	0.004**	0.000**	0.000**	0.000**

Interaction between factors

Factors	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**
Seasons × Cup materials	0.195	0.659^{NS}	1.109	0.293^{NS}	0.030	0.863^{NS}	2.266	0.143^{NS}	0.145	0.706^{NS}
Seasons × Substrates	4.348	0.001**	2.033	0.075^{NS}	2.281	0.063^{NS}	48.844	0.000**	1.801	0.143^{NS}
Cup materials× Substrates	3.097	0.010*	2.142	0.062^{NS}	7.669	0.000**	58.229	0.000**	6.650	0.000**
Seasons × Cup materials× Substrates	0.873	0.500^{NS}	1.164	0.328^{NS}	2.091	0.084^{NS}	55.355	0.000**	7.185	0.000**

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test, '**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of three replicated value and 'NS' non-significant.

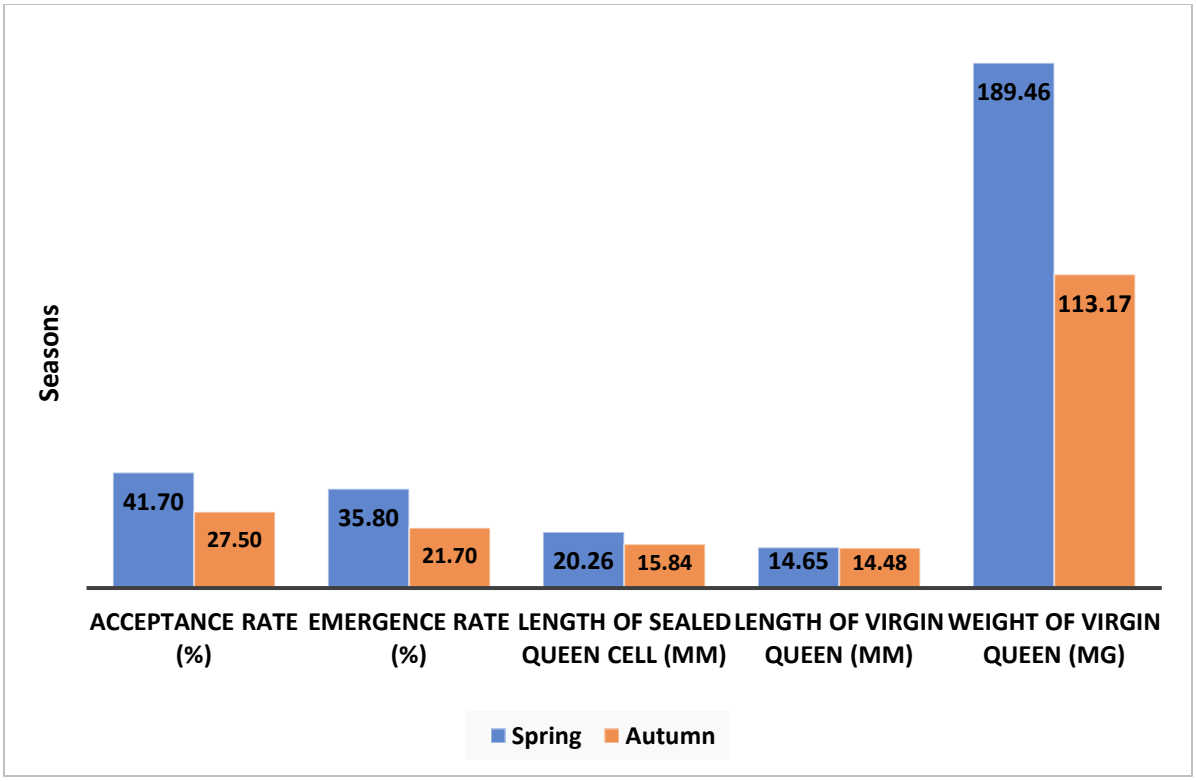


Fig. 4.19: Comparative impact of seasons on larval acceptance, queen emergence rates, and morphometric traits of *A. mellifera* L. queens

