DOI: 10.1515/JAS-2017-0019 J. APIC. SCI. VOL. 61 NO. 2 2017

DE GRUYTER OPEN

Original Article

EFFECT OF DIFFERENT SUBSTRATES ON THE ACCEPTANCE OF GRAFTED LARVAE IN COMMERCIAL HONEY BEE (*APIS MELLIFERA*) QUEEN REARING

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Received: 8 April 2017; accepted: 27 October 2017

Abstract

The need for the increased production of honey bee (*Apis mellifera*) queens has led beekeepers to use different substrates in artificial queen cups where larvae destined to become queens are deposited (grafting). However, not enough scientific evidence exists that indicates that this practice is useful and what substance offers the best results. This study was conducted to determine with the Doolittle queen rearing method the acceptance rate of larvae deposited on different substrates during grafting and to determine if the sugar content and pH of the substrates used affect the acceptance of larvae in cell builder colonies. The evaluated substrates were coconut water, apple nectar, royal jelly, cola soda and distilled water, plus control (without substrate). Grafted larvae of the six treatments were introduced into cell builder colonies and their acceptance verified after 72 h. Apple nectar provided the highest rate of larvae acceptance with 81.06%, followed by cola soda with 62.93%, coconut water with 60.90%, royal jelly with 57.82% and distilled water with 58.99%. The larvae acceptance rates of all substrates were significantly higher than the control, which had an acceptance rate of 47.04%. No significant relationship was found between the sugar content of the substrates and larvae acceptance. However, although not significant, a high negative correlation was found between the substrate pH and the number of accepted larvae (Rho = - 0.90, p = 0.07). These results suggest that the use of liquid acidic substrates during larvae grafting, in particular apple nectar, may increase the production of honey bee queens.

Keywords: *Apis mellifera*, grafting, larvae, queen rearing, substrates

INTRODUCTION

The honey bee (*Apis mellifera*) queen is almost in all cases the single mother of bees in honey bee colonies (Winston, 1987). The queen's egg-laying rate tends to be high during her first year of life but decreases as she ages (Laidlaw & Page, 1997). Therefore, one of the most effective management practices to maintain populous, healthy and productive colonies is frequent requeening. In general, requeening colonies once a year is recommended as a good general practice, particularly in latitudes where queens lay all-year round. This practice however

requires the massive production and supply of artificially reared queen bees.

The process of artificial queen rearing is a practice that has revolutionized the beekeeping industry because, in relation to natural queen replacement (supersedure), introducing a new, mated queen, into a colony shortens the time gap within which eggs are not laid and new bees are produced. Newly mated queens of selected strains also help maintain colony genetic diversity, health and productivity and are less prone to swarm compared with older queens (Laidlaw & Page, 1997; Guzman-Novoa, 2007). Conversely, if a queen is naturally superseded by her workers, it will take 25 to 30 days for a new queen to be reared, mated and developed to initiate egg laying. However, when a new, young and fertilized queen is introduced into a colony, this time gap is shortened to less than five days (Winston, 1987). To have young mated queens readily available for requeening requires that they be artificially raised.

Various practices are used to simulate natural conditions to rear queens. The most efficient and widely used methods worldwide involve the Doolittle grafting technique which enables large-scale production levels (Doolitle, 1889). Grafting is the physical transfer of larvae from worker cells of a selected breeder colony into artificially made queen cell cups. The cups are attached to wooden bars on grafting frames, which are introduced into cell builders strongly populated colonies in which the queen has been purposely removed. The queenless condition stimulates the workers to rear queens, and the introduced larvae are fed a diet of royal jelly and develop into queens. Adult queen bees emerge sixteen days after a fertilized egg is laid or twelve days after the larvae are grafted (Laidlaw & Page, 1997).

Since there is a great need for the massive production of queens for the beekeeping industry, queen breeders have tried to optimize rearing practices by implementing such techniques as the placement of different substrates in queen cups before larvae are grafted into them. With this practice, larvae acceptance by the workers in queen cell builder colonies is increased through the provision of a moist environment and food for the grafted larvae. Keeping a humid environment during the grafting process is known to be important for the prevention of larvae dehydration (Laidlaw & Page, 1997; Curbelo et al., 2009).

