Vector Relations

# Honeybee Foraging Behavior, In-Hive Survival of Infectious, Pollen-Borne Blueberry Leaf Mottle Virus and Transmission of the Virus in Highbush Blueberry

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## ABSTRACT

Boylan-Pett, W., Ramsdell, D. C., Hoopingarner, R. A., and Hancock, J. F. 1991. Honeybee foraging behavior, in-hive survival of infectious, pollen-borne blueberry leaf mottle virus and transmission of the virus in highbush blueberry. Phytopathology 81:1407-1412.

Factors associated with honeybees that facilitate the spread of pollen-borne blueberry leaf mottle nepovirus (BBLMV) to highbush blueberries were examined. These factors included the longevity of infectious BBLMV in the honeybee colony and in-hive pollen transfer within and between colonies. Blueberry leaf mottle virus remained infectious for at least 10 days within colonies of the honeybee, which is the primary pollinator. Flowering blueberry plants were caged for various periods with colonies of honeybees derived from a blueberry farm where a high percentage of bushes were infected by BBLMV. Twelve of 84 plants later tested positive for BBLMV by enzyme-linked immunosorbent assay. Counts of pollen grains washed from "house bees" (bees that never left the colony) resulted in an average of 5,149 pollen grains per bee, indicating that in-hive pollen-virus transfer occurs and can be a source of spread of virus-contaminated pollen within a colony. Evidence of pollen transfer between colonies and to a lesser extent between apiaries was also documented by observing the drifting (wandering far away from the bee's hive and/or visiting other hives) behavior of honeybees. Only 42.6% of the total foragers of a colony originated from their own colony and 2.4% of them were from colonies located 600 m apart. This drifting phenomenon could contribute to the maximum spread of BBLMV by bee-to-bee contact and multiple colony visits.

Additional keywords: Apis mellifera, Vaccinium corymbosum.

Associations between plant viruses and pollen have been known for over fifty years (4). Since that time, virus-pollen relations have been established for at least 46 different viruses (4). The two virus groups most often associated with this biological means of transmission are those belonging to the ilarvirus (12) and nepovirus (17) groups. In early studies of nepoviruses, Callahan (1) reported that elm mosaic nepovirus (a strain of cherry leaf roll virus) was transmitted to elm seedlings (Ulmus spp.) via pollen. Lister and Murant (21) observed transmission of raspberry ringspot and tomato black ring nepoviruses to raspberry, Rubus idaeus L., mother plants and seedlings via the pollen from infected plants. Raspberry bushy dwarf virus was shown to be transmitted by hand pollination from raspberry to raspberry, and the virus was shown to spread in the field using "infector" plants (26). Gilmer and Way (15) provided conclusive evidence that prune dwarf ilarvirus and prunus necrotic ringspot (PNRSV) ilarvirus were transmitted by pollen to seeds produced by healthy sour cherry trees (Prunus cerasus L.). The transmission of virus from

tree to tree via pollen was documented by George and Davidson (14) and Davidson (6) in studies involving bee and hand pollination of flowers. Cherry leaf roll virus from walnut was shown to be transmitted to nuts and seedlings of English walnut (*Juglans regia* L.) by hand pollination with diseased pollen (25). Cherry leaf roll virus was also shown to infect birch (*Betula pendula*) embryos as a result of hand pollination with infected pollen (5). Virus movement from plant to plant via pollen may have a devastating effect in a perennial crop in which all plants could become infected over time.

In studying the virus-pollen interaction, plant virologists have focused mainly on associating a particular virus with pollen using electron microscopy or pollen washes, followed by enzyme-linked immunosorbent assay (ELISA). Little information is available on the spread of virus via pollen from plant to plant. One exception is a study by Mink (24) that investigates stored pollen in honeybee colonies and the long-distance spread of PRNSV from California to Washington sweet cherries as a result of interstate movement of hives.