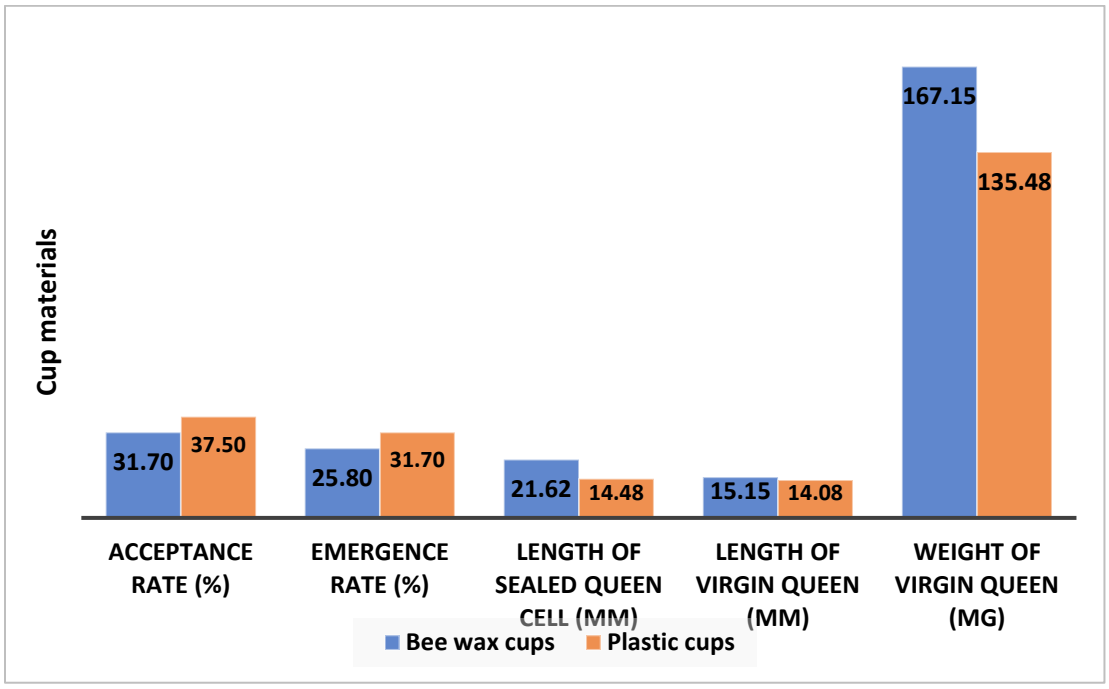


Fig. 4.20: Comparative impact of cup materials on larval acceptance, queen emergence rates, and morphometric traits of *A. mellifera* L. queens

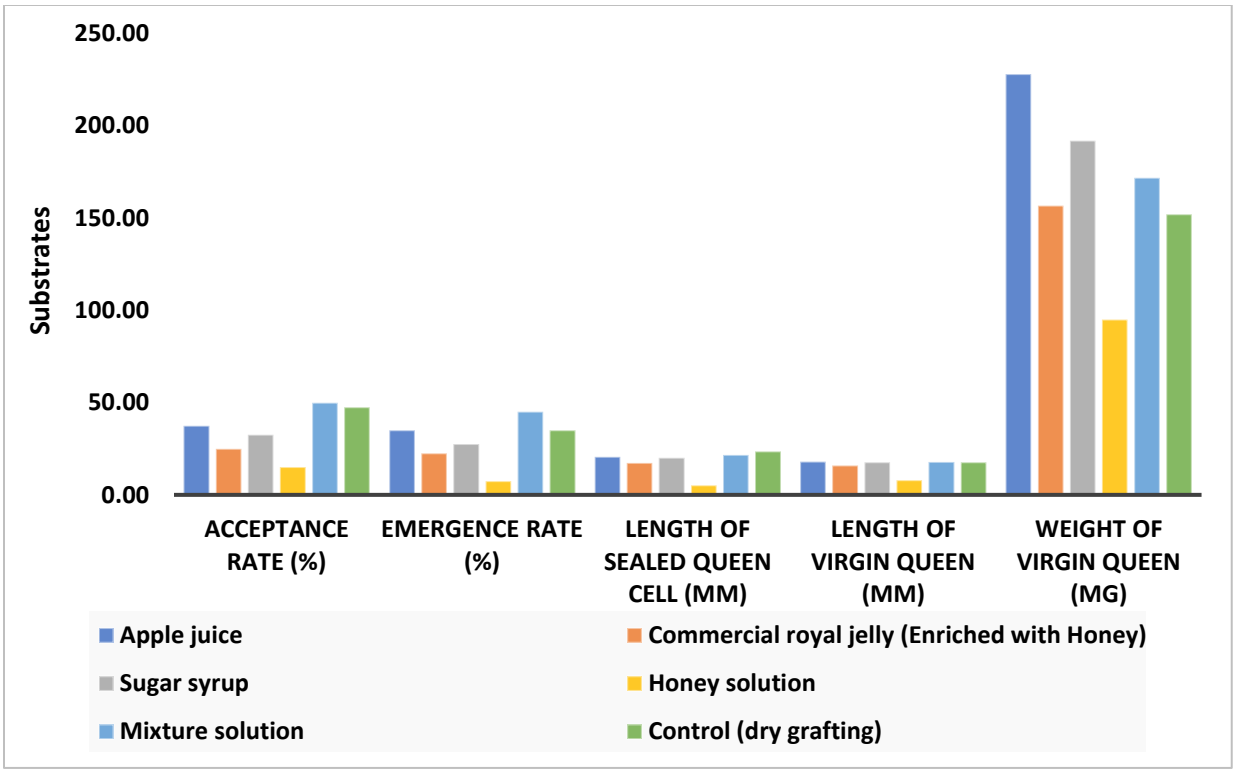


Fig. 4.21: Comparative impact of substrates on larval acceptance, queen emergence rates, and morphometric traits of *A. mellifera* L. queens

4.6. Comparative impact of grafting folds on larval acceptance and queen emergence rates of the *A. mellifera* L.:

The larval acceptance and queen emergence rates were significantly higher in plastic cups compared to the bee wax cups during the Spring season (Table 4.12), (Fig. 4.22). The findings of the present study are consistent with those recorded by Dhaliwal et al., (2017), who observed the highest larval acceptance rates in plastic cups when employing the Doolittle grafting method. No significant differences were found in the queen cup materials during the Autumn season (Fig. 4.23). No significant differences were observed in the larval acceptance rates among the substrates in Spring season, with the highest larval acceptance rates in apple juice (31.67 %) (Table 4.12), (Fig. 4.24). The results of the present study agreed with those reported by Contreras-Martinez et al. (2017), which might be attributed to the substrate's acidic characteristics. On the other hand, significant differences were reported in the queen emergence rates among the substrates during the Spring season, with the highest emergence rate of queens in apple juice (25.00 %) (Table 4.12), (Fig. 4.24). The larval acceptance rates

demonstrated highly significant differences among the substrates, while no significant differences were observed in the queen emergence rates during the Autumn season (Fig. 4.25). This might be due to the unfavorable conditions and floral scarcity.

