Insect visitation and pollination of a culturally significant plant, Hopi tobacco (*Nicotiana rustica*)

**Highlights**

- Open pollination benefits the reproduction of culturally significant plant, *Nicotiana rustica*
- Fruit weight of *N. rustica* is significantly higher with diurnal pollination
- Small bees are the most frequent floral visitors to *N. rustica*
- A new plant-pollinator interaction between *N. rustica* and Halictid bees is reported
Insect visitation and pollination of a culturally significant plant, Hopi tobacco (Nicotiana rustica)

Shelby D. Gibson,1,3,* Kennedy S. Halvorson,2 Lisa Myers,2 and Sheila R. Colla2

SUMMARY
Nicotiana rustica is a monecious member of the Solanaceae family, distributed across North America where it is grown for ceremonial purposes. Flowers of N. rustica open in the morning and are receptive to pollen until the following day. This study investigates the role of diurnal and nocturnal pollinators in reproductive success (seed weight, seed set, seediness) and insect visitation rate in N. rustica in eastern North America using floral bagging techniques. Results show that N. rustica benefits most from open (open day and night) and day open (closed at night) pollination. Fruit weight was higher with the presence of diurnal pollinators; fruit set and seediness were unaffected. Video recordings show that the most abundant floral visitors are small bees. These results provide information that will be useful in making management decisions surrounding the continued growth of this culturally significant plant species.

INTRODUCTION
Intimately connected to many Indigenous cultures and considered sacred, Hopi tobacco (N. rustica L.) continues to play an important role in traditional, ceremonial, spiritual, social, and medicinal customs across North America.1–3 The plant’s importance cannot be overstated; Haudenosaunee and Cree creation stories feature tobacco as among the first plants gifted to the earth, and in Anishinaabe culture, Semaa/Asemaa (tobacco) is a kind of bagijigan, or offering, meant to foster relationships between humanity and the spiritual world.4 Bagijigan has been referred to as the currency of life and are included at many daily and special events.4 While commercial tobacco (Nicotiana tabacum) has sometimes been used for ceremonial purposes, many people in the past decade have been working to re-establish the practice of growing and increasing the availability of traditional tobacco species like N. rustica, in an effort to reduce recreational usage, promote ceremonial use, and rekindle sacred relationships.5 Tobacco also plays an important role in Indigenous research methodologies, where offering the plant to the land upon which the research will take place, or to the participants within the study, can be an integral step in conveying the respect and commitment necessary to begin the research in a “good way”6(p32). Better elucidating the ecology of N. rustica and filling knowledge gaps surrounding the plant’s reproduction represents one way to help ensure its continued growth on the landscape and promote its cultural use in North America.

N. rustica L. (Alternate names: wild tobacco, Aztec tobacco, Native tobacco, Zuni tobacco, Mapacho, Semaa, Asemaa) is a dicot member of the Solanaceae family7 (Figure 1). N. rustica is a forb/herb with an annual life cycle, introduced to Canada and the lower 48 states of the United States through Indigenous trading routes.7,8 N. rustica is a very recent hybrid (~0.5 million years) from Nicotiana undulata and Nicotiana paniculata.9 N. rustica is a monoecious plant that was reported to produce up to two-thirds of its seeds through self-pollination, although it is also known to be visited by bees.10 Rates of self-pollination were later noted to differ based on the relative distance between the stigma and ring of anthers (heterostomy or herkogamy).11 Based on findings by Breese,11 rates of outcrossing varied between 0 and 70%, and were inferred to be under genetic control responding to changing environmental conditions. In a study in Delhi, India, N. rustica flowers were reported to open at 8:00 a.m. with the release of nectar available to pollinators.12 Anther dehiscence occurs a couple of hours prior to anthesis and self-pollination occurs during the process of anthesis.13 Flowers remain receptive to pollen until the following day.12 It has been documented repeatedly in the literature that flowers open >12 h are exposed to both diurnal and nocturnal pollinators as well as diurnal and nocturnal pollinators as well as diurnal and nocturnal pollinators.
Some species of Nicotiana are known to be night-time pollinated. However, little is known about the pollination of *N. rustica*, such as when pollination occurs.