In the apiculture literature, many studies pertaining to honeybee foraging behavior and pollination have been reported. These studies have utilized several techniques to ascertain the origins of bees foraging on particular plants. Gary et al (13) used a magnetic retrieval system to study the distribution of honeybees foraging almond trees during pollination. He concluded that the distance honeybees flew to forage was dependent on the foraging competition within the orchard. Bees foraged at a greater distance from their colony when the number of colonies at their apiary was high. Other researchers have used radioisotopes (20), genetic markers (29), and fluorescent pigment (19) to study the foraging behavior of honeybees in a variety of pollinating systems. The drifting (wandering) of bees has been studied between adjacent colonies in an apiary (10). However, according to an extensive literature search, no drifting studies have been done over long distances, and no studies have involved an infectious plant virus associated with pollen.

Blueberry leaf mottle virus (BBLMV) is classified as a nepovirus (27,28). Infective virus was associated with pollen collected from diseased blueberry, and BBLMV antigen was detected in triturated pollen (2). It has been shown that the honeybee (*Apis mellifera* L.) can transmit BBLMV from blueberry to blueberry during pollination (3). BBLMV causes a devastating disease to at least three cultivars of highbush blueberry (Jersey, Rubel, and Blueray). The symptoms are most severe in Rubel. Stems are killed to the ground. Sparse regrowth from the crown exhibits leaf distortion and mottling. Yield is nil. In Jersey, stem-kill occurs, but leaf symptoms are very mild. The regrowth from the crown exhibits shortened internodes and rosetting of leaves which are small and yellow-green instead of deep green. Yield is greatly reduced. The symptoms in Blueray are similar to Jersey. Blueray is of minor importance in Michigan. Jersey is the most important cultivar in Michigan because it makes up 45% of the total acreage. The disease causes extreme reduction of the bearing surface of bushes, 75–100% crop loss, and eventual bush death. The causal virus has been detected and serologically diagnosed in over 15 fields in the state (D. C. Ramsdell, *unpublished data*).

The importance of the honeybee as a pollinator for highbush blueberries has been well documented (9). Michigan blueberry growers utilize colony numbers ranging from 2.5 to 10 per hectare to ensure adequate pollination (22). Due to the necessity of the honeybee for pollination and proper fruit development, it is impossible to remove the vector from the blueberry system. Therefore, we must understand the factors that promote the spread of BBLMV, so possible means of decreasing the spread of the virus can be devised.

The factors associated with the honeybee colony and the foraging bee that contribute to the spread of BBLMV include, but are not limited to: longevity of infectious BBLMV within a colony; the in-hive transfer of virus and/or pollen from bee to bee; and the transfer of virus between colonies via honeybee drifting (movement of foragers from their own colony to adjacent colonies). This study was initiated to examine the role of honeybee

colonies and foraging bees in the spread of BBLMV to highbush blueberry (*Vaccinium corymbosum* L.) under field conditions and to develop strategies to minimize the spread of virus in spite of the necessary widespread use of honeybees in commercial blueberry culture.

## MATERIALS AND METHODS

**BBLMV longevity and transmission.** At the start of bloom, three bee colonies were placed at an Ottawa Co., MI, blueberry farm near West Olive known to have a large number of cv. Jersey blueberry bushes infected with BBLMV. The colonies were kept at this location until petal fall (for 2 wk), at which time they were returned to East Lansing, MI, and individually placed in cages with seven healthy flowering 2-yr-old potted Jersey blueberry bushes. Seven additional flowering bushes were introduced to each cage on days 3, 6, and 10 of the study. All plants were kept in the cage until petal fall, a period of 2 wk. Additionally, a control cage containing a colony that had not been used for blueberry pollination was established at the same time. Four healthy flowering bushes were placed in this cage following the same time schedule as noted above. A total of 100 potted, healthy, virus-tested plants were used in the experiment. Eighty-four bushes were caged with colonies from the infected field, and 16 bushes were placed in the control cage.

At the petal fall stage the plants were removed from the cages and placed in a greenhouse until leaf drop in the autumn (a period of 4 mo), at which time they were placed in cold storage for vernalization for 8 wk at 4 C. On their removal from cold storage, (approximately 10 mo after exposure to bees), all plants were allowed to develop leaves and were tested for BBLMV using an established ELISA protocol (2) with the following alteration: the leaf tissue was homogenized in extraction buffer, filtered through cheese cloth, and the filtered extract was collected and kept at 5 C overnight before plating. This procedure did not alter the ELISA absorbance ($A_{405nm}$) values of the healthy controls (compared to samples directly plated after filtering) but did produce higher absorbance values from the infected samples. These higher absorbance values may have been due to the release of virus particles that were sequestered or bound to plant material. Leaf tissue from plants that tested ELISA-positive for BBLMV was mechanically inoculated onto *Chenopodium quinoa* Willd. for verification of virus infection. The infected blueberry tissue was ground with a mortar and pestle in a 0.01 M phosphate buffer (pH 7.2, containing 2% nicotine [v/v]) and rubbed to Carborundum-dusted plants. Plants that developed symptoms typical of BBLMV infection 7–10 days after inoculation were tested by ELISA.