Beekeepers worldwide have empirically assayed different substrates, but very few studies have been conducted to test the effect of substrates on larvae acceptance, and so far, only water, royal jelly and mixtures of these substrates with other substances have been evaluated. For example, Macicka (1985) compared grafting larvae into dry queen cups (no substrate) and into cups with a royal jelly primer, and found that larvae acceptance was higher when royal jelly was used. In Mexico, beekeepers in many regions claim that they get higher numbers of queens when empirically using different substances as grafting substrates (Contreras-Escareño et al., 2013). The most commonly used substances by Mexican beekeepers are coconut water, apple nectar, cola soda and sugar syrup, but there have been no scientific studies that compared these substances as grafting primers (substrates). Therefore, the objective of this study was to evaluate the effect of several of the above substances as grafting substrates on the acceptance of larvae following the Doolittle queen rearing method. Additionally, it was assessed if the sugar content and acidity level of the substrates tested were related to larvae acceptance after grafting.

MATERIAL AND METHODS

Study area

This study was conducted between February and April 2015 at the experimental apiary of the University of Guadalajara's Southern Coast Centre in the municipality of Autlán de Navarro in the state of Jalisco, Mexico (19° 40' N, 104° 19' W). This location has a semidry, tropical climate, without defined seasonal changes, an average annual temperature of 23.5°C and an average annual precipitation of 719.8 mm (Comisión Nacional del Agua, 2016).

Queen cell builder colonies and treatments

Six queen cell builder colonies kept in jumbo-sized brood chambers (modified Dadant: 41 x 51 x 29 cm) were prepared by removing their queens and equalizing them to each contain five frames of emerging capped brood and three frames with honey and pollen. The adult bee population of each colony covered eight frames on both sides, which was equivalent to a population of approximately 31680 bees (Delaplane, Van der Steen, & Guzman-Novoa, 2013). These conditions were maintained throughout the study through the frequent addition of frames from healthy colonies. In addition, each colony was artificially fed with 0.5 l of 50% sucrose solution (one part sucrose, one part water) and 50 g of a protein supplement elaborated with 70% low-fat soybean flour, 20% pollen and 10% honey. Both the syrup and the protein supplement were provided to each cell builder colony twice a week (each time a grafting was performed).

Before larvae were grafted, a small drop (approx. 5 µL) of one substrate was placed at the bottom of each of the fifteen plastic queen cups. Six treatments were compared, including five for different substrates and one for control (without substrate). The treatments were coconut water (T1), apple nectar (Jumex®, Ecatepec, Mexico; T2), royal jelly (T3), distilled water (T4), cola soda (Coca Cola Classic®, Atlanta, USA; T5) and control (dry queen cups without substrate, T6). The royal jelly used in this study was produced in the same apiary in a different colony using standard methods (Caron & Connor, 2013) and used at 100% undiluted. The cola pop, coconut water and apple nectar were purchased at a grocery store. The composition of the substrates tested is mainly water and sugars.

After primed with a substrate (except for the control), each queen cup received a larva (<24 h) grafted from a comb of a randomly selected colony using a standard metal grafting tool. The same source of larvae was used for all graftings in each occasion. Since groups of fifteen cups were used for each treatment at each grafting occasion, a total of 90 queen cups were used. These 90 cups were installed on the bars of two grafting frames that were introduced into one cell builder colony and six of them were used in each grafting. Initially, five preliminary graftings were done to establish the queen rearing conditions in the cell builder colonies. The data of these graftings were not used for the analysis. After the preliminary graftings, eighteen experimental graftings were performed per cell builder colony, for a total of 1620 grafted larvae per treatment (9,720 for all treatments). The acceptance or rejection of larvae was corroborated and recorded 72 h post grafting. An average number of accepted larvae and a percent acceptance rate were calculated with the data collected.