Pollination effectiveness, parental plant genetics, and environmental conditions are all known to affect the size of *N. rustica* seeds. Pollination effectiveness refers to the % of conspecific pollen deposited on stigmas and has a positive correlation with high-quality seed production in *N. rustica*. Understanding how to achieve optimal seed production is useful in growing healthy plants, and plants being grown for seed should be grown under optimal controlled conditions. The few available breeding system studies performed with *N. rustica* have shown both self-incompatibility, and self-compatibility. The plant has shown to have low to intermediate reliance on pollination based on studies of hand-pollination in a greenhouse setting; however, the pollination biology of *N. rustica* remains poorly studied. Raguso et al. suspected bees or moths as *N. rustica*’s main pollinators based on the plant’s morphology. Tiedge & Lohaus characterized *N. rustica* as a day-time flowering plant pollinated by bees in the family Apidae, citing the work of Raguso et al. and that the plant has (1) flowers open during the day and at night, (2) corollae which are white, yellow, green, or pink, (3) and low scent intensity. Note, however, Raguso et al. found scent increased 2-fold at night and was predominantly composed of benzaldehyde, a known chemical attractant for both pollinators and florivores. With these plant-pollinator relationship predictions based on the currently contested theory of pollination syndromes, it is important to complete a study directly observing the ecological interactions of *N. rustica*. There have been no studies that document pollinator activity on *N. rustica* in a field setting with the aim of determining important groups of pollinators for the plant’s reproduction.

This study examines the role of diurnal and nocturnal pollinators in the reproductive success of *N. rustica* using fruit weight (g), fruit set (%), and seediness (estimated average number of seeds per pod). Here it is hypothesized that *N. rustica* benefits most from diurnal pollination, based on the previous work and predictions of Raguso et al. and Tiedge & Lohaus, as it is known to flower during the day and have floral...
traits compatible with pollinators active in the daytime. It is predicted that plants that receive diurnal pollination will have higher reproductive success than those that receive nocturnal pollination. Accordingly, insect visitation is predicted to peak during the day.

RESULTS

Bagging experiment
Four treatments were included in the bagging experiment (Figure 2). Fruit weight, fruit set, and seediness all varied significantly with pollination treatment (Figure 3). Fruit weight was not independent of pollination treatment which varied significantly between treatments (P=<2e-16, F = 54.19, df = 3). A post-hoc test showed day open, night open, and open to have higher fruit weight than closed. Day open had significantly higher fruit set than night open, as did open. Day open and open were not significantly different from each other. Fruit set also differed between treatments (p = 0.0281, F = 3.258, df = 3). Open and closed were the only two treatments that differed significantly. Lastly, seediness varied with pollination treatment (p = 0.0109, F = 3.327, df = 4). Day open, night open, and open all differed significantly with the closed treatment. Seediness also was higher in Round 1 than Round 2 (p = 0.000292, F = 7.38, df = 57).

Insect visitation rate
A total of 781 plant-flower visitor interactions were documented during 334 h of video recording. Small bees were the most common visitor (59% of interactions), and bumble bees were the second most common visitor (30%) (Figure 4). The least common visitors were honeybees (1) and butterflies (1) (Figure 4). These visitors were most often caught on the video footage interacting with the flowers of N. rustica (Figure 5). The average visitation rate was highest in the late morning sessions and lowest at night (Figure 6). Voucher specimens collected were identified to species level with the three most common species being Augochlorella aurata (28), Lasioglossum perpunctatum (12), and Bombus impatiens (7) (Table 1). Insect visitation rate differed significantly between pollination treatment (p = 0.0109, F = 3.327, df = 4, 0.05954). The main difference was late morning which was significantly higher than night (p = 0.0229704). Insect visitation rate also differed significantly between round with Round 1 having significantly higher IVR than Round 2 (p = 1.08^15, F = 67.22, df = 734).
DISCUSSION