**In-hive pollen transfer.** One drone bee and four house bees (nonforaging bees) were collected from colonies used for blueberry pollination in a commercial field near Dougles, MI, as described later. To distinguish house bees from foragers, colonies were fitted with fluorescent pigment dispensers that marked bees as they entered or left the colony. The dispensers were a modification of those previously described (8). Bees that were not marked with pigment were considered to be house bees. The bees were individually stored in vials and kept frozen until pollen counts were made.

The pollen was rinsed from the bees by adding 20 ml of 50% ethanol to the vial containing the bee. The vial was then gently agitated so that the bee was washed with ethanol. Then, the bee was removed and the ethanol-pollen mix was filtered through a 0.45-μm filter. Increasing concentrations of ethanol (75, 95, and 100%) were passed through the filter to dehydrate the pollen and prepare the sample for scanning electron microscopy (SEM). After the last 100% ethanol treatment the filters were fixed to a 10-mm aluminum stub with a "Double Stick" tab and placed in a desiccator to dry. The stubs were gold-coated for 3 min at 20 mA (about 21 nm of gold layer) and examined using a JEOL 35CF SEM (15 kV, magnification of ×400). The stubs were scanned and all pollen was counted. Blueberry pollen was readily identified by the fact that it is in tetrads, which have

a mean diameter of 33.4 $\mu$m and a diameter range of 24–41 $\mu$m (23). Pollen exine patterns can be used to determine a particular cultivar if one has access to a scanning electron microscope.

**Honeybee drift within and between apiaries.** Two apiaries (six colonies at each location) were established in a single row facing south (2 m spacing between colonies) at an Allegan Co., Michigan blueberry farm (Fig. 1) near Douglas. One apiary (colonies labeled 1–6) was located at the northern 2-ha planting made up equally of cvs. Rubel and Jersey. The other apiary (colonies labeled 7–12) was located 600 m away at the southern 1.5-ha planting and also included equal amounts of cvs. Rubel and Jersey. The two plantings were separated by a stream and wooded area. The odd-numbered colonies were fitted with dispensers that marked the thorax of foraging bees with fluorescent colored pigment as the bees entered or left the colony. Each marker colony's dispenser marked the bees with a colored pigment that was unique for that particular colony, allowing for the determination of the forager's origin. A forager is defined as any bee that flew from the colony. The even-numbered colonies were also fitted with dispensers but contained no pigment. Three 1-min counts recording the number and color of marked foragers as well as the number of unmarked foragers entering or leaving each even-numbered colony (unmarked colonies) at the two sites were made on 6 days of the 12-day blueberry flowering period. A mean of 54.4 bees were counted and classified at each observation.