The larval acceptance rates demonstrated no significant difference among the grafting folds (First, Second, and Third) (Table 4.12), (Fig. 4.26), (Fig. 4.27). However, the emergence rate of queens showed highly significant differences among the grafting folds, with the highest emergence rate of queens in the second fold (24.17 %) during the Spring season (Table 4.12), (Fig. 4.26). This might be due to floral abundance and changes in weather conditions, such as temperature and relative humidity. The larval acceptance and queen emergence rates showed highly significant differences among the grafting folds (First, Second, and Third), with the highest larval acceptance and queen emergence rates in the first fold (15.83 %, 14.167 %), respectively, during the Autumn season (Table 4.12), (Fig. 4.26), (Fig. 4.27).

No significant differences were recorded between the queen cup materials and the substrates in the larval acceptance and queen emergence rates during the Spring and Autumn seasons. Highly significant differences were found between the queen cup materials and grafting folds in the larval acceptance rates during the Spring season (Table 4.12). However, no significant differences were found in the queen emergence rates during the Spring season. No significant differences were found between the queen cup materials and grafting folds in the larval acceptance and queen emergence rate during the Autumn season. Highly significant differences were observed between the substrates and grafting folds in the larval acceptance and queen emergence rate during the Spring season (Table 4.12). There was no significant difference observed in the larval acceptance and queen emergence rates during the Autumn season. This might be due to the variation in the environmental conditions between the Spring and Autumn seasons. Significant differences were reported among the queen cup materials, substrates, and grafting folds in the larval acceptance and queen emergence rates during the Spring season. No significant differences were observed in the larval acceptance and queen emergence rates during the Autumn season (Table 4.12).

Table 4.12: Comparative impact of grafting folds on larvae acceptance and queen emergence rates of the *A. mellifera* L.

Seasons	**Spring season**		**Autumn season**	
Parameters / **Factors**	**Acceptance rates (%)**	**Emergence rates (%)**	**Acceptance rates (%)**	**Emergence rates (%)**
Cup materials				
Bee wax cups	15.56^{b}	11.67^{b}	7.78^{a}	7.22^{a}
Plastic cups	28.33^{a}	20.56^{a}	10.56^{a}	7.22^{a}
$\mathbf{F_{value\ (1,\ 324)}}$	10.510	6.031	0.875	0.000
$\mathbf{P_{value}}$	0.001**	0.015*	0.350^{NS}	1.00^{NS}
Substrates				
Apple juice	31.67^{a}	25.00^{a}	8.33^{b}	6.67^{ab}
Commercial royal jelly (enriched with honey)	21.67^{ab}	15.00^{ab}	8.33^{b}	8.33^{ab}
Sugar syrup	23.33^{ab}	16.67^{ab}	6.67^{b}	6.67^{ab}
Honey solution	13.33^{b}	5.00^{b}	1.67^{b}	0.0^{b}
Mixture solution	26.67^{ab}	21.67^{a}	8.33^{b}	8.33^{ab}
Control (dry grafting)	15.00^{b}	13.33^{ab}	21.67^{a}	13.33^{a}
$\mathbf{F_{value\ (5,\ 324)}}$	2.070	2.469	3.341	1.698
$\mathbf{P_{value}}$	0.069^{NS}	0.033*	0.006**	0.135^{NS}

Grafting folds								
First	16.67[a]		12.50[b]		15.83[a]		14.167[a]	
Second	25.83[a]		24.17[a]		5.83[b]		5.00[b]	
Third	23.33[a]		11.67[b]		5.83[b]		2.50[b]	
$F_{value\ (2, 324)}$	1.927		4.971		5.043		6.920	
P_{value}	0.147[NS]		0.007**		0.007**		0.001**	
Interaction between factors								
Factors	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**
Cup materials × Substrates (5, 324)	1.699	0.137[NS]	0.0942	0.454[NS]	0.371	0.868[NS]	0.102	0.992[NS]
Cup materials × Grafting folds (2, 324)	9.914	0.00**	2.285	0.103[NS]	1.821	0.164[NS]	0.892	0.411[NS]
Substrates × Grafting folds (10, 324)	4.240	0.00**	2.992	0.001**	0.756	0.671[NS]	1.163	0.315[NS]
Cup materials × Substrates × Grafting folds (10, 324)	2.166	0.020*	2.116	0.023*	1.317	0.220[NS]	0.841	0.590[NS]

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test, '**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of ten replicated value and 'NS' non-significant.

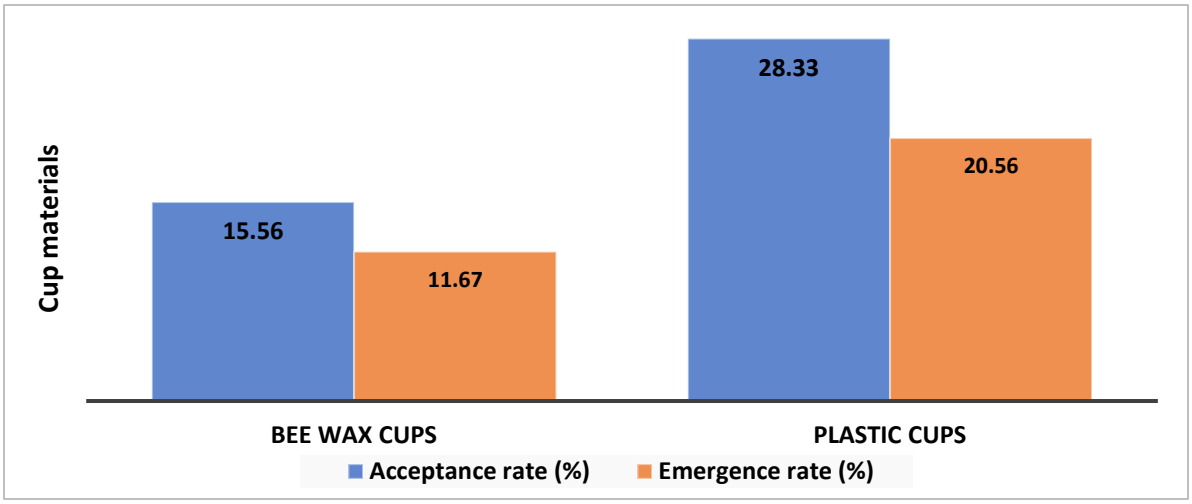


Fig. 4.22: Comparative impact of cup materials on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Spring season

