# SOYBEAN AND BEES

Decio Luiz Gazzoni

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*Ministry of Agriculture, Livestock and Food Supply*

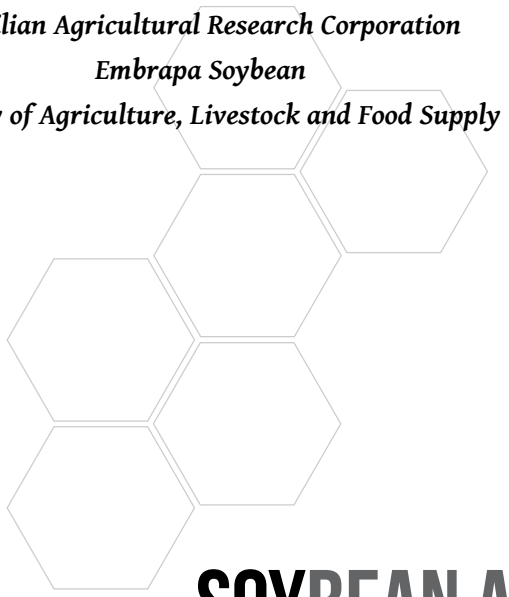

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Photo: Paulo Robson de Souza

This book is dedicated to my long time friend **Dr. Arnildo Pott**, a former Embrapa Scientist, presently a Botany Professor at the Universidade Federal do Mato Grosso do Sul. He is a world recognized and widely known botany scholar, whose wisdom, knowledge, scientific contribution, dedication and hardworking attitude I have always admired.

# FOREWORD

Brazil, aligned with international efforts, has conducted several studies and activities concerning conservation and sustainable use of pollinators. The loss of primary habitat of these agents, particularly the elimination of native vegetation for multiple uses, ranks as one of the greatest threats to ecosystem services of pollination. In terms of public policies, the Brazilian Forest Law, for instance, can potentially shift the game in favor of the environmental pollination service, by providing shelter and food for pollinators. Embrapa is committed to the study and the quantification of this contribution.

At Embrapa, the growing attention to this issue underscores the need to expand the use of technology, processes and structures to ensure that pollination services are favored. Studies are being developed for continuous monitoring and characterization of pollinators and their contribution to the agricultural production systems used in the country. Special consideration is required to monitor and study the consequences of deforestation and the inappropriate use of pesticides in agriculture, as well as other processes that might pose a disadvantage to the environmental service of pollination.

Furthermore, Embrapa understands that these have great value to agricultural production and the environment. The balance of ecosystems depends on soil fertility, cleanness of water and air, waste decomposition and recycling, as well as the slowdown in the extreme weather events and natural disasters. Therefore, every effort to understand and ensure the integrity of environmental services highly ranks as beneficial to human existence on the planet.

Small-scale farming, conducted in small spaces or in organic production systems, can be performed without using pesticides, under certain circumstances. On the other hand, pesticides constitute an important input for large-scale agriculture, due to operational and economic reasons, although its application should follow the recommendations of Good Agricultural Practices. Therefore, production systems should be improved to favor the action of natural enemies and pollinators.

Consequently, the management of agricultural areas must rely on practices that favor the presence and permanence of beneficial insects in the cropping fields, with an emphasis on the populations of pollinators. The adoption of good farming practices such as integrated pest management often means lower production costs and less risk of disruption of ecosystems. It is also worth mentioning that Embrapa, historically, keeps guidelines to support the

continuous development and use of pest management programs, aiming to minimize the pest damage to the crops, the production costs and the negative environmental impact.

In accordance with the historic environmental concern of Embrapa embedded on the technologies developed by its scientists, its research program recently incorporated a research arrangement – a set of correlated projects – called POLIAGRO, which aims to harmonize existing and future production systems comprising the environmental service of pollination. Other arrangements of research projects such as ABELHA and SA (Environmental Services) largely overlaps with the central theme of the POLIAGRO.

The POLIAGRO will integrate existing research networks, or create new ones, involving institutes and universities in Brazil and abroad, under the coordination of Embrapa, generating processes and technologies to help farmers and subsidize public policies, focusing on creating a favorable environment for the pollination service. Besides being a widespread demand in society, the studies to be conducted under POLIAGRO are fully online with concerns of farmers and their organizations, as well as government agencies such as the Brazilian Ministries of Environment, Agriculture, Livestock and Food Supply, and Science and Technology.

The *Soybeans and Bees* book aims to deeply review and discuss the pollination process in soybeans, whose recent increasing productivity, founded on technological innovations, depends on efficient crop management. Soybean is the most important crop in the country, occupying large areas, constituting the highest consumption of pesticides – aspects that can negatively affect the ecosystem service of pollination, which, on the other side, benefits other cultures growing in adjacent agricultural landscape. An aspect that should also be considered is that soybeans is a cleistogamic plant, with low rate of cross pollination, but some studies point out to benefits when bees consistently visit their flowers, an aspect that must be definitively clarified.

The author correctly argues the need to expand the domain of diversity and seasonal abundance of pollinating bees in relation to differential morphological behavior among soybean cultivars, which is crucial to set up a mitigation strategy for the negative impact of pest control actions upon the pollination service.

In my opinion, the effort represented by this book is justified by the economic and environmental importance of soybean production, a crop that has expressively grown over the past three decades and accounts for almost 50% of the grain areas in Brazil. Mainly cultivated in the Central West and South regions, soybean enables a complex consisting of beans, meal and oil, and stands as one of the most traded products in the Brazilian trade balance. The

Ministry of Agriculture, Livestock and Food Supply foresees a 45% increase in Brazilian soybean production by 2019, supported by the expansion of both international demand and domestic consumption, not only for food production but also for industrial purposes, including biodiesel production, a reason for continuously improving the sustainability of soybean production systems.

The effort made by Dr. Decio Luiz Gazzoni in the organization of this book is widely welcomed. With detailed information, presented in an objective, didactic and illustrated way, this publication offers producers, technicians, students, government officials and other interested citizens data and reflections indispensable to expanding the knowledge of the interactions between pollination by bees and soybean cultivation. Performing the harmonization of the ways, means and times needed by the pollination service, it will also be possible to program the application of pesticides in such a way not to harm the pollinators as well as to mitigate present and potential damage to the equilibrium of ecosystems.

***Maurício Antônio Lopes***
President of Embrapa

# PREFACE

Soybeans (*Glycine max* (L.) Merrill) are host of several insect pests along its cycle, since germination to the maturity stage. Exception made for the germination to seedling stage, soybean yield and the quality of seeds and grains are far more affected during the reproductive stage when pods are present, as compared to the vegetative or flowering stages. Pests attacking soybeans during the vegetative stage are chiefly defoliators (lepidopterous and coleopterous), while pod feeders (stinkbugs and pod feeders or pod borers) are more important from pod set to maturity. During the flowering period, before pod set, only defoliator insect pests are considered harmful to soybeans. Nevertheless, this is not true for indeterminate cultivars, because there is an overlapping period of ca. 15-20 days of blooming with pod set and fill and even the beginning of seed fill. In this case, pod feeders, especially stinkbugs, may show up large populations, beyond the action level, requiring a pest control measure. In this moment, care should be taken to avoid or minimize the impact over pollinators.