The sugar content (°Brix) of the substrates was measured with a refractometer (Misco Instruments, model DFR123, USA). The pH of the substrates was determined with a pH meter (Hanna Instruments, model HI 991002, Rom). Five measurements during randomly selected graftings were performed for each substrate. Averages and SD were obtained from these five measurements.

Statistical analyses

To analyze the effect of the different treatments on larvae acceptance, the response variable data were subjected to a Poisson model regression analysis because the data (number of accepted larvae) had such distribution. The statistical model used was:

$\ln E(y) = \ln (n\, l) = \ln n + b_0 + b_1$ cell builder 2 $+ b_2$ cell builder 3 $+ b_3$ cell builder 4 $+ b_4$ cell builder 5 $+ b_5$ cell builder 6 $+ b_6$ coconut water $+ b_7$ apple nectar $+ b_8$ royal jelly $+ b_9$ cola pop $+ b_{10}$ distilled water, where $\ln E(y)$ is the number of expected accepted larvae in a log scale; n is the number of treated larvae in a log scale; l represents a function that defines the larvae acceptance rate in relationship with the prediction factors (cell builder and treatment) in the log scale; b_0 is the constant regression coefficient; b_1, b_2, b_3, b_4, b_5 are the regression coefficients for each level of the factor cell builder, respectively; b_6, b_7, b_8, b_9, b_{10} are the regression coefficients for each level of the factor treatment, respectively. The reference levels for the model are the cell

Table 1
Average number of accepted larvae 72 h after being grafted onto different substrates ± SD

Treatments	No. accepted larvae ± SD
Coconut water	9.13 ± 1.13
Apple nectar	12.14 ± 1.89
Royal jelly	8.64 ± 1.32
Distilled water	8.85 ± 1.17
Cola soda pop	9.43 ± 1.75
Control (without substrate)	7.38 ± 1.54

builder 1 and the control treatment.
In addition to the above analysis, the relationship between accepted larvae and °Brix and pH of the substrates was determined with a Spearman rank correlation analysis to find out if the sugar content and pH of the substrates tested influenced larvae acceptance. All the statistical analyses were performed using the R software, version 3.3.2 (R Development Core Team, Auckland, New Zealand).

RESULTS

Percentage of accepted larvae

Apple nectar was the substrate that most increased the acceptance of grafted larvae (81.06%), followed by cola soda (62.93%) and coconut water (60.90%). The substrates that least influenced the acceptance of grafted larvae were distilled water (58.99%) and royal jelly (57.82%). However, all substrates were associated with a higher rate of larvae acceptance than the control treatment (47.04%).

Number of accepted larvae

Similar to the acceptance rates, the substrate associated with the highest average number of accepted larvae was apple nectar, followed by cola soda, coconut water, distilled water, royal jelly and the control. The average number of accepted larvae ± SD for all treatments are shown in Tab. 1. The median and distribution of the larvae acceptance data are shown in a box plot (Fig. 1). The highest median corresponded to apple nectar (12) and the lowest to the control treatment (7). The statistical analysis indicates that there were no significant differences between cell builder colonies for the average number of larvae accepted per grafting ($p>0.05$), and thus, it is inferred that the cell builder colonies did not influence the response variable.
However, the treatments did affect the response variable. The average number of accepted larvae that were treated with apple nectar greatly exeeded of all the other treatments ($p<0.01$) by 29%, 33%, 37%, 40% and 64%, the number of accepted larvae for the cola soda, coconut water, distilled water, royal jelly and control treatments, respectively. The number of accepted larvae did not significantly differ among the treatments of cola soda, coconut water, distilled water, and royal jelly ($p>0.05$), but all of them had a significantly higher number of accepted larvae than the control treatment ($p<0.01$), which had the lowest number of accepted larvae.