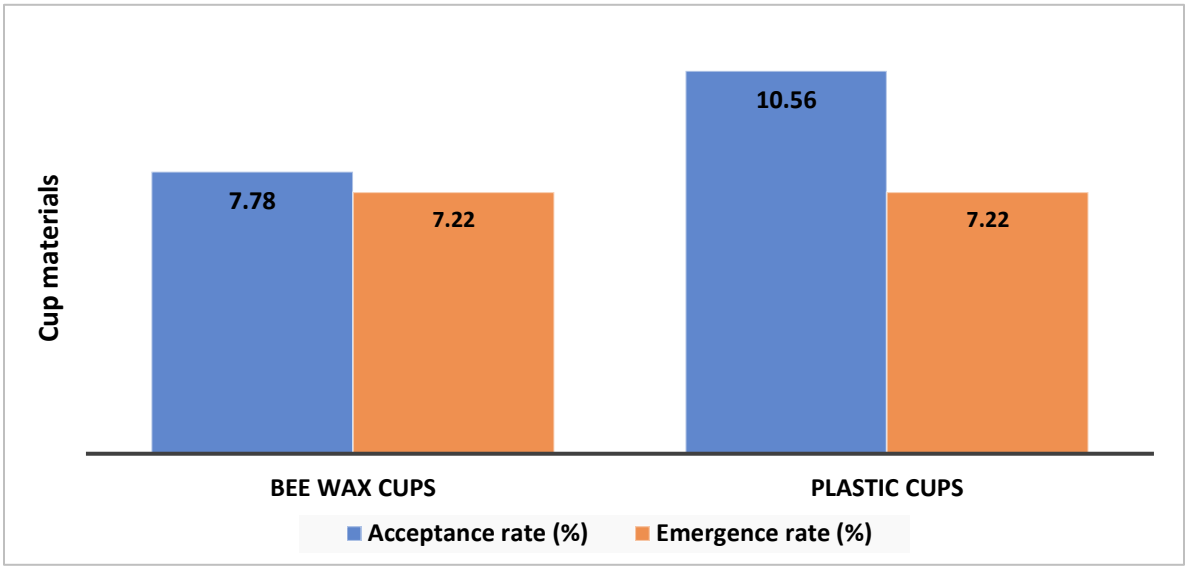


Fig. 4.23: Comparative impact of cup materials on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Autumn season

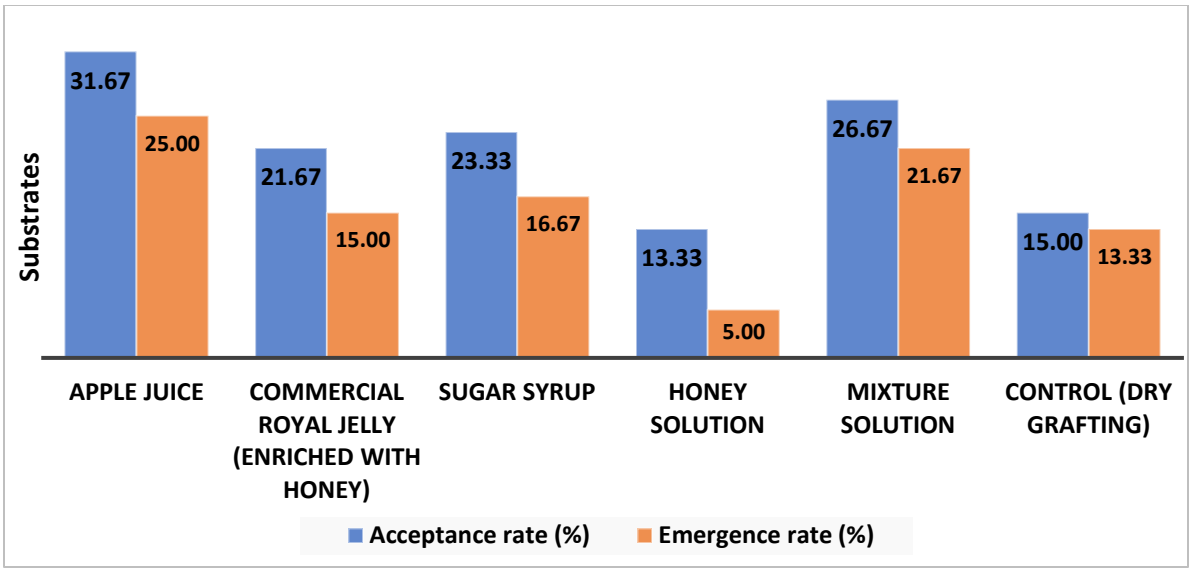


Fig. 4.24: Comparative impact of substrates on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Spring season