The soybean plant is hermaphrodite, producing perfect flowers with functional male and female parts. The anthers produce pollen and ovules develop in the ovary in the same flower. If ripe pollen lands on a receptive stigma, the pollen can grow a tube through the style and the pollen nucleus with all of the genetic information can travel through the tube and combine with the egg in the ovule. The ovary protects and nourishes the zygote and supports the development of the embryo, endosperm and seed coat of the seed. In the base of the flower there is a nectary, which produces nectar, a highly nutritive chemical compound that attracts pollinators.

Soybeans are considered autogamous, cleistogamous and self-pollinating plants. When soybean flowers open, they are normally self-pollinated; the stigma of the pistil is completely covered by the anthers of the stamen, making it very difficult for exogenous pollen to reach it. The cross pollination is mentioned in the literature as normally occurring at rates around 2%, being the wind pollination negligible. Cross-pollination on soybeans is mediated by pollinators, normally insects, and especially bees. Bees collect nectar as their major source of energy (carbohydrates) and pollen as the major protein source.

Soybean flower abscission is very high, as the number of harvested pods are in the range of 10-20% of the number of opened flowers. According to the revised literature, abscising flowers were mostly all fertilized and usually contained proembryos that had undergone two or three cell divisions. In this case, apparently there is no interference of insect pests or lack of pollination on the abscission of soybean flowers.

The fertilization of soybean flowers usually occurs one day before or in the very day of flower opening, which theoretically reduces the dependency on pollination by insects, which is largely supported in the literature. Cross-pollination on soybeans usually do not surpass 2%, and is mediated by pollinating insects, normally honeybees. The bees concentrate its foraging on soybeans from 9 am to 3 pm.

Nevertheless, some authors have determined that assisted pollination by honeybees increases the soybean yield, especially due to a higher number of pods and more seeds per pod. Some studies on USA and Brazil found out that, when soybeans are caged with honeybees' colonies inside, yield could increase from 10 to 50%, as compared to caged soybean plots absent of bees. When a soybean yield increase was observed in the presence of bees, the number of filled pods, and the number of seeds per pod were higher than plots grown with absence of bees. There are also references mentioning that no yield difference was found on soybeans cultivated on the presence or absence of bees.

The literature mention a dominance of the honeybee, *Apis mellifera*, foraging on soybean fields, but several native species are also found. There is a clear need to establish the seasonal diversity and abundance of pollinating bees foraging on soybeans, to support the strategy for mitigating the impact of pest control actions upon the pollination service.

A single soybean flower remains open for 1-2 days. The blooming period of soybeans lasts about 15 days for determinate cultivars, and up to 30 days for indeterminate ones. In the case of determinate cultivars, when pods set, the blooming period finishes. In the case of indeterminate cultivars, blooming period extends during pod set and pod development, and might partially overlaps the seed filling stage. As for the present moment, indeterminate varieties are far more cultivated in Brazil, as compared to determinate ones.

These differential blooming behaviors between soybean cultivars is crucial for the compatibility of pollination on soybeans with the control of insect pests. Leaf feeder pests (coleopterons or caterpillars) can attack soybeans along the whole cycle, from early seedling stage up to physiological maturity. Pod feeders (caterpillars or stinkbugs) are considered pests only when pods are larger than 0.5 cm long. On determinate varieties, there is no need to control pod feeder pests during blooming, which is not true for indeterminate ones, given

the pest abundance and the level of action recommend by IPM practices. For these cultivars, a period of ca. two weeks is extremely critical, due to the overlapping of flowers and pods, at the same time, on soybean plants.

Honeybee visitation to a flower can be considered a two-stage process. At first involves orientation from a larger distance and then, secondarily, close-range orientation during which the bee alights and probes for nectar. Bees are guided to particular flowers by floral aroma, color, and shape. Floral aroma, color, and shape all therefore appear to influence initial honeybee visitation and provide foraging landmarks, which honeybees utilize to optimize foraging on a specific plant species.

Nectar is a powerful attractant of bees to a given flower. Nectar is a complex of carbohydrates, basically a solution of fructose, glucose, or sucrose in water with minute amounts of many other plant compounds like other carbohydrates, amino acids, proteins, mineral ions, organic acids, vitamins, lipids, antioxidants, glycosides, alkaloids, and flavonoids. Content of carbohydrate in the nectar may vary, from 4 to 60%, depending on species and on environmental conditions, and there is also a variation according to the time of the day, what may determine foraging hours.

The importance of olfaction in recruitment of forager honeybees has been well documented. Honeybees have large numbers of antennal placoid sensilla, which are the principal chemoreceptors for floral aromas. Indeed, it has been suggested that olfaction plays a more important role in forager recruitment than the dance maneuvers observed in colonies. Scent is more important in conditioning honeybees than color, form, or time of day, as honeybee discrimination was greater with a change of scent than with a change of flower pattern or shape.

At first, it is not fairly acceptable that increasing soybean yields would depend on cross-pollination, considering that on cleistogamic plants, when the flower opens it is normally fertilized. Natural cross-pollination in soybeans has been estimated to be low, ranging from about 0.03% to 3.62%. This low cross-pollination would indicate that the entomophilous pollination would represent very low impact on soybean yield. However, there is a controversy on the contribution of pollinators for increasing the yield and seed quality on soybeans. Some authors have found that the presence of pollinators, especially honeybees, in open field environment, or on caged soybeans, lead to the increment of soybean yield. This is still an open issue, because it is not the expected behavior of a cleistogamic, self-pollinating plant, with an average of only 2% cross-pollination. When larger soybeans yields were found, no physiological evidences were stated to support the findings.

In order to minimize the adverse impact upon pollinators of pest control measures that cannot be avoided, studies should be developed to define the most appropriate strategies. Among other should be mentioned that, during this period, it is paramount to strictly observe the IPM recommendations, controlling pests when absolutely necessary to avoid yield or seed quality reduction. Minimum rates of insecticides with less impact on pollinators should be used. Pesticide application should preferentially be performed during periods of the day when bee populations on soybean fields are lower, or even absent, especially early on the morning, late on the afternoon or at night. Additionally, components of the production systems deterrents to pollinators should be adequate, while those favoring the natural pollination services must be reinforced.

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# SOYBEAN CYCLE

Soybean (*Glycine max* (L.) Merrill, family Fabaceae, subfamily Faboideae) hosts several insect pests along its cycle, since germination to the maturity stage. Exception made for the germination to seedling stage, soybean yield and the quality of seeds and grains are far more affected during the portion of the reproductive stage when pods are found on the plants, as compared to the vegetative or flowering stages. Pests attacking soybeans during the vegetative stage (Table 1 and Figure 1) are chiefly defoliators (lepidopterous and coleopterous). Scientists have definitively demonstrated that soybeans can withstand severe defoliation rates, even up to 100%, prior to blooming, without loss of yield or quality. This ability demonstrates a sound resilience to these biotic stresses, given abiotic environmental (temperature and water) stresses are not present, and adequate cultural practices (soil management; plant nutrition; weeds, nematodes and diseases control) are observed (GAZZONI et al., 1978).