This study confirms that fruit weight, fruit set, and seediness are all increased in a field setting when *N. rustica* is exposed to open pollination. The difference between diurnal and nocturnal pollination is reflected in the difference in fruit weight between flowers open for pollination during the daytime compared to those open overnight. Fruit weight did not differ between the unbagged, continuously open flowers and flowers only open during the daytime, which has been previously reported in the literature, and highlights the importance of diurnal pollinators. Adler et al. found that in a greenhouse setting *N. rustica* had an intermediate reliance on pollinators, where the plant was self-compatible but had increased reproductive success when exposed to pollination.

Fruit set and average seediness per pod do not reflect a similar trend. Flowers available for pollination overnight versus during the daytime do not significantly differ for the attributes of fruit set and average seediness per pod. The difference in fruit set between unbagged flowers and those continuously bagged to exclude pollinators shows that fruit set is increased significantly by insect pollination. However, there is no clear difference between the timing of pollination, since the flowers that were always available to be pollinated were not found to be significantly different from those that were only available overnight or during the daytime. Neither diurnal nor nocturnal pollinators are the dominant pollinator group.

All treatments with flowers open to visitors had on average higher seediness than treatments excluding pollinators. There is, however, no difference between the remaining treatments, indicating that neither day nor nighttime pollinators are more effective. While *N. rustica* plants were able to produce seeds without pollination (closed treatment), treatments open to pollination had a significantly higher number of seeds, consistent with the observations that the plants are self-compatible, but benefit from insect pollination. One factor affecting seed set may be that since *N. rustica* flowers open in the morning, the flowers are freshest and most receptive to pollen at this time. It is also possible that continuous nectar secretion during bagging could have led to artificially inflated nectar levels upon unbagging, which may have influenced the results. Seediness was significantly higher earlier in the season, corresponding with a significantly higher IVR at that time as well. This information helps to better characterize the assemblage of pollinators visiting *N. rustica* and contributing to the plant’s pollination.

Based on the findings of the video recordings, it may be the case that the diurnal visitors show an increase in fruit weight caused by their sheer abundance. It has been reported that while nocturnal visitors may be more effective per-visit, diurnal visitors show a higher reproductive success due to their abundance and frequency of visitation. Small bees had the highest abundance based on their visitation rate, and therefore may be the most effective visitors contributing to pollination. Small bees were the most commonly documented visitor, making up 59% of all recorded interactions. Since visitation rates can serve as a measure of abundance, we can conclude that small bees are the most abundant pollinators of *N. rustica*. While video recording was limited to dawn and dusk (crepuscular activity), when assessed in combination with the results of the fruit weight measures, it would appear that small bees are significant pollinators of *N. rustica*. Visitation peaked during late morning which is potentially related to when the anthers dehisced and pollen availability is highest, as has been the case in similar studies. Based on observations, the visits of bees were shorter in duration than those by moths. There was one video session that lasted all night due to a full moon, however it showed no moths during the middle portion of the night. It would appear that the nocturnal pollinators primarily are active just after dusk. A previous study using video recording of nocturnal visitors found a peak of activity between 21:00 and 24:00 p.m., a time frame frequently represented by the videos in this study. Awareness of the timing of bee activity may also be important for creating best management practices for growing *N. rustica* in a crop setting where insecticides may be used.