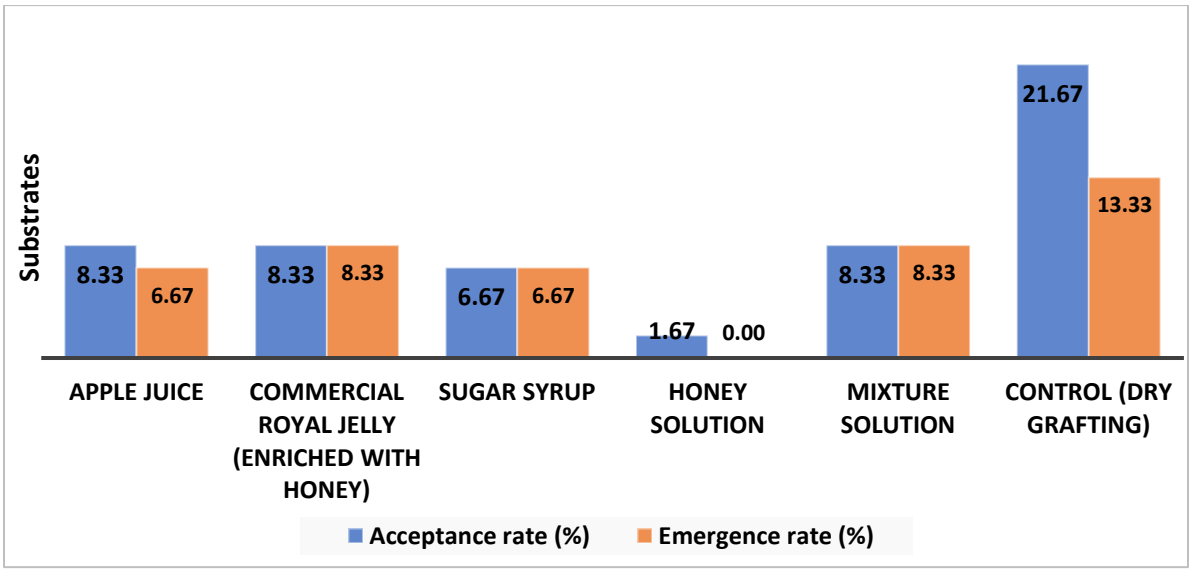


Fig. 4.25: Comparative impact of substrates on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Autumn season

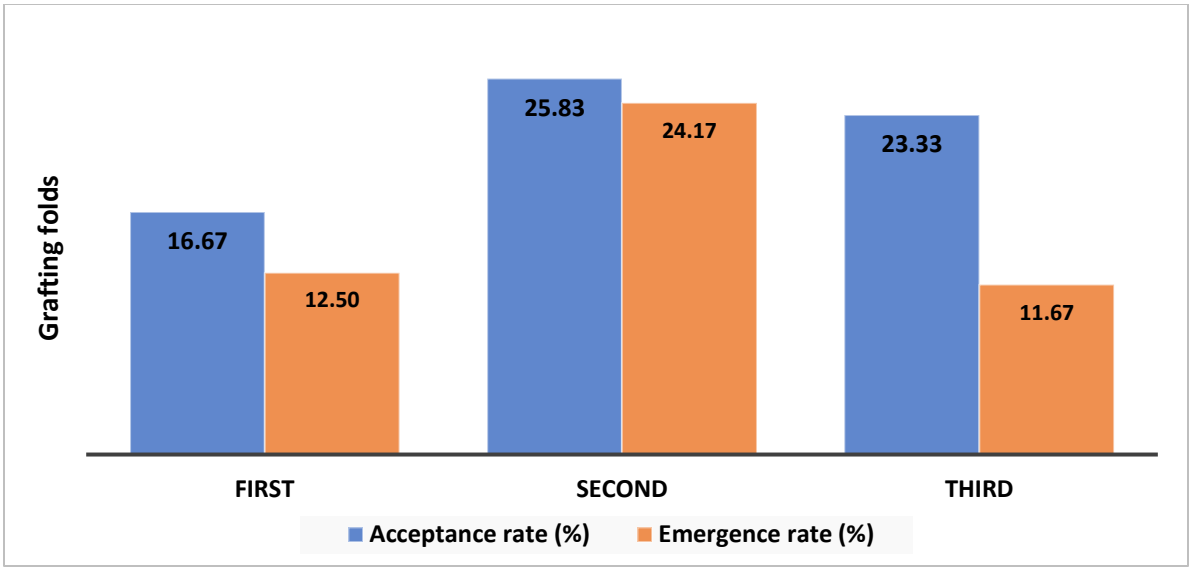


Fig. 4.26: Comparative impact of grafting folds on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Spring season

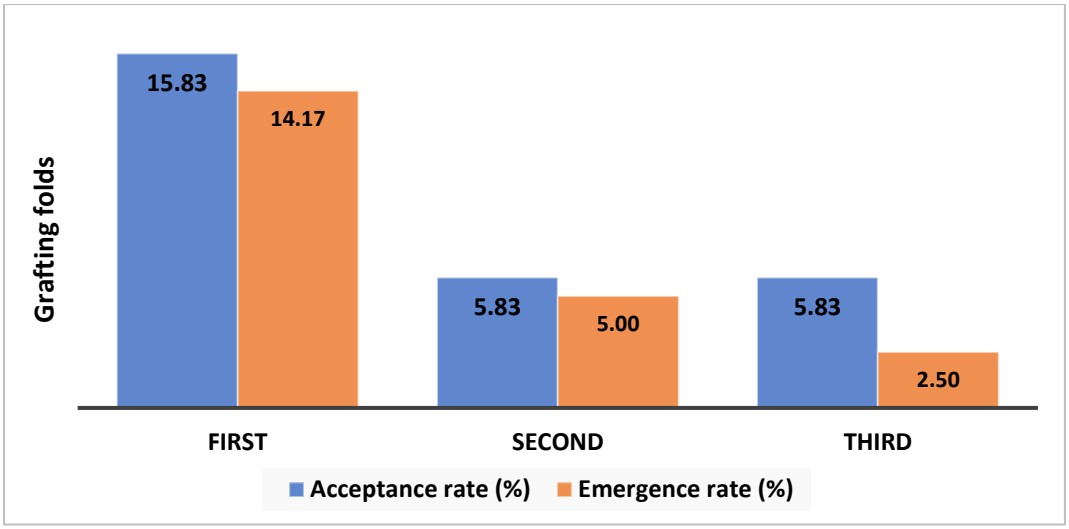


Fig. 4.27: Comparative impact of grafting folds on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Autumn season