**Table 1.** Soybean growth stages.

**a. Vegetative stages**

| Stage | Stage title | Description |
|---|---|---|
| VE | Emergence | Cotyledons above the soil surface |
| V1 | First node | Fully developed leaves at unifoliolate nodes |
| V2 | Second node | Fully developed trifoliolate leaves at node above the unifoliolate nodes |
| V3 | Third node | Three nodes on the main stem with fully developed leaves beginning with the unifoliolate nodes |
| Vn | $n_{th}$ node | "n" nodes on the main stem with fully developed leaves beginning with the unifoliolate nodes. |

**b. Reproductive stages**

| Stage | Stage title | Description |
|---|---|---|
| R1 | Beginning bloom | One open flower at any node on the main stem |
| R2 | Full bloom | Open flower at one of the two uppermost nodes on the main stem with a fully developed leaf |
| R3 | Beginning pod | Pod 5 mm (3/16 inch) long at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R4 | Full pod | Pod 2 cm (3/4 inch) long at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R5 | Beginning seed | Seed 3mm (1/8 inch) long in a pod at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R6 | Full seed | Pod containing green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R7 | Beginning maturity | One normal pod on the main stem that has reached its mature pod color |
| R8 | Full maturity | Ninety-five percent of the pods have reached their mature pod color |

Source: Fehr and Caviness, 1971; 1977.

Photos: Decio Luiz Gazzoni

VE. (Germination and cotyledon).

V1. Unifoliolate.

V3. Two trifoliolate open.

V4. Three trifoliolate open.

V6. Five trifoliolate open.

**Figure 1.** Soybean vegetative stages.

Soybeans become more susceptible to insect pests attack during the reproductive stage, especially after blooming (pods are present on the plants). During blooming, the plant still withstands large defoliation rates (up to 50%), being more susceptible to foliar area loss-

es from pod set to physiological maturity (R7) (GAZZONI and MINOR, 1979; GAZZONI and MOSCARDI, 1998).

Nevertheless, insect attacking pods and seeds are far more noxious to the soybean plant than defoliators are. Their importance is limited to the R3-R7 soybean growth stages (Figures 2 and 3). Their attack might result in loss of entire pods and seeds, or reduced seed weight. The recovery ability of soybean plant decreases as pods increase in size, or according the development of seeds (FRASER at al, 1982).

The attack of stinkbugs to soybean seeds may lead to irreversible damage when attacking bugs affect the hypocotyl-radicle axis, which prevents the seed or affects seedling emergence (CORSO, 1977).

Sometimes, bug attacks can trigger physiological unbalance, and affected plants do not adequately complete its cycle, slowing the maturity process, causing leaf retention and troubling mechanical harvesting (SILVA and RUEDELL, 1983). Bugs are also responsible for disease transmission, since the location where mouthparts penetrate the seeds allows intrusion of pathogenic organisms in seeds, like the fungus *Nematospora coryli* and also species of bacteria. Besides yield reduction, severe attacks of stinkbugs result in reduced oil content and increased grain protein content (CORSO and PORTO, 1978).

It is important to mention that stinkbugs may colonize soybean plants in various stages of development. However, the ability to cause damage is restricted to its attack directly to pods and seeds, thus no noxious effects were observed prior to pod set and close to harvest. It is frequently observed even considerable populations of these insects prior to the blooming stage, increasing progressively in the reproductive phase, with an exponential growth, even accelerated at the end of the crop cycle, especially in the case of medium and late maturity cultivars, which remain longer in the field. This population increase is not only due to insects coming from eggs laid on the same field where they are observed. In most cases, population growth is due to the intense migration of adult insects from recently harvested crops, in search of better shelter, feeding and reproduction conditions.

Soybean can stand a given level of stinkbug population without reducing its yield or seed quality. A pioneer and probably the most important and conclusive study on this area was conducted by Villas Boas et al. (1990), who studied for seven consecutive years the effects of different populations of bugs on soybean yield and seed quality. The authors found that on plots where the action level for controlling the pest were populations up to four bugs/m of soybean row, there was no statistical difference in the productivity and quality of seeds, as compared to plots with no stinkbugs (zero population). When the action level was set over four bugs/m, the yield consistently decreased, being also affected the seed viability and vigor.

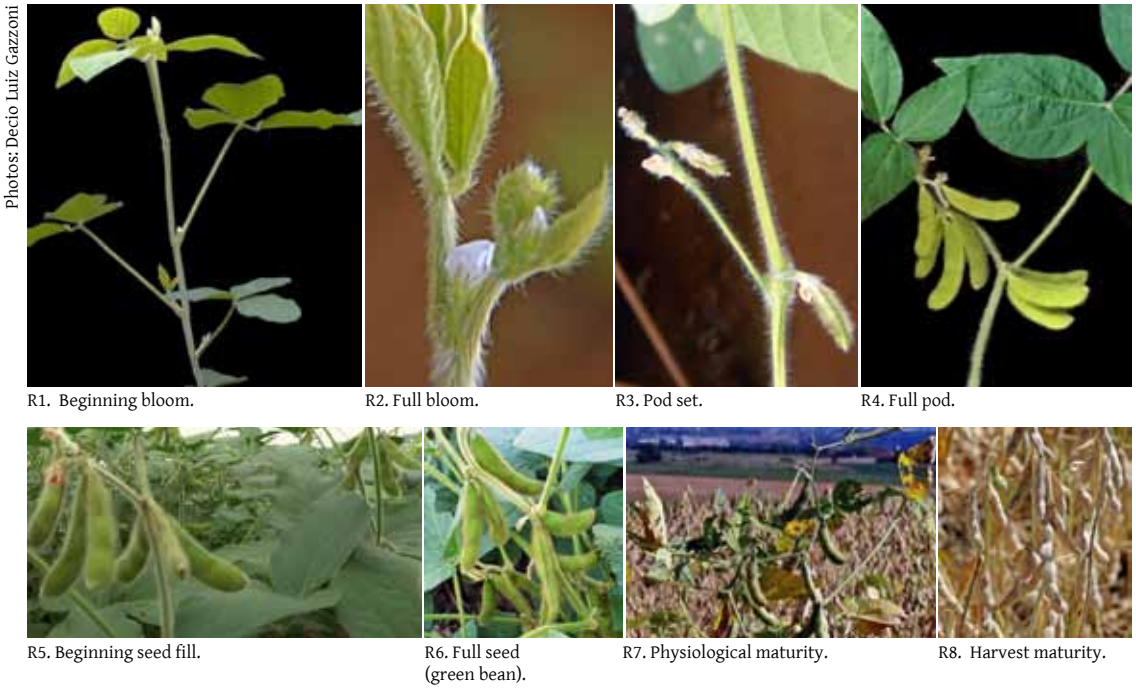


**Figure 2.** Soybean reproductive stages.


**Figure 3.** Pods and seeds size (mm) from stages R3 to R8 of soybean development.

Luckily for the growers - and for the pollinators - during the blooming period, when bees use to forage on soybeans, pod and seed feeder pests usually are not important – because pods and seeds are not yet present on the plants. However, while this is quite true for determinate soybean cultivars, the situation is more complex in indeterminate ones, due to the overlapping of blooming, pod set and pod fill stages, which are quite distinct along the cycle on determinate varieties. While blooming is over at the end of R3 stage for determinate cultivars, flowers may still be observed on the plants up to the R6 stage on indeterminate cultivars.