°Brix and pH of the substrates

Distilled water had the lowest level of °Brix (2.01 ± 0.62), whereas apple nectar, cola pop and coconut water had levels of 12.35 ± 0.74, 12.15 ± 1.02 and 9.15 ± 0.96 °Brix, respectively. Royal jelly had the highest °Brix level (26.2 ± 0.94). With regards to pH, apple nectar had a mean value of 3.50 ± 0.08, whereas the values for cola soda, coconut water, distilled water and royal jelly were, 2.91 ± 0.08, 5.36 ± 0.27, 5.56 ± 0.39 and 5.78 ± 1.24, respectively.

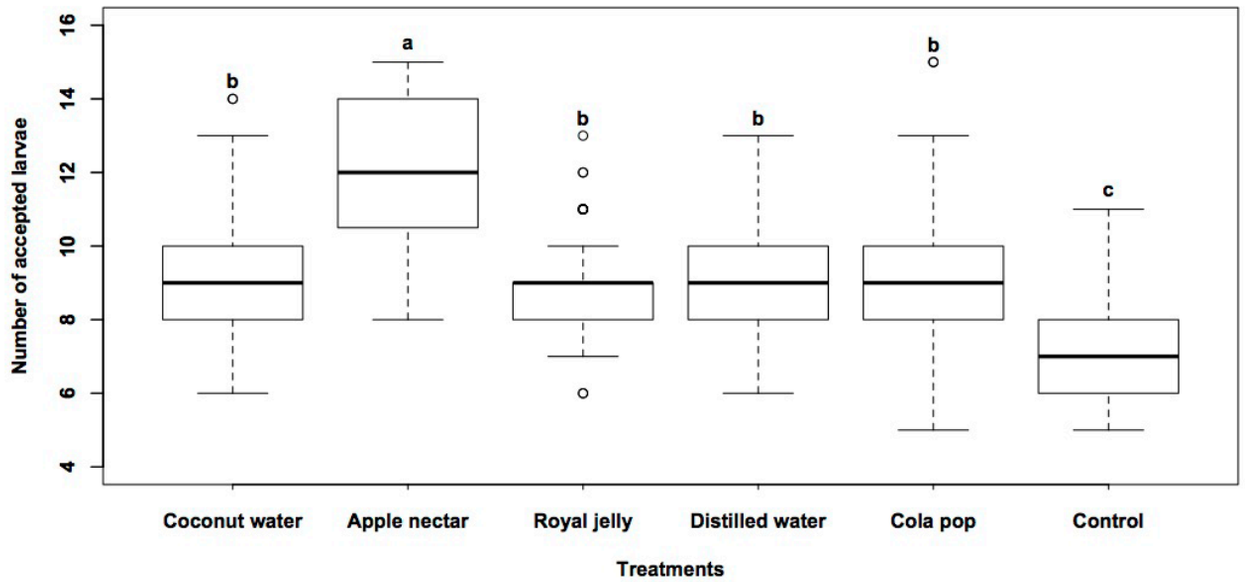

Fig. 1. The box plot shows the values for the number of accepted larvae 72 h after being grafted onto five different substrates (coconut water, apple nectar, royal jelly, distilled water and cola soda) or directly into queen cups without substrate (control). The values shown are minimum, maximum, quartile 1 (Q_1, 25% of the data), median (50% of the data), quartile 3 (Q_3, 75% of the data) and the inter-quartile range. The data outside the whiskers are atypical. Different letters above the boxes indicate significant differences between treatments based on a regression analysis using the Poisson model.

Relationship between °Brix or pH of the substrates and accepted larvae

No significant correlations were found between the °Brix of the substrates and the number of accepted larvae ($p>0.05$). However, a high and negative correlation was found between the pH of the substrates and the number of accepted larvae, which was close to being significant (Rho = - 0.90, p = 0.07).