4.7. Comparative impact of grafting bar level on larvae acceptance and queen emergence rates of the *A. mellifera* L.:

No significant differences were observed between the beewax and plastic cups in the acceptance larvae and emergence of queen rates during the Spring and Autumn seasons (Table 4.13). Highly significant differences were observed in the larval acceptance and queen

emergence rates among the substrates, with the maximum larval acceptance and queen emergence rates in the mixture solution (75.00 %, 65.00 %), respectively, during the Spring season (Table 4.13). On the other hand, highly significant differences were reported in the larval acceptance rates among the substrates. No significant differences were observed in the queen emergence rates among the substrates during the Autumn season (Table 4.13).

No significant differences were found between the grafting bar level (up and down levels) in the larval acceptance and queen emergence rates during the Spring season (Fig. 4.28). In contrast, significant differences were observed in the larval acceptance (36.67%) and queen emergence rates (30.00%) during the Autumn season (Table 4.13), (Fig. 4.29). Significant differences were observed between the queen cup materials and the substrates in the larval acceptance and queen emergence rates during the Spring season. In contrast, no significant differences were observed during the Autumn season (Table 4.13).

No significant differences were found between the queen cup materials and grafting bar level in the larval acceptance and queen emergence rates during the Spring and Autumn seasons (Table 4.13). No significant differences were observed between the substrates and grafting bar level in the larval acceptance and queen emergence rate during the Spring and Autumn seasons. No significant differences were observed among the queen cup materials, substrates, and grafting bar level in the larval acceptance and queen emergence rates during the Spring and Autumn seasons (Table 4.13). Therefore, the larval acceptance and queen emergence rates are not affected by the grafting bar level during both the Spring and Autumn seasons.

The comparative impact of grafting bar level on larvae acceptance and queen emergence rate of the *A. mellifera* L. was not discussed because no literature review was available; therefore, these findings were not discussed.

Table 4.13: Comparative impact of grafting bar level on larvae acceptance and queen emergence rates of the *A. mellifera* L.

Seasons	Spring season		Autumn season	
Parameters / Factors	Acceptance rates (%)	Emergence rates (%)	Acceptance rates (%)	Emergence rates (%)
Cup materials				
Bee wax cups	41.67^{a}	31.67^{a}	23.33^{a}	21.667^{a}
Plastic cups	43.33^{a}	41.67^{a}	31.67^{a}	21.667^{a}
$F_{value\ (1, 96)}$	0.039	1.500	1.220	0.000
P_{value}	0.843^{NS}	0.224^{NS}	0.272^{NS}	1.000^{NS}
Substrates				
Apple juice	50.00^{ab}	50.00^{ab}	25.00^{b}	20.00^{ab}
Commercial royal jelly (enriched with honey)	25.00^{b}	20.00^{bc}	25.00^{b}	25.00^{ab}
Sugar syrup	45.00^{ab}	35.00^{abc}	20.00^{b}	20.00^{ab}
Honey solution	25.00^{b}	15.00^{c}	5.00^{b}	0.66^{b}
Mixture solution	75.00^{a}	65.00^{a}	25.00^{b}	25.00^{ab}
Control (dry grafting)	35.00^{b}	35.00^{abc}	65.00^{a}	40.00^{a}
$F_{value\ (5, 96)}$	3.365	3.467	4.654	2.051
P_{value}	0.008**	0.006**	0.001**	0.078^{NS}

Grafting bar level								
Up	43.33[a]		31.667[a]		18.33[b]		13.33[b]	
Down	41.667[a]		41.667[a]		36.67[a]		30.00[a]	
$F_{value\ (1, 96)}$	0.039		1.500		5.902		5.128	
P_{value}	0.843[NS]		0.224[NS]		0.017*		0.026*	
Interaction between factors								
Factors	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**	**F_{value}**	**P_{value}**
Cup materials × Substrates $_{(5, 96)}$	2.863	0.019*	2.700	0.025*	0.517	0.763[NS]	0.123	0.987[NS]
Cup materials × Grafting bar level $_{(1, 96)}$	0.039	0.843[NS]	0.667	0.416[NS]	0.439	0.509[NS]	0.821	0.367[NS]
Substrates × Grafting bar level $_{(5, 96)}$	1.357	0.247[NS]	0.900	0.484[NS]	0.751	0.587[NS]	0.574	0.719[NS]
Cup materials × Substrates × Grafting bar level $_{(5, 96)}$	0.792	0.558[NS]	0.867	0.507[NS]	1.376	0.240[NS]	1.928	0.097[NS]

[a]Similar letter marked by common letters are not significant according to Duncan's Multiple Range Test, '**' significant level at 0.01, '*' significant level at 0.05, 'SE' standard error of five replicated value and 'NS' non-significant.