Since small bees were the most common visitor, pollination of *N. rustica* could be increased in the field by providing unmanaged land nearby for bee nesting and additional foraging resources, a suggestion made...
by other studies which have found bees to be the most effective pollinators of a particular plant. The most common species of voucher specimen collected was *A. aurata*. The conservation status of this species in Ontario is Secure, however globally it has not been evaluated. *A. aurata* is a ground-nesting eusocial species. In terms of providing suitable habitat, The Xerces Society recommends leaving bare patches of sandy soil for use by ground nesting bees. The second most common species collected from *N. rustica* was *L. perpunctatum*. *Lasioglossum* is a genus exhibiting a full range of social behavior and often are ground-nesters. Notably, these species are both members of the Halictidae family, revealing a pollination network different from Tiedge & Lohaus’ Apidae prediction. Providing suitable ground-nesting habitat in the form of bare patches of sandy soil will support wild bee populations as a whole and ensure the effective pollination of *N. rustica*.

Interestingly, although all of the sites in this study were located within a wider agricultural landscape, only one honeybee was seen visiting *N. rustica*. This suggests that the use of managed honeybees to increase the pollination of *N. rustica* plants may not be appropriate and that the plants benefit from wild bee activity. Further research is required to understand why honeybees were not common visitors of *N. rustica*. Since there is a mismatch between conservation science and management actions for bees with regard to bee

![Floral visitor abundance](image)

**Figure 4. Floral visitor abundance**
The overall abundance of each floral visitor category recorded during diurnal (274 h) and nocturnal (60 h) video recording of *N. rustica* between July and September 2020 across all sites.

![Most common floral visitors](image)

**Figure 5. Most common floral visitors recording during the insect visitation recordings**
Left to right: small bees, bumble bees, moths.
It is important to highlight the role of wild bee pollinators on *N. rustica* in the field. This information may be useful for ensuring the continued reproduction of this culturally significant plant which provides foraging resources for wild bees. Since many wild bees have been found to be in decline, or remain data deficient, understanding the role of wild bees to plants of importance can help to preserve both the plant and the pollinator together.

**Conclusion**

The results of this study highlight the importance of wild bees to the pollination of a culturally significant medicine plant, *N. rustica*. During this experiment *N. rustica* was able to set fruit without pollination (closed treatments), confirming that the plant is self-compatible, including when grown in a field setting in eastern North America. However, fruit weight (g) was significantly increased by any kind of treatment where flowers were unbagged and open to floral visitors, indicating that *N. rustica* benefits from insect pollination. With the Indigenous use of traditional tobacco species like *N. rustica* in ceremony and research increasing, this study broadens the general ecological knowledge of *N. rustica* and can help to inform potential strategies for improving the growth and harvest of this sacred plant. Those interested in cultivating *N. rustica* may find

**Figure 6. Average insect visitation rate**

Using a time-lapse video camera between July and September 2020 at all three sites, the average insect visitation rate was recorded on *N. rustica* during different times of day. Significant difference was found between late morning and night (p > 0.05). Error bars represent standard deviation.

**Table 1. Species list of voucher specimens collected on *N. rustica***

| Visitor                  | Count |
|--------------------------|-------|
| Augochlorella aurata     | 28    |
| Lasioglossum perpunctatum| 12    |
| Bombus impatiens         | 7     |
| Lasioglossum tegulare    | 5     |
| Augochlora pura          | 3     |
| Lasioglossum michiganense| 3     |
| Lasioglossum versans     | 3     |
| Bombus vagans            | 3     |
| Halictus ligatus         | 1     |
| Lasioglossum oblongum    | 1     |
| Halictus rubicundus      | 1     |
| Megachile frigida        | 1     |
| Sphecodes heraclei       | 1     |
better success if their management practices consider the habitat needs of small wild bees like A. aurata and L. perpunctatum, who require bare sandy soil patches for their ground nests, rather than seeking out the more commonly used and commercially available pollination services from managed honeybees. This research also reveals a previously unknown plant-pollinator relationship, that of the Halictidae family and N. rustica, and adds to the growing body of literature highlighting the importance of preserving wild pollinators and their respective ecosystem services (both ecological and cultural) on the landscape.