In order to avoid negative impacts of the control of soybean pests on bees and other pollinators, it is crucial to understand the reproductive anatomy and physiology of the soybean plant, as well as the synchrony of pest attack and its damages according to the stage of soybean development. Concepts like pest monitoring, levels of damage and action as well as diversification of pest control strategies, play an important role to reach this target.

In addition, it is important to review details of the anatomy and physiology of soybean reproduction in order to understand the unique resilience of soybeans to insect pest damage, not only during the vegetative but also during the reproductive stage, especially while pods are not present on the plants. This specificity will help to design Pest Management practices, fine-tuning the strategy in order to consider the minimum impact on pollinators visiting soybeans.

# TYPES OF REPRODUCTIVE STRUCTURE OF PLANTS

## MONECIOUS AND DIOECIOUS PLANTS

The pollination process is largely linked to the type of floral structure of the plants. Relating to its reproductive constitution, some plants have flowers that are not perfect, i.e., they do not have both male and female reproductive parts in the same structure. Instead, they produce male flowers that have only stamens or female flowers that have only pistils.

Monecious plants (Figure 4) have separate male and female flowers rather than perfect flowers, both on the same plant. Corn is a typical monecious plant. It has two types of flowers that develop at different parts of the plant. The male flower forms at the top of the plant and is called the tassel, while the female is located inside the ear, a structure that will late contain the seeds, positioned on the middle part of the stalk.

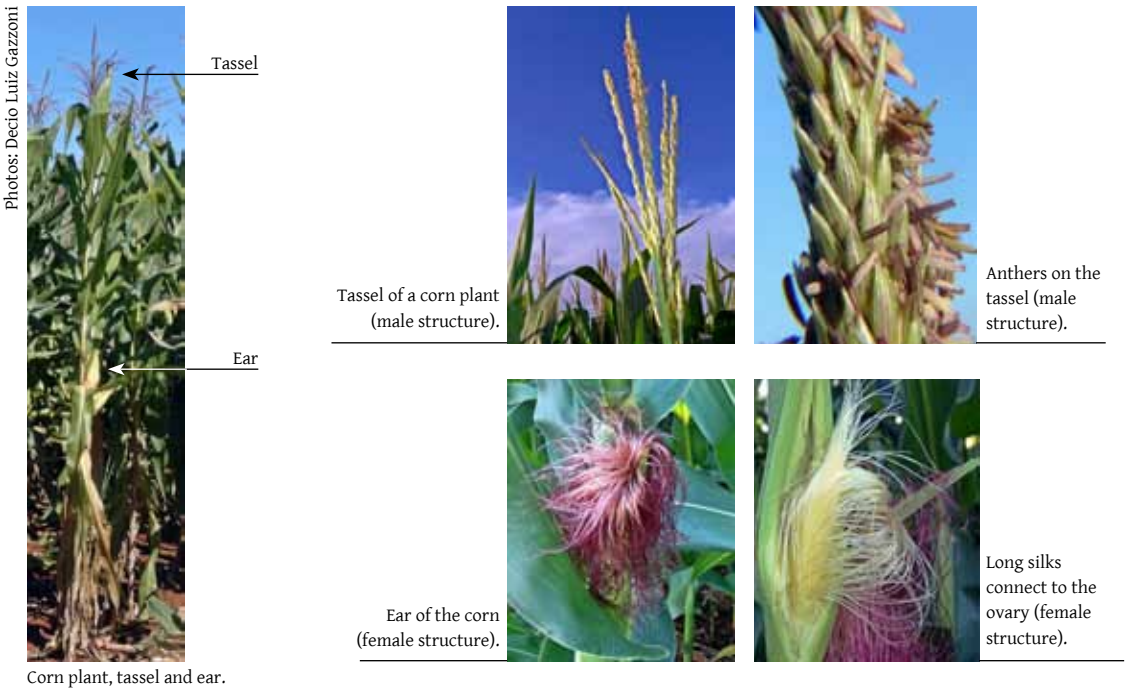


**Figure 4.** Parts of the reproductive structure of a monecious plant (corn).

The tassel starts to develop inside the plant and is composed of hundreds of male flowers, which contain stamens. Emergence occurs about one day before it is mature. Just after the emergence, it starts producing and shedding the pollen. The female structure is located inside the future ear, and pollen is conducted through the pollen tube inside the ear hairs or silks (GEITMANN and RAVISHANKAR, 2007).

For some plant species, called dioecious, each individual plant is either a male or a female. The flowers of dioecious plants will have stamens but no pistils, or the reverse. These male or female specific flowers, however, are present on separate plants, not on separate parts of the same plant. An example of dioecious plant can be observed on Figure 5.

Photos: Decio Luiz Gazzoni


**Figure 5.** Example of a dioecious plant, *Actinidia deliciosa* (Kiwi). A) Female, B) Male and C) Fruit.

# PERFECT FLOWERS

There are hermaphrodite plants, like soybeans, with perfect flowers comprising specific reproductive organs targeting to make and distribute the male gametes; produce the female gamete; and to receive the male gamete.

Flowers with functional male and female organs are considered as perfect flowers (Figure 6) because everything needed to produce a seed by sexual reproduction is present. In the same flower, there are anthers that produce pollen and ovules developing in the ovary. When ripe pollen lands on a receptive stigma, a pollen tube grows through the style. The pollen nucleus, with all the genetic information, travel through the tube and combine with the egg in the ovule. The ovary protects and nourishes the zygote and supports the development of the embryo, endosperm and seed coat of the seed.

The most visually attractive component of the flower is the petal. The set of petals of a flower is called corolla. The attractiveness of the petals is the main motivation for growing a non-food plant, from a human perspective. Petals also attract insects and plants can benefit from visiting insects, in order to move pollen within and between flowers. Pollinators are attracted to a specific flower by its color, form and size of the petals. Sepals are the structure beneath the petals, and can look like the petals, but they function as the protective layer around the unopened flower.