DISCUSSION

This is one of few studies that have been conducted to test and compare substrates for their effect on the acceptance of grafted larvae during the queen rearing process. Apple nectar was associated with the highest percentage of accepted larvae and the acceptance rate of larvae treated with this substrate was significantly higher than those for larvae subjected to all of the other treatments ($p<0.01$). This result is novel because for the first time apple nectar is tested and resulted in a successful grafting substrate. Therefore, this result supports the recommendation of using apple nectar for grafting purposes during queen rearing. Moreover, all the other substrates tested significantly increased larval acceptance compared to dry grafting. These results demonstrate that the use of liquid substrates improves the acceptance of grafted larvae for queen rearing purposes. The reason why liquid substances are beneficial during queen rearing may be explained by the fact that these substances provide moisture, thus preventing dehydration of the grafted larvae (Laidlaw & Page, 1997; Emsen, Dodologlu, & Gene, 2003; Cobey, 2005). Royal jelly has been the most commonly tested grafting substrate in previous studies (Ebadi & Gary, 1980; Pickard & Kitner, 1983; Macicka, 1985; Gene, Emsen, & Dodologlu, 2005; Chhuneja & Gill, 2014), whose results for royal jelly have similarities and differences with those of this study. For example, Ebadi and Gary (1980) compared two grafting substrates, pure royal jelly and a mixture of 90% royal jelly and 10% pollen and found the highest rate of larvae acceptance with the royal jelly treatment (93.3%). The larvae acceptance rate for the royal jelly treatment in this study was lower (57.82%) than that found by Ebadi and Gary (1980) and also slightly lower than the 75.6% found for the same substrate by Macicka (1985). Macicka (1985) and Gene et al. (2005) reported that when larvae were grafted

without substrate the acceptance rate was low, ranging from 41.3 and 53%, which is similar to the 47.04% of the control treatment in this study. In a more recent study, Chhuneja and Gill (2014), compared graftings with and without royal jelly as a substrate. The larvae acceptance rates were 36.7% and 22.2%, for the treatments with and without substrate, respectively. These results are lower than those found in this study but coincide with our results in that the substrate (royal jelly) improves larvae acceptance relative to grafting without it. The similarities and differences in grafting results between this and previous studies may be influenced by many variables including the composition and conservation of royal jelly, grafting skill of different persons, type of cell cups, environmental conditions and population of cell builder colonies (Doolittle, 1889; Skowronek & Skubida, 1988; Cobey, 2005; Zheng, Hu, & Dietemann, 2012). It is thus important that future studies optimize these variables to increase grafting success.

The relationships between pH and °Brix of the substrates with the acceptance rate of grafted larvae were not significant. The literature does not report studies about the effect of these variables on the acceptance of grafted larvae during queen rearing processes, so future studies on this matter are warranted. However, the high and almost significant negative correlation between the substrates' pH and the number of accepted larvae found in this study (Rho = - 0.90, p = 0.07) indicates that acidity in substrates might increase larvae acceptance after grafting. The lack of significance of this analysis was likely due to the low sample size (n = 5), and therefore future studies should be conducted with a larger number of samples.

The reasons why apple nectar yielded the best results as grafting substrate are unknown and remain to be investigated in further field and laboratory studies. However, apple nectar might have been more stable and conserved for a longer time in the relatively warm and dry environment of cell builder colonies (30 – 35°C) than the other substrates tested. Apple nectar might have provided a more lasting effect than the other substrates because of its higher hygroscopic properties provided by its sugar content and components in the fruit pulp such as pectin (Berk, 2016). Hygroscopic fruit components are not found in such substrates as coconut water or cola soda. Additionally, apple nectar has chemical preservatives and acids, as well as other fruit pulp components that might make this substrate less palatable to the bees than cola soda or coconut water. It is possible that the royal jelly, cola soda or coconut water deposited at the bottom of queen cups had been rapidly consumed by the workers in the cell builder colonies, which would result in a faster loss of moisture for the grafted larvae compared with apple nectar. These substrates might have also evaporated at a faster rate than apple nectar.

In conclusion, the results of this study indicate that the use of liquid acidic substrates increase the acceptance of grafted larvae during queen rearing. Apple nectar in particular could be used to further increase the production of queen honey bees.

ACKNOWLEDGEMENTS

This study was funded by the Research Coordination Office of the University of Guadalajara's Southern Coast Centre.

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