Limitations of the study

Our study was limited to crepuscular insect visitation (dawn and dusk) for the video recordings. Future research may include video recordings throughout the night which would provide a better understanding of the nocturnal visitators of N. rustica.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization, S.D.G., L.M., and S.R.C.; methodology, S.D.G. and S.R.C; investigation, S.D.G; formal analysis, S.D.G.; writing – original draft, S.D.G; writing – review & editing, S.D.G., K.S.H, and S.R.C.; funding acquisition, S.D.G., L.M., and S.R.C.; supervision, S.R.C.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited data      |        |            |
| Raw and analyzed data | This paper[^1] | ![DOI](https://doi.org/10.17632/kdc9fgfbrm.1) |
| Experimental models: Organisms/strains |        |            |
| *Nicotiana rustica* L. | Urban Harvest | N/A |
| Software and algorithms |        |            |
| RStudio | RStudio Team[^4] | (Version 3.6.2) |

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources can be directed to and will be fulfilled by the lead contact of this manuscript, Shelby Gibson (shelbydgibson@gmail.com).

Materials availability

This study did not generate any new unique materials or reagents.

Data and code availability

The original datasets for this manuscript have been deposited at Mendeley Data and are publicly available as of the date of publication. The DOI is listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant materials and site description

This study was conducted at three sites located near Ottawa, Ontario, Canada. Plot A was in Lanark, ON, and Plot B was in Pakenham, ON. Each plot was 20ft x 20 ft. Plot C was in Arnprior, ON – this site was too small for a breeding experiment but was used for the video recording. Round 1 of the experiment was conducted at Plot A between July 27th–August 9th, 2020. Round 2 of the experiment was conducted at Plot B September 11–18th, 2020. Experimental rounds were conducted during peak blooming which varied by location based on differences in external factors such as soil, sun, and wind exposure. *N. rustica* seeds were sourced from Urban Harvest (http://uharvest.ca/shop/) and were placed on top of the soil after the last frost in spring 2020 (May 25). Plots A and B were planted with 175 seeds. Seeds were watered until germination. Flowers bloomed late July into late September.

Land acknowledgement

This research was undertaken on the traditional territory of many First Nations. The sites located in Tkaronto, including the campus of York University, are located on the traditional territory of the Anishinabek Nation, the Haundenosaunee Confederacy, the Huron-Wendat, and the Métis. The current treaty holders in this location are the Mississaugas of the New Credit First Nation, and the land is subject to the Dish with One Spoon Wampum Belt Covenant. The study sites are located on unceded Algonquin territory and are subject to Treaty 27 and Treaty 27 1/4. We acknowledge the generations of caretaking by the many Indigenous peoples who have and still do call these places home.

METHOD DETAILS

Bagging experiment

To determine if there was a difference between diurnal and nocturnal pollinators, the bagging experiment included four treatments: open (open both day and night), day open (bag removed during daytime), night open (bag removed during nighttime), and closed (no visitation). Flower buds were bagged using small
mesh bags prior to anthesis to ensure no pollination had occurred prior to treatment.32,34,45,46 Bags remained in place until blooming was completed. Bags were made of Organza fabric (estimated 1-mm mesh size) and were 5 cm x 7 cm in size. Sunrise was between 4:39 AM and 4:55 AM during Round 1, and between 5:38 AM and 5:47 AM during Round 2. Sunset was between 19:24 PM and 19:43 PM during Round 1, and between 18:10 PM and 18:24 PM during Round 2. Bags were placed on the flowers prior to opening and bags were removed during the day to allow diurnal pollinators and removed at night to allow nocturnal pollinators (Figure 2). Bags were placed on the day open and night open treatments within 1 hour of the time of sunrise or sunset, coinciding with the changeover between diurnal and nocturnal pollinator activity.15,24 Flowers were randomly chosen to be included. The number of flowers on each branch was recorded every day throughout the experiment, and the number of pods developed was counted upon collection.19 Tobacco fruits were harvested once mature, approximately 21 days post-flowering.12,47 Reproductive success of the plants was determined by fruit set (number of fruits/number of flowers), fruit weight, and seediness.34,46 Seediness was estimated by weighing all seeds from all pods on one plant, dividing this by the total number of pods to get an average mass of seeds per pod (g), and counting the number of seeds within a subset of the sample (0.05g). The number of seeds in the subset of the sample (0.05g) was then factored against the average mass of seeds per pod (g), giving an average seediness per pod. This method was used because the seeds were too small to be counted by a seed counter, and N. rustica produces approximately 250–300 seeds per capsule.12