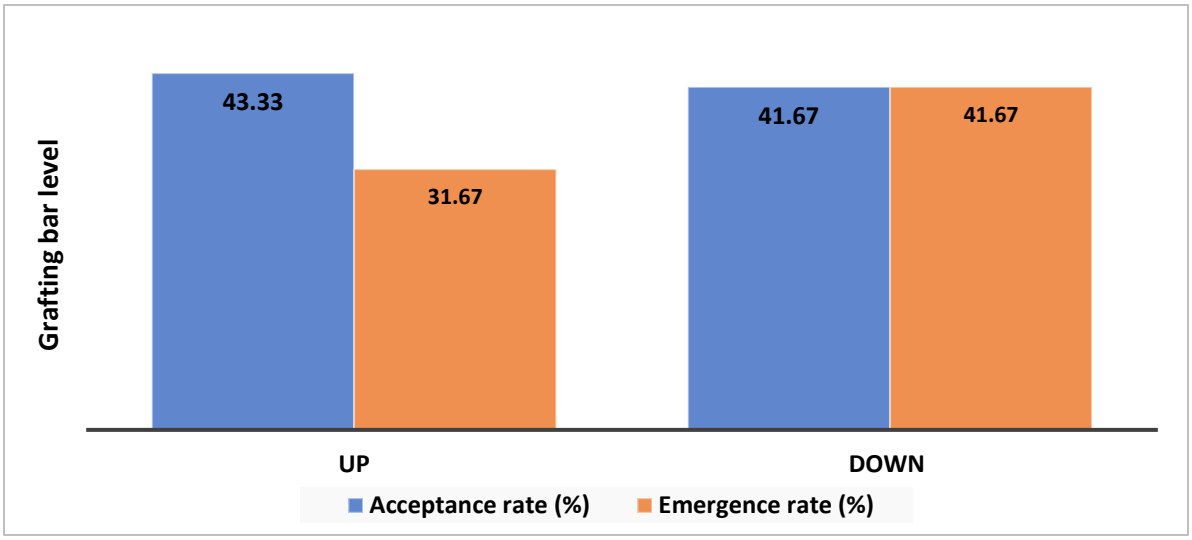


Fig. 4.28: Comparative impact of grafting bar level on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Spring season

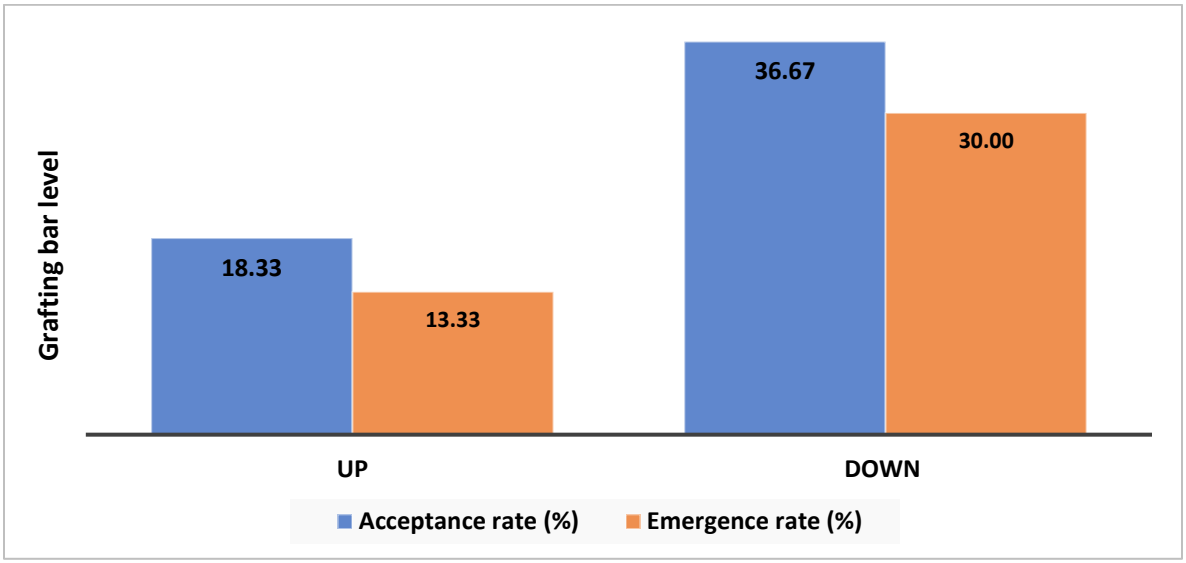


Fig. 4.29: Comparative impact of grafting bar level on larvae acceptance and queen emergence rates of the *A. mellifera* L. during Autumn season

4.8. Behavior of honey bee workers during queen-rearing using the Doolittle grafting method in different processes:

During the queen-rearing process using the Doolittle grafting method, distinct behavioral responses were observed among the worker bees. Worker bees diligently prepared queen cups by thoroughly polishing them with wax and secretions, ensuring a clean and smooth surface that encourages larval development (Fig. 4.30). In other words, a significant number of queen cups were polished by worker bees, indicating their acceptance of the provided queen cells and readiness for queen rearing. The polishing process resulted in a high percentage of queen cups with the characteristic smooth, polished surface (Fig. 4.30).

A. Before polishing B. After polishing

Fig. 4.30: Polishing process

Immediately after grafting, nurse bees exhibited heightened attention to the introduced larvae. Within the first few hours, a significant aggregation of nurse bees around the grafted queen cups was evident, indicating active inspection and acceptance behavior (Fig. 4.31). This initial clustering behavior is critical, as it reflects the colony's assessment of larval quality and environmental readiness for queen rearing. Acceptance of grafted larvae was marked by the substrates with apple juice and mixture solution (33.33%) and (50%) during the spring season, respectively, which began within 2-3 days of grafting in most colonies, signifying the success of grafting.