## RESULTS

**BBLMV longevity and transmission.** ELISA tests indicated that twelve of the 84 healthy Jersey plants that were caged with colonies previously exposed to BBLMV became infected. Figure 2 shows the percentage of the total number of infected plants corresponding to the day the plants were introduced into the cage. There is no apparent trend with respect to the day the plants were caged with the infected colonies and the number
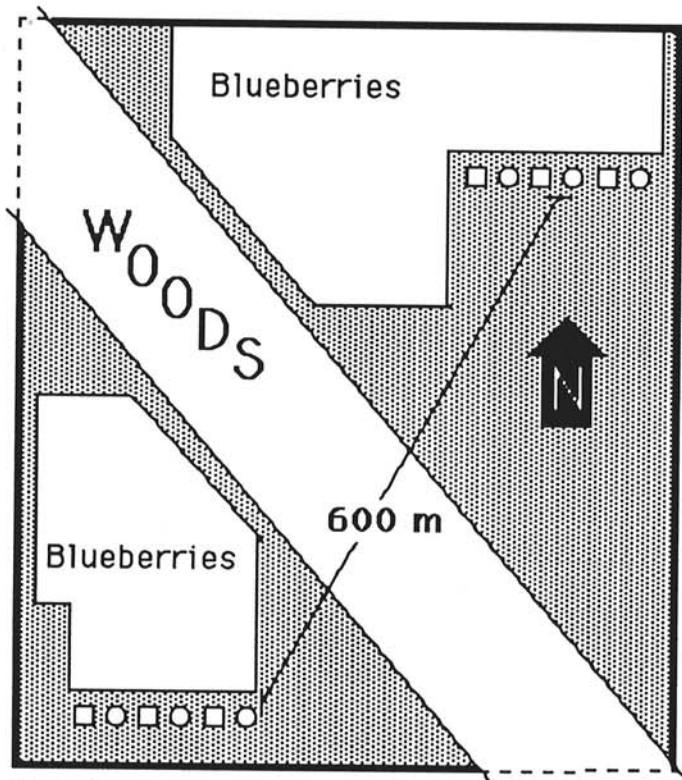
of plants that became infected. None of the blueberry plants showed symptoms of blueberry leaf mottle disease. This is not unexpected, because plants may remain symptomless for as long as 4 yr (28). The results demonstrate that BBLMV was infectious and accessible to bees for transmission to healthy plants for at least 10 days in the colony.

The ELISA absorbance values of the 12 infected blueberry bushes, the day the bushes were placed in the cage, and the results of the mechanical inoculation of leaf tissue from BBLMV-infected blueberry to *C. quinoa* are shown in Table 1. Only six of the 12 *C. quinoa* plants, which were mechanically inoculated with plant tissue from blueberries that tested ELISA-positive for BBLMV, were infected. High summertime greenhouse temperatures (up to 34 C) may have interfered with maximum transmission of BBLMV to *C. quinoa*. None of the 16 plants that were caged with the control colony were infected.

**In-hive pollen transfer.** The house bee pollen counts from four individuals resulted in counts of 6,034, 5,872, 5,631, and 3,059, or a mean count of 5,149 (SD = 1,403) pollen grains per bee. A nonworker drone bee also had a pollen count of 7,024 grains. These bees were not marked with fluorescent pigment and had not left the colony. Thus, any pollen found on these bees was the result of in-hive pollen transfer. More than 90% of the pollen found on bees was blueberry pollen.

**Honeybee drift within and between apiaries.** A total of 3,115 drifting foragers were observed on the 6 days of data collection. Figure 3 shows the percentage of marked and unmarked foragers found at the entrances of the unmarked colonies within each apiary location. The data are means of all counts made during the flowering period. The colonies placed in the row (colonies 2 and 4 at the north apiary and colonies 8 and 10 at the south apiary) had 62.6 and 57.4%, respectively, of their foragers originating from colonies other than the unmarked colonies. The colonies at the ends of the row (colonies 6 and 12) had the greatest number of unmarked foragers. However, 46% of the foragers from colony 2 and 32.4% of those from colony 12 originated
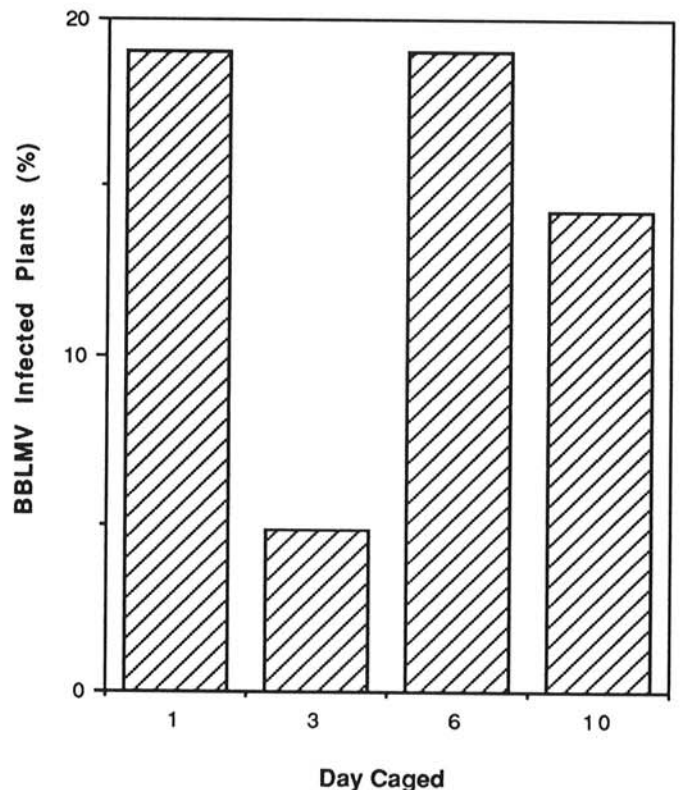