**Insect visitation rate and video recordings**

A time lapse video camera48 was used to record insect visitation.32 The camera was supported by a tripod and footage was recorded to a memory card. Continuous recording provides a near-complete documentation of floral visitors throughout blooming.46 Floral visitations and behaviour (i.e., visitation rate) were determined by analysing video recordings.32,48,49 Insects were tallied into the following categories: honey bee, bumble bee, small bee, fly, butterfly/moth, beetle, other, unless they could be identified to species.32,34 Crepuscular recordings included dawn and dusk when there was some limited natural light available. Insects that landed on the outside of the flower were not included in analysis. Only insects that entered and contacted the reproductive parts of the flower were included. Video recordings occurred around the time of the bagging experiment: July 30-August 9 (Plot A), August 31-September 9 (Plot B), and August 18-August 25 (Plot C). Video recordings were conducted earlier than the bagging experiment to ensure high-quality video, as significant dew began to form on the camera lens as the season progressed making the recordings difficult to interpret. Therefore, it is possible that the floral visitors are different between the bagging experiment at Plot B and the video recording at this location. In addition, bagging and video recordings occurred on the same plant, but not the same flowers, to avoid interference with the video by bags or at changeover time. A collection of voucher specimens was collected randomly from N. rustica throughout blooming during the time of video recordings (n = 70).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Generalized linear modelling**

Statistical analysis was conducted in RStudio (Version 3.6.2)48 on fruit weight, fruit set, and seediness amongst the various pollination treatments, as well as insect visitation rate at various times of day. The two rounds of the experiment were combined for pollination analysis to provide the most robust results, however the effect of round on fruit weight, fruit set, and seediness was also assessed. Fruit weight was assessed using a generalized linear model (GLM) fit to a Gaussian distribution with an identity link function. Fruit weight was the continuous response variable while pollination treatment was the fixed effect variable. Fruit set (non-continuous) was assessed using a GLM fit to a binomial distribution with logit link function. Seediness was the continuous response variable while pollination treatment was the fixed effects variable. For fruit weight, fruit set, and seediness, a Tukey post-hoc test was used for pairwise comparisons between treatments.50 This was then repeated using round as the fixed effect variable.

Average visitation rate was calculated as (# of visits/# of flowers/# of sites).32,34 An average visitation rate was found for each time of day (early morning, late morning, early afternoon, late afternoon, nighttime). The times of day were separated as follows: Round 1: Night (19:24pm-4:55am), Early AM (4:55am-9:00am), Late Morning (9:00am-12:00pm), Early Afternoon (12:01pm-16:00pm), Late Afternoon (16:01pm-19:24pm). Round 2: Night (18:10pm-5:47am), Early AM (5:47am-9:00am), Late Morning
(9:00am-12:00pm), Early Afternoon (12:01pm-16:00pm), Late Afternoon (16:01pm-18:01pm). Round 3: Night (19:07pm-5:10am), Early AM (5:10am-9:00am), Late Morning (9:00am-12:00pm), Early Afternoon (12:01pm-16:00pm), Late Afternoon (16:01pm-19:07pm). Insect visitation rate (IVR) was assessed using a GLM fit to a Poisson distribution with log link function. IVR was the continuous response variable while time of day was the fixed effects variable. A Tukey post-hoc test was used for pairwise comparisons between treatments. This was then repeated to determine significance of round on IVR.