Fig. 4.31: Significant aggregation of nurse bees around the grafted queen cups

Worker bees displayed highly organized provisioning behavior, characterized by the frequent deposition of royal jelly into the artificial queen cups (Fig. 4.32). The intensity and frequency of royal jelly feeding increased significantly between 12 to 24 hours post-grafting, corresponding with the period of most active larval development. This provisioning behavior reflects the colony's prioritization of queen development, as larvae destined to become queens require abundant and sustained royal jelly feeding. Differences in provisioning intensity were also linked to environmental factors and colony strength, suggesting that worker responsiveness is modulated by colony condition.

Fig. 4.32: Frequent deposition of royal jelly into the artificial queen cups

In addition to feeding behavior, workers demonstrated robust queen cell maintenance activities. Continuous inspection, cleaning, and repair of the artificial queen cups were observed throughout the rearing period (Fig. 4.33). This maintenance ensures hygienic conditions and structural integrity of the developing queen cells, which are critical for successful pupation. Moreover, guarding behavior by older workers around the queen cells was recorded, particularly after the capping stage, indicating a protective response during the vulnerable pupal stage (Fig. 4.34).

The behavioral patterns of inspection, feeding, maintenance, and selection are vital to the success of the grafting process and provide insights into the colony's intrinsic capacity to evaluate and rear replacement queens under controlled interventions.

Fig. 4.33: Queen cell maintenance activities, inspection, cleaning, and repair of the artificial queen cups

Fig. 4.34: Guarding behavior by older workers around the queen cells

Chapter 5
Summary and Conclusions

CHAPTER - 5

SUMMARY AND CONCLUSIONS

The research investigated the **"Evaluation of Different Substrates and Queen Cup Materials on the Artificial Rearing of Honey Bee Queen (*Apis mellifera* Linnaeus) Using Grafting Method"** in between than February to April 2024 (Spring season) and in between than September to November 2024 (Autumn season) at the Apiculture Area, Department of Entomology, School of Agriculture, Lovely Professional University, Phagwara, Punjab, India.

The Doolittle grafting method was used to conduct the experiment. Larvae aged 24 – 48 hours were selected from a strong and gentle breeder colony to use for grafting. Three queenless Langstroth hives were prepared as queen cell builder colonies for grafting. Before grafting, queen cups were primed with five different substrates: apple juice (S_1), commercial royal jelly (enriched with honey) (S_2), sugar syrup (S_3), honey solution (S_4), and a mixture of all four (S_5). control (dry grafting method) (S_6).

Three types of queen cups (beeswax, paraffin wax, and plastic cups) were used. Wax cups were prepared using a silicone mold method, while plastic cups were commercially purchased. A total of 180 larvae were grafted for all treatments, distributed across three cell builder colonies. Larval acceptance was recorded after three days, and queen emergence was assessed 11–12 days post-grafting.

Grafting was performed three different folds each season, Spring (24/03/2024, 27/03/2024, 25/04/2024) and Autumn (04/10/2024, 07/10/2024, 11/10/2024). Each grafting frame had two levels (upper and lower bars). Ten replications of each substrate were divided evenly between the two levels. Measurements of sealed queen cells length, virgin queen length, and virgin queen's weight at emergence were taken using a digital vernier calliper and a weighing balance.

5.1. Concluding remark:

- In Spring, queen larval acceptance and queen emergence rates were significantly influenced by both the substrate and cup materials used. The mean larval acceptance rates were highest with the mixture solution substrate (50.00%) and plastic cups (43.33%), while paraffin wax cups recorded no acceptance. Similarly, the mean queen emergence rates were highest with the mixture solution substrate (43.33%) and plastic cups (41.66%). Morphometric traits such as the length of sealed queen cells and length of virgin queens were significantly greater in bee wax cups compared to plastic cups. Queens reared in bee wax cups also had higher weights at emergence, although differences were often statistically non-significant except for certain substrates like the honey solution.
- During the Autumn season, overall mean larval acceptance and queen emergence rates were lower compared to Spring. The control group (dry grafting) showed the highest mean larval acceptance (43.33%) and queen emergence (26.67%). Morphometric measurements, including the length of sealed queen cells and queen weight at emergence, were also reduced in Autumn compared to Spring. However, bee wax cups consistently produced queens with better morphometric traits than plastic cups.
- Comparative seasonal analysis indicated that queen rearing performance was significantly better in Spring than in Autumn. Spring queens had higher mean larval acceptance (41.70%), queen emergence (35.80%), longer sealed queen cells (20.26 mm), and greater queen weight at emergence (189.46 mg). However, there was no significant seasonal difference in the length of virgin queens.
- Comparative impact of grafting folds indicated that the first grafting fold had better larval acceptance and queen emergence in Autumn, while the second grafting fold performed better in Spring.
- Comparative impact of grafting bar level indicated that a lower grafting bar level showed significantly higher larval acceptance (36.67%) and queen emergence

(30%) in Autumn. In contrast, there were no significant differences between the grafting bar level (up and down levels) during the Spring season.

- Apple juice and mixture solution substrates encouraged faster and higher mean larval acceptance rates (33.33% and 50%, respectively, during Spring).

5.2. Future scope:

- The current investigation offers valuable insights into the effect of different seasons, substrates, and queen cup materials on larval acceptance, the queen emergence rates, and morphometric traits.
- Further research is recommended to evaluate these additional variables and develop more refined protocols for efficient and sustainable queen production.
- Future research should focus on specific substrates, such as apple juice, and conduct studies to determine the ideal concentration for high-quality queen rearing and its consequences in various agroclimatic zones.
- Further research is recommended to examine the specific type and properties of beeswax used in the preparation of artificial queen cups, as this factor may influence larval acceptance, the queen emergence rates, and morphometric traits.

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