**Fig. 1.** Arrangement of north (top) and south (bottom) apiaries used for examining the drift of honeybees within and between apiaries. The north apiary colonies are numbered 1–6 from left to right. The south apiary colonies are numbered 7–12 from left to right. Squares represent marker colonies (odd-numbered colonies) and the circles represent the unmarked colonies (even-numbered colonies). Colonies are separated by 2 m within the apiary and 600 m between apiaries.



**Fig. 2.** Percentage of the total number of caged plants (21 per time period) that tested ELISA-positive for blueberry leaf mottle nepovirus (BBLMV). Flowering Jersey plants were caged 1, 3, 6, and 10 days after cages containing colonies and BBLMV-infected pollen were established.

from neighboring colonies.

The drifting of bees was not confined to the apiary where the colonies were located. Figure 4 shows the percentage of bees that originated from one apiary and were part of the foraging force observed at the unmarked colonies in the second apiary 600 m away. The results are a mean of all counts made during the study.
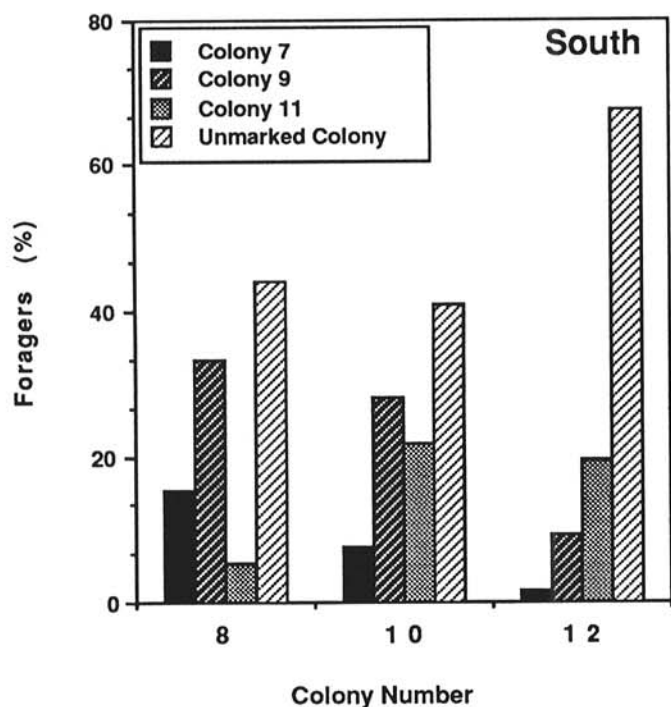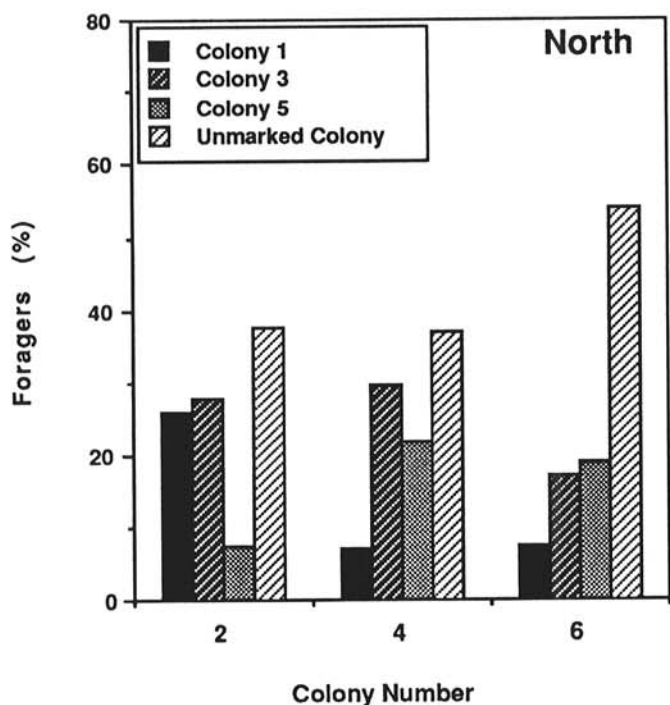
The number of bees that drifted between apiaries declined over the duration of the experiment. However, bees that originated 600 m away were found in all colonies, except one, 12 days after the colonies were established.