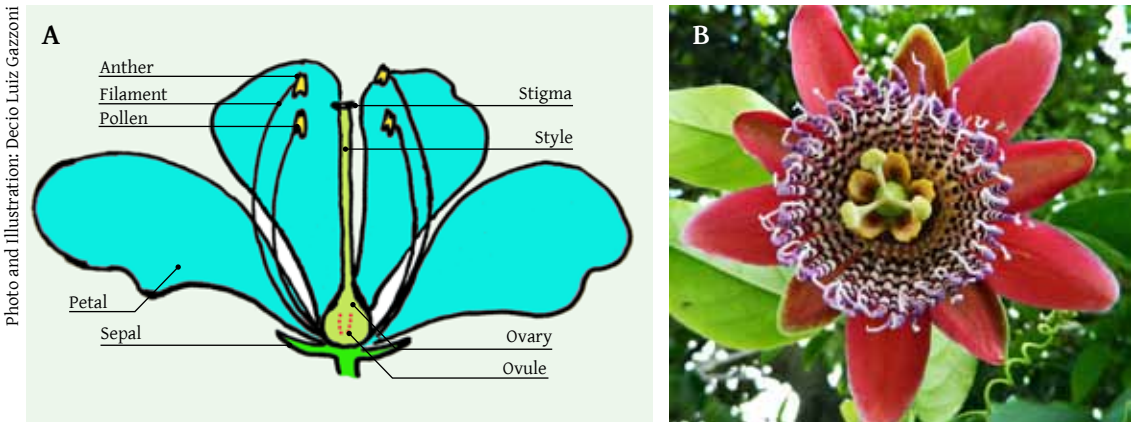


Photo and illustration: Decio Luiz Gazzoni

**Figure 6.** Schematic description (A) and an example of a perfect flower (B), *Passiflora edulis* (Passion fruit).

Perfect flowers have structures called stamens that produce the pollen (male gametes). A stamen consists of the anthers, where the pollen is produced, and filaments, which support the anther. The female reproductive structure, called the pistil, is composed by: a) the stigma, where pollen will land; b) the style, supporting the stigma and where the pollen develops the pollen tube; and c) the ovary where eggs (female gametes) are located and the seed develops. The ovary is supported by the pedicel and may contain several ovules. Each ovule has the female egg cell that combines with the pollen to form the seed embryo, plus other cells that will develop into the seed endosperm and seed coat. The parts of a perfect flower are always the same, but there are many versions of perfect flowers. For example, some perfect flowers have a structure that keeps the stamens separated from the pistil until an insect visits the flower.

# FLOWERS: STRUCTURE, ANATOMY AND MAJOR EVENTS

A flower is the reproductive structure found in flowering plants of the angiosperms. The biological function of a flower is to effect reproduction, usually by providing a mechanism for the union of sperm with eggs. Flowers may facilitate outcrossing (fusion of sperm and eggs from different individuals of the same species) or allow selfing (fusion of sperm and egg from the same flower). Some flowers produce diaspores without fertilization (parthenocarpy). Flowers contain sporangia and are the site where gametophytes develop. Flowers give rise to fruit and seeds. Many flowers have evolved to be attractive to animals, so as to cause them to be vectors for pollen transfer.

## PARTS OF THE FLOWER

The essential parts of a flower can be divided in a) the vegetative part, consisting of petals and associated structures in the perianth; and b) the reproductive or sexual parts. A stereotypical flower consists of four kinds of structures attached to the tip of a short stalk. Each of these parts is arranged in a whorl on the receptacle (Figure 7). A whorl is an arrangement of either sepals, petals, leaves, stipules or branches that radiate from a single point and surround or wrap around the stem. The four main whorls (starting from the base of the flower or lowest node and working upwards) are as follows:

**a) Vegetative (perianth)**

**I. Calyx**: the outermost whorl consisting of units called sepals; these are typically green and enclose the rest of the flower in the bud stage;

**II. Corolla**: the next whorl toward the apex, composed of units called petals, which are typically thin, soft and colored to attract animals that help the process of pollination.

Photo: Decio Luiz Gazzoni

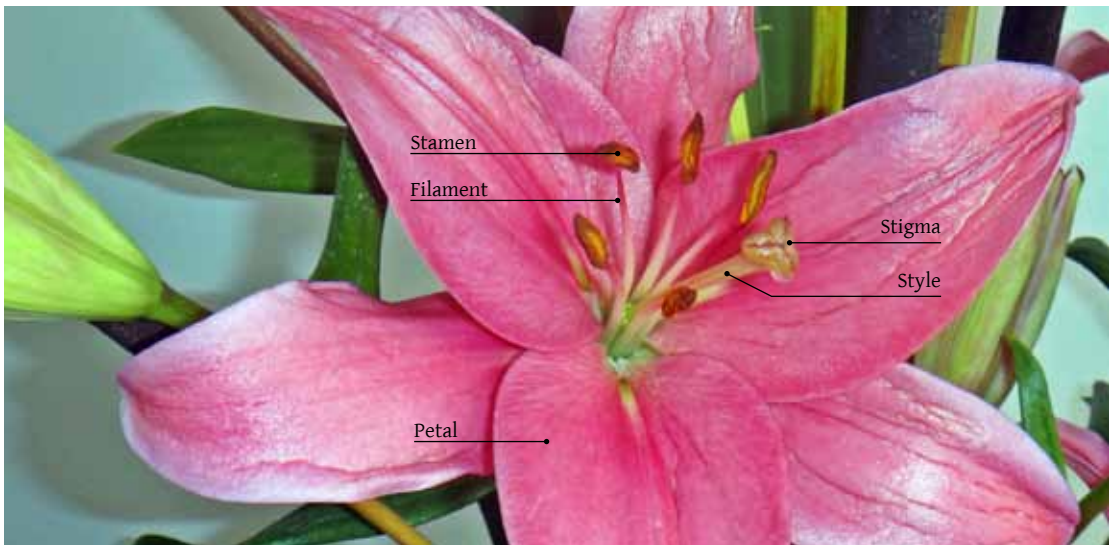


**Figure 7.** Parts of a perfect flower.

**b) Reproductive**

**I. Androecium** (from Greek *andros oikia*: man's house): the next whorl (sometimes multiplied into several whorls), consisting of units called stamens. Stamens consist of two parts: a stalk called a filament, topped by an anther where pollen is produced by meiosis and eventually dispersed (RODRIGUEZ-RIAÑO et al., 1999).

**II. Gynoecium** (from Greek *gynaikos oikia*: woman's house): the innermost whorl of a flower, consisting of one or more units called carpels (SATTLER, 1974). The carpel or multiple fused carpels form a hollow structure called an ovary, which produces ovules internally. Ovules are megasporangia and they in turn produce megaspores by meiosis, which develop into female gametophytes. These give rise to egg cells. The gynoecium of a flower is also described using an alternative terminology wherein the structure one sees in the innermost whorl (consisting of an ovary, style and stigma) is called a pistil. A pistil may consist of a single carpel or a number of carpels fused together. The sticky tip of the pistil, the stigma, is the receptor of pollen. The supportive stalk, the style, becomes the pathway for pollen tubes to grow from pollen grains adhering to the stigma (SATTLER, 1974).

Flowers may show variations, according to the presence, or absence, of the different parts above mentioned, in the same floral structure (sepal, petal, stamen and pistil). A flower is called complete if all four floral organs are present in the same flower structure, while an incomplete flower lacks one or more of them. As an example, flowers of the family Poaceae are incomplete - lacking both sepals and petals - and chiefly anemophilous. One can deduce that an anemophilous plant do not need to attract pollinators, therefore evolution lead to an incomplete flower.

## STRUCTURE OF A FLOWER

In spite that plant species show a wide variation in floral structure, Figure 8 shows a general example of the structures of a flower. The four main parts of a flower described above are generally defined by their positions on the receptacle and not by their function. Many flowers lack some parts, or their parts may be modified into other functions and/or look like what is typically another part. In some families, the petals are greatly reduced and, in many species, the sepals are colorful and petal-like. Other flowers have modified stamens that are petal-like (PRENNER, 2010).

Specific terminology is used to describe flowers and their parts (SATTLER, 1988). Many flower parts are fused together; fused parts originating from the same whorl are connate, while fused parts originating from different whorls are adnate. When petals are fused into a tube or ring that falls away as a single unit, they are sympetalous (also called gamopetalous). Connate petals may have distinctive regions: the cylindrical base is the tube, the expand-

ing region is the throat and the flaring outer region is the limb. A sympetalous flower, with bilateral symmetry with an upper and lower lip, is bilabiate. Flowers with connate petals or sepals may have various shaped corolla or calyx, like campanulate, funnelform, tubular, urceolate, salverform or rotate (SATTLER, 1988).

Photos: Decio Luiz Gazzoni


**Figure 8.** Details of the structure of a perfect flower. A) Cross cut of a perfect flower; B) Details of the ovary and the nectaries; C)/D) Side and top view of the reprodutory structure.

Many flowers have a symmetry. When the perianth is bisected through the central axis from any point, symmetrical halves are produced, forming a radial symmetry. These flowers are also known to be actinomorphic or regular, e.g. rose or trillium. When there is only one line to bisect flowers that produces symmetrical halves, the flower is said to be irregular or zygomorphic, e.g. snapdragon or most orchids (SATTLER, 1988).

Flowers may be directly attached to the plant at their base (sessile—the supporting stalk or stem is highly reduced or absent). The stem or stalk subtending a flower is called a peduncle. If a peduncle supports more than one flower, the stems connecting each flower to the main axis are called pedicels. The apex of a flowering stem forms a terminal swelling which is called the torus or receptacle (SATTLER, 1988).

In those species that have more than one flower on an axis, the collective cluster of flowers is termed an inflorescence. Some inflorescences are composed of many small flowers arranged in a formation that resembles a single flower. The common example of this is most members of the very large composite (Asteraceae) group. A single daisy or sunflower, for example, is not a flower but a flower head - an inflorescence composed of numerous flowers (or florets). An inflorescence may include specialized stems and modified leaves known as bracts (SATTLER, 1988).

## FLOWER DEVELOPMENT

A flower develops on a modified shoot or *axis* from a determinate apical meristem (determinate meaning the axis grows to a set size). It has compressed internodes, bearing structures that in classical plant morphology are interpreted as highly modified leaves. Detailed developmental studies, however, have shown that stamens are often initiated more or less as modified stems (caulomes) that in some cases may even resemble branchlets. Taking into account the whole diversity in the development of the androecium of flowering plants, it is found a continuum between modified leaves (phyllomes), modified stems (caulomes), and modified branchlets (shoots) (SATTLER, 1988).

The transition to flowering is one of the major phase changes that a plant makes during its life cycle. The transition must take place at a time that is favorable for fertilization and the formation of seeds, hence ensuring maximal reproductive success. To meet these needs a plant is able to interpret important endogenous and environmental cues such as changes in levels of plant hormones and seasonable temperature and photoperiod changes.

The transition from vegetative to floral meristems in higher plants is programmed by the coincidence of internal and environmental signals. Since the decade of the 1930´s, the Florigen (or flowering hormone) Theory is in place, surrounded by controversy. Florigen is referred to as a hormone-like molecule that would be responsible for triggering and controlling flowering in plants. According to the theory, florigen would be produced in the plant leaves, and its target would be the buds or the shoot apical meristem, transforming them on flowers. It is known to be graft-transmissible, and even functions between species.

Authors like Turck et al. (2008) assure that the florigen is produced in the leaves in reproductively favorable conditions, and acts in buds and growing tips, in order to induce a number of different physiological and morphological changes. However, up to today, the exact nature of florigen is still a mystery even after Huang et al. (2005) having proposed that florigen is the FT (Flowering Locus T) mRNA, while Lifschitz et al. (2006) attributes the mobile flower-inducing signal to a product of the FT mRNA.

According to the theory, in response to changes in the photoperiod, systemic signals (florigen) induce flowering at the shoot apex. Although the florigen paradigm was conceived in photoperiod-sensitive plants, it is accepted that the signal is common to all plants, as it would be activated by different stimuli. As an example, tomato is a day-neutral plant, with sympodial and modular organization of its shoots and thus with reiterative regular vegetative/reproductive transitions. According to Lifschitz and Ashed (2006), the Single Flower Truss, a regulator of flowering-time and shoot architecture, encodes the tomato orthologous of FT, a major flowering integrator gene.

The molecular interpretation of these signals is performed through the transmission of the complex signal (florigen?) involves a variety of genes, including 'Constans', 'Flowering Locus C' and 'Flowering Locus T' (KIM et al., 2008). The first step of the transition is the transformation of the vegetative stem primordia into floral primordia (SEARLE et al., 2006). This occurs as biochemical changes take place to change cellular differentiation of leaf, bud and stem tissues into tissue that will be transformed in reproductive organs. Growth of the central part of the stem tip stops or flattens out and the sides develop protuberances in a whorled or spiral fashion around the outside of the stem end. These protuberances develop into the sepals, petals, stamens, and carpels.

In most plants, including soybeans, once this process begins, it cannot be reversed and the stems develop flowers, even if the initial start of the flower formation event was dependent of some environmental cue, meaning that once the process begins, even if that cue is removed the stem will continue to develop a flower.

The molecular control of floral organ identity determination is well understood in some species. In a simple model, three gene interact in a combinatorial manner to determine the developmental identities of the organ primordia within the floral meristem. They are called A, B and C-gene functions. In the first floral whorl, only A-genes are expressed, leading to the formation of sepals. In the second whorl both A- and B-genes are expressed, leading to the formation of petals. In the third whorl, B and C genes interact to form stamens and in the center of the flower C-genes alone give rise to carpels. The so-called ABC theory was first described by Haughn and Somerville (1988).

# POLLEN

Pollen is a fine to coarse powder containing the microgametophytes of seed plants, which produce the male gametes (sperm cells). Individual pollen grains are small enough to require magnification to observe its structural details. Pollen grains have a hard coat made of sporopollenin that protects the gametophytes during the process of their movement from the stamens to the pistil of flowering plants or from the male cone to the female cone of coniferous plants. If pollen lands on a compatible pistil or female cone, it germinates, producing a pollen tube that transfers the sperm to the ovule containing the female gametophyte (TWELL, 2014).

Pollen itself is not the male gamete. Each pollen grain contains vegetative (non-reproductive) cells (only a single cell in most flowering plants but several in other seed plants) and a generative (reproductive) cell. In flowering plants, the vegetative tube cell produces the pollen tube, and the generative cell divides to form the two sperm cells (TWELL, 2014). Pollen in plants is used for transferring haploid male genetic material from the anther of a single flower to the stigma of another in cross-pollination. In a case of self-pollination, this process takes place from the anther of a flower to the stigma of the same flower.

The pollen is produced in the microsporangium present in the anther of an angiosperm flower, male cone of a coniferous plant, or male cone of other seed plants. Pollen grains come in a wide variety of shapes (normally spherical), sizes, and surface markings, which are characteristic of the species. Wind-borne pollen grains can be as large as about 90–100 μm (PLEASANTS et al., 2001).

In angiosperms, during initial flower development, the anther is composed of a mass of cells that appear undifferentiated, except for a partially differentiated dermis. As the flower develops, four groups of sporogenous cells form within the anther. The fertile sporogenous cells are surrounded by layers of sterile cells that grow into the wall of the pollen sac. Some of the cells grow into nutritive cells that supply nutrition for the microspores that form by meiotic division from the sporogenous cells (TWELL, 2014).

In a process called microsporogenesis, four haploid microspores are produced from each diploid sporogenous cell (microsporocyte, pollen mother cell or meiocyte), after meiotic division (ALBERTSEN and PALMER, 1979). After the formation of the four microspores, which are contained by callose walls, the development of the pollen grain walls begins. The callose wall is broken down by an enzyme called callase and the freed pollen grains grow in size, develop their characteristic shape, and form a resistant outer wall, called the exine, and an inner wall, called the intine. The exine is what is preserved in the fossil record (OWEN, 2014).

In the microgametogenesis, the unicellular microspores undergoes mitosis and develops into mature microgametophytes containing the gametes. In some flowering plants, germination of the pollen grain often begins before it leaves the microsporangium, with the generative cell forming the two sperm cells. The exine often bears spines or warts, or it is sculptured following various patterns, and the character of its markings is of value on Taxonomy for identifying genus, species, or even a cultivar. The spines may be less than a micron in length referred to as spinulose (scabrate), or longer than a micron referred to as echinate. Various terms also describe the sculpturing such as reticulate, a net like appearance consisting of elements separated from each other by a lumen (OWEN, 2014).

The pollen grain surface is covered with waxes and proteins, which are held in place by structures called sculpture elements present on the surface of the grain. According to Owen (2014), the outer pollen wall is composed of two layers, which prevents the pollen grain from shrinking and crushing the genetic material during desiccation. These two layers are the tectum and the foot layer, which is just above the intine. The tectum and foot layer are separated by a region called the columella, which is composed of strengthening rods. The outer wall is constructed with a resistant biopolymer called sporopollenin. While the pollen grain is moving from the anther to the stigma, the pollen wall protects the sperm from drying out and against solar radiation.

The pollen tube passes through the pollen grain wall by way of structures called apertures (OWEN, 2014). The apertures are various modifications of the wall of the pollen grain that may involve thinning, ridges and pores. They allow shrinking and swelling of the grain caused by changes in moisture content.

On studying pollinators, specially their habits and preferences, it is paramount to identify the pollen form different sources. They normally can be differentiated by its physical appearance. According to Owen (2014), elongated apertures or furrows in the pollen grain are called colpi or sulci, while apertures that are more circular are called pores. Pollen may be referred to as inaperturate (apertures absent) or aperturate (apertures present). The aperture may have a lid (operculum), hence is described as operculate. The orientation of furrows (relative to the original tetrad of microspores) classifies the pollen as sulcate or colpate. Sulcate pollen has a furrow across the middle of what was the outer face when the pollen grain was in its tetrad. If the pollen has only a single sulcus, it is described as monosulcate. Colpate pollen has furrows other than across the middle of the outer faces. Eudicots have pollen with three colpi (tricolpate) or with shapes that are evolutionarily derived from tricolpate pollen. The evolutionary trend in plants has happened from monosulcate to polycolpate or polyporate pollen.

## FERTILIZATION

A pollen tube is part of the male gametophyte of seed plants. It acts as a conduit to transport the male gamete cells from the pollen grain, either from the stigma (in flowering plants) to the ovules at the base of the pistil, or directly through ovule tissue in some gymnosperms. In maize, this single cell can grow longer than 30cm to traverse the length of the pistil.

Angiosperm reproduction is a complex process that includes several steps that may vary among species. Pollen is produced by the stamen, the male reproductive organ of the flower. Each pollen grain contains a vegetative cell, and a generative cell that divides to form two sperm cells. The pollen is delivered by the opening of anthers allowing transferring pollen grains to the pistil, the female reproductive organ. Pollination is usually carried out by wind, water or insects. The ovaries hold the ovules that produce the female gamete: the egg cell, which waits in place for fertilization.

Once a pollen grain settles on a compatible pistil, it germinates in response to a sugary fluid secreted by the mature stigma. Lipids at the surface of the stigma stimulate pollen tube growth if it is a compatible pollen grain. Plants that are self-sterile inhibit the pollen grains from their own flowers to develop pollen tubes.

The presence of multiple grains of pollen stimulates quicker pollen tube growth in some plants. The vegetative cell then produces the pollen tube, a tubular protrusion from the pollen grain, which carries the sperm cells within its cytoplasm. The sperm cells are the male gametes that will join with the egg cell.

In order to reach the ovule, the germinated pollen tube must drill its way through the nutrient-rich style and curl to the bottom of the ovary. Once the pollen tube successfully attains an ovule, it delivers the two sperm cells with a burst. One of them fertilizes the egg cell to form an embryo, which will become the future plant. The other one fuses with both polar nuclei of the central cell to form the endosperm, which serves as the embryo's food supply. Finally, the ovary will develop into a fruit and the ovules will develop into seeds.

Studies had been developed (MESSERLI et al., 2000) to comprehend how the pollen tube responds to extracellular guidance signals to achieve fertilization. It is accepted that pollen tubes react to a combined sets of chemical, electrical, and mechanical cues, during the journey through the pistil. Anyway, it is not clear how these cues work or how they are processed internally. Moreover, the sensory receptors for external cues have not been identified yet. Nevertheless, several aspects have already been identified as central in the process of pollen tube growth. The actin filaments in the cytoskeleton, the peculiar cell wall, secretory vesicle

dynamics, and the flux of ions are fundamental features accepted as crucial, but whose role has not yet been completely elucidated (MASCARENHAS; MACHLIS, 1964; ROBINSON, 1985; CHEBLI; GEITMANN, 2007; OKUDA; HIGASHIYAMA, 2010).

# POLLINATION

Pollination is the process of transferring the pollen from the stamen to the pistil of plants, a parallel to the sexual conjunction in animals. In general, flowers can be divided between three broad groups of pollination methods:

**Entomophilous:** flowers attract insects, sometimes other animals like bats or birds to transfer pollen to the pistil of the same flower or to another one. Often they are specialized in shape and have an arrangement of the stamens that ensures that pollen grains are transferred to the bodies of the pollinator when it lands in search of its attractant (such as nectar, pollen, or a mate). In pursuing this attractant from many flowers of the same species, the pollinator transfers pollen to the stigmas—arranged with equally sharp precision—of all the flowers it visits. Many flowers rely on simple proximity between flower parts to ensure pollination. Others have elaborate designs to ensure cross-pollination and avoid self-pollination.

**Anemophilous:** flowers use the wind to move pollen from one flower to the next, sometimes from male to female structures on the same flower. In this case, they have no need to attract pollinators and therefore tend not to have large blossoms. Whereas the pollen of entomophilous flowers tends to be large-grained, sticky, and rich in protein (a "reward" for pollinators), anemophilous flower pollen is usually small-grained, very light, and of little nutritional value to insects, though it may still be gathered in times of dearth of more suitable food. Surprisingly, honeybees and other bees actively gather anemophilous corn pollen, though it is of little value to them.

**Self-pollinated**: In some plants, like soybeans, flowers are self-pollinated before the flowers open. In other plants, flowers never open even after being self-pollinated. These two types of flowers are called cleistogamous.

The anatomy and design of a flower will dictate how pollination will function to successfully produce seeds. Some plants are self-pollinated, because they have perfect flowers with the stamens and pistils developing in perfect synchrony. In this case, the anther efficiently sheds pollen onto the stigma, as they mature at the same time. This is considered the case of soybeans and other species of the family (Fabaceae), as shown on Figure 9.

Photos: Decio Luiz Gazzoni


**Figure 9.** Examples of perfect flowers of Fabaceae (soybean botanic family). A) Pea, B) Bean and C) Soybean.

Some plants have perfect flowers but the pollen tube will not grow through the style if pollinated by pollen of the same plant. These plants have self-incompatible flowers, so they must cross-pollinate to produce seeds. When a bee or other pollinator explores the flower for nectar, specialized petals are tripped and outsprings the stamen. This action showers the insect with pollen, which will be carried to other flowers of the same species where the insect forage. Therefore, insects visiting these kinds of flowers promote cross-pollination, with the pollen from one plant landing on the stigma of another – no matter if both are perfect flowers.

Two major processes are present for pollination of perfect flowers:

**a) Cleistogamy**, when the pollination takes place in unopened flowers, like soybean, considered a typical self-pollinating plant;

**b) Chasmogamy**, when the pollination takes place in open flowers.

Meanwhile, there are some evidences that even cleistogamic plants, like soybean, might benefit from pollination by insects under certain circumstances, thus incrementing its yield.

Once confirmed, agricultural practices that might affect pollination should be adapted to result in higher soybean yields, while protecting the natural service of pollination.

## FERTILIZATION: SEQUENCE OF EVENTS

Once a viable pollen grain lands on a receptive stigma, a series of sequential and concatenated events are deflagrated, starting with the germination of the pollen grain, inducing the formation of the pollen tube, which emerges and grows. The growing pollen grain moves inside the pollen tube, towards the ovary.

The formation of the pollen tube make it necessary to digest the female tissue, by way of hydrolytic enzymes, as the tube moves down towards the stigma and style. The digested tissue becomes a source of nutrients for the pollen tube.

Should be noticed that two types of nucleus are present in the pollen, and both the vegetative tube and generative nuclei of the pollen grain pass into the pollen tube, which is stimulated by a sugary substance secreted by the stigma.

The pollen tube does not reach the ovary in a straight line, instead grows near the style and curls to the bottom of the ovary and then near the receptacle. At the end, the pollen tube enters the ovary through a tiny pore called micropyle. Finally, the micropyle bursts into the embryo sac, when the male nucleus fuses with the nucleus of the egg and forms a diploid zygote, known as true fertilization or syngamy.

In the sequence, the other male nucleus enters the embryo sac and fuses with secondary nucleus, generating a triploid nucleus called the primary endosperm nucleus. This process is called triple fission.

After fertilization, the resulting embryo undergoes a sequence of mitotic divisions, forming the seed, as well as the endosperm nucleus also divides itself forming the endosperm cells, providing nutrients for the developing embryo. At the end, a fruit is formed and, in case of fruits with multiple seeds, multiple pollen grains are necessary to fertilize each ovule.

Sometimes a double fertilization is present, a variant complex mechanism of fertilization in angiosperms, represented by the conjunction of two male gametes with a single female gametophyte. While one sperm nucleus fertilizes the egg cell, the second joins

with the two polar nuclei of the megagametophyte. This way, a haploid sperm combine with a haploid egg, resulting in a diploid zygote, whereas another male nucleus fuse with other two haploid polar nuclei of the megagametophyte, generating a triploid nucleus. This last one is called the primary endosperm nucleus, which will later develop the endosperm.

In gymnosperms, the male gametes are microgametophytes, developing from sperm cells produced by microspores. On the female side, gametes present on the ovule develops from megagametophytes, and produces multiple archegonia. As described for angiosperms, pollen grains are transported from pollen cone to the ovule via pollinating process, anemophilous or entomophilous. There, the pollen grains enter the ovule through micropyle and mature inside the female gametophyte, to produce the sperm cells. After the fertilization, an embryo is formed in the female gametophyte, resulting in a seed surrounded by a protecting or nutritional coat (GEORGE et al., 1979).

# SOYBEAN REPRODUCTIVE DEVELOPMENT

## SOYBEAN GROWTH TYPES

Following the period of vegetative growth, which varies depending upon cultivar, latitude and associated environmental conditions such as day length and temperature, and the cultural practices (MIKSCHE, 1961), the soybean plant enters the reproductive stage, during which axillary buds develop into clusters of 2 to 35 flowers each. Soybeans flowers can be white or purple (Figure 10).

Photos: Decio Luiz Gazzoni


**Figure 10.** Soybean flowers have different colors. A) Purple flower; B) White flower.

Alexandrova and Alexandrova (1935) and Bernard and Weiss (1973) first summarized literature pertaining to inheritance of color pigments in soybeans. Regardless of green parts of the plant, color pigments occur in flowers, pods, pubescence, seed coat and hilum. Flower colors

are white and purple, controlled by a single gene pair (*W1,w1*) with purple (*W1*) completely dominant over white (*w1*) (JOHNSON and BERNARD, 1962; WOODWORTH, 1923). Some other loci, which cause variation between purple and white flower colors, have been reported, being designated as (*W2,w2*), (*W3,w3*), and (*W3,w4*).

According to more recent studies of Palmer et al. (2004) and Takahashi et al. (2008), flower color of soybean is primarily controlled by six genes (*W1*, *W2*, *W3*, *W4*, *Wm* and *Wp*). When *W1* is present, soybean genotype with *W3W4* produce dark purple flowers; with *W3w4* the flowers shows dilute purple or purple throat; with *w3W4* flowers are purple; and with *w3w4* plants show near white flowers. Flower color of genotypes with allelic combination W1w3w4 was indistinguishable from those with white flowers (*w1,w1*) under many environments, suggesting that environments affect flower color under this allelic combination (HARTWIG AND HINSON, 1962). Yan et al. (2014) described two new variants (near white and light purple), concluding that complete loss-of-function of DFR2 gene leads to near white flowers. A new allele of the *W4* locus, *w4-lp* regulates light purple flowers.

There are two types of stem growth types and floral initiation in soybean (DZIKOWSKI, 1936; GUARD, 1931; WILLIAMS, 1950; FLORES and ESPINOZA, 1977). One type is the indeterminate stem, in which the terminal bud continues growing during most of the season, including the reproductive stage. In this type, the inflorescences are axillary racemes (Figure 11a) and the plant at maturity has a sparse and rather even distribution of pods on all branches, diminishing its frequency toward the tip of the stem or branches. The stem may sometimes appear to have a terminal inflorescence, in fact a series of small one or two flowered axillary inflorescences crowded together by the short internodes at the stem tip.

The second type is the determinate stem, in which the vegetative activity of the terminal bud ceases when it becomes an inflorescence (Figure 11b). This type has both axillary racemes and a terminal raceme, and at maturity has pods distributed along the stem as well as a rather dense terminal cluster of pods.

The node of the first flower is related to the development stage of the plant. Since cotyledonary nodes, the primary leaves and the first two or three trifoliolate leaves are usually vegetative, the first flowers appear at nodes five, six or higher, depending on the cultivar, environmental conditions and cultural practices. Flowers form progressively toward the tip of the main stem and also toward the tips of the branches (on branched cultivars).

Both flowering onset and duration is a function of cultivar, time of planting and latitude. On older cultivars, flowering period may extend from 3 to 5 weeks for determinate types or even longer for indeterminate ones (BORTHWICK; PARKER, 1938; HARDMAN, 1970).

Photos: Decio Luiz Gazzoni