**Fig. 3.** Percentage of marked foragers (from odd-numbered colonies) within north and south apiaries found at entrances of the even-numbered unmarked colonies (See Fig. 1 for diagram of apiaries and colonies within them). Apiaries were located 600 m apart. Results are the mean of six, 3-min counts made over 12 days of the blueberry flowering period. "Unmarked colony" refers to bees that either were entering their own unmarked colony or bees from a nearby unmarked colony.

TABLE 1. Infection results of healthy potted Jersey blueberry bushes that were caged over time with bees and a bee hive obtained from a blueberry leaf mottle nepovirus (BBLMV)-infected blueberry field and presumed to contain BBLMV-infected pollen

| | ELISA values ($A_{405 nm}$) | | |
|---|---|---|---|
| Day[a] | Healthy controls[b] | Caged plants exposed to infected pollen[c] | Infectivity on *Chenopodium quinoa*[d] |
| 1 | 0.108 | 0.169 | − |
| 1 | 0.108 | 0.200 | + |
| 1 | 0.253 | 0.538 | + |
| 1 | 0.253 | 0.613 | + |
| 3 | 0.330 | 0.378 | − |
| 6 | 0.253 | 0.683 | + |
| 6 | 0.108 | 0.168 | − |
| 6 | 0.113 | 0.432 | − |
| 6 | 0.330 | 0.372 | − |
| 10 | 0.253 | 0.536 | + |
| 10 | 0.330 | 0.394 | − |
| 10 | 0.253 | 0.570 | + |

[a] Healthy 2-yr-old potted Jersey plants (three, seven-bush replicates/time period) were placed in mesh cages with a hive containing bees and BBLMV-infected pollen at day indicated after the infected pollen source was placed in the cage. Plants shown here are the 12 bushes that developed infections based on ELISA (enzyme-linked immunosorbent assay).

[b] Mean $A_{405 nm}$ value + 3 SD of four healthy control plants placed in a cage with bees and a hive containing uninfected pollen.

[c] Mean $A_{405 nm}$ value (duplicate wells) of leaf extracts from each test plant after exposure to bees carrying BBLMV-infected pollen, followed by incubation in isolation for at least 6 mo.

[d] Results of mechanical inoculation with triturated plant tissue from ELISA positive test bushes to *Chenopodium quinoa*. Infectivity was based on development of terminal leaf mottle/collapse symptoms characteristic of BBLMV in *C. quinoa*, followed by confirmatory tests by ELISA.
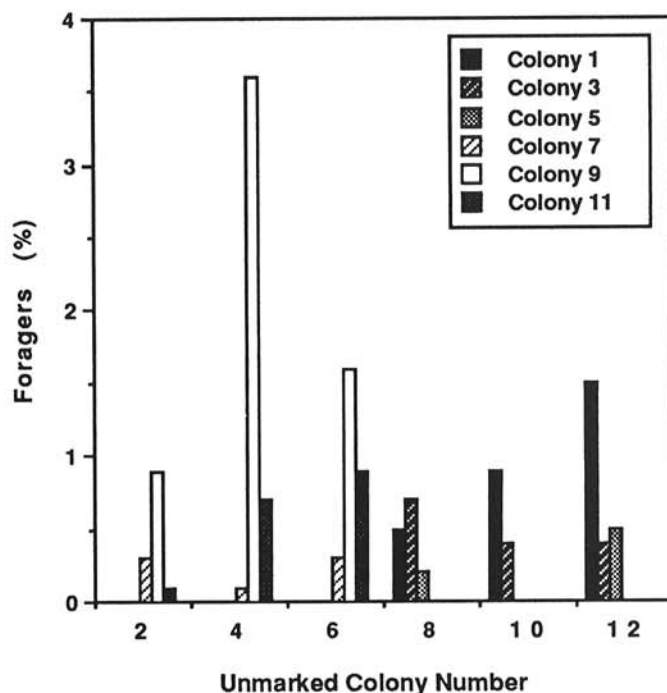


**Fig. 4.** Percentage of foreign foragers (marked honeybees) originating 600 m away that were found entering unmarked colonies. Colonies 1–6 were located at the northern apiary and colonies 7–12 were located at the southern apiary.

# DISCUSSION

Michigan is a major producer of highbush blueberries (7,300 ha) with a high concentration of farms in the southwest region of the state. Many of these farms border one another, and the spread of a pollen-borne virus via honeybee colonies used for pollination is probable. The results presented in this paper provide information on the behavior of the honeybee colony and foraging bees that could facilitate the spread of BBLMV.

The phenomenon of bee-to-bee spread of virus-infected pollen, coupled with visits by bees to several colonies, some as far as 600 m, could lead to rapid spread of BBLMV from one or more infection foci in a plantation.

The longevity of infectious BBLMV within a honeybee colony would allow for long distance spread of the virus. Infected colonies used for pollination in one area of the state and then moved to a farm in a later blooming area could transfer the virus over 100 miles. In a previously reported study by Mink (24), the long distance transfer of PNRSV from California to Washington was examined. He found that 20 of 40 hives tested contained PNRSV in infectivity tests on *C. quinoa* with pollen that had been stored in hive cells for 3 days. In the same study, a hive from a California almond and cherry orchard containing infected trees was caged with a healthy cherry tree in Washington within 24 h after leaving the California orchard. No apparent transmission of PNRSV occurred. In the present study, stored pollen was not tested for virus, because pollen stored in the cells of the hive is inaccessible to bees for possible virus spread. Stored pollen is used as a protein source for the larvae of the colony (16). Instead, the BBLMV-infected colonies were allowed to pollinate healthy plants in a cage. Plants placed in the cage up to 10 days after the colonies were removed from the virus field source became infected with BBLMV. These results indicate that the colony serves as a reservoir of virus; bees inadvertently move virus-infected pollen from insect to insect in the hive. The result is spread of BBLMV from the hive to previously healthy bushes.

Our results are in agreement with those of Free and Williams (11), who conducted extensive studies determining the number of pollen grains on nonforaging bees. The transfer of pollen within the hive from bee-to-bee has also been documented in an apple orchard system (7). Because the virus is associated with pollen and pollen exchange occurs between bees, the spread of virus from one plant to any plant in the foraging range of the colony is possible. The foraging range of honeybees is dependent on many environmental factors and conditions within the colony but may be as great as 4 km (11). Thus, virus transferred between bees foraging in opposite directions could spread 8 km from the infected plant.

The large number of drifting foragers observed during this study would suggest that virus transfer occurs not only within a single colony, but between all colonies within an apiary. The drift of foragers between apiaries would also promote the spread of BBLMV to adjacent blueberry fields.

It is not known whether BBLMV was vectored by bees that injured blossom tissues during pollination, thus allowing direct inoculation by deposition of infected pollen in wounds, (pollen bears the virus both externally and internally [2]), or by ovule infection following fertilization by infected sperm.

The current recommendations for the control of BBLMV is to rogue out all infected plants as soon as the virus is detected visually or by ELISA. This study indicates that additional steps can be taken to minimize the spread of BBLMV to highbush blueberries. Honeybee drift can be minimized by arranging colonies of an apiary in a serpentine pattern, or in a circle, with all colony's entrances facing different directions (18). This arrangement may give bees a better reference as to the location of their own hive. However, no sure method of controlling long range drifting is known. The prior location of the colonies used for pollination must be known and colonies used previously in blueberries or located near blueberry plantations should be avoided. The most effective means of controlling the spread of BBLMV is to identify all infected plants by ELISA tests, kill

and remove them, and replant with a cultivar resistant to BBLMV. Presently, the only cultivars known to be susceptible to BBLMV are Jersey, Rubel, and Blueray. Studies are underway to identify suitable virus-resistant replacement cultivars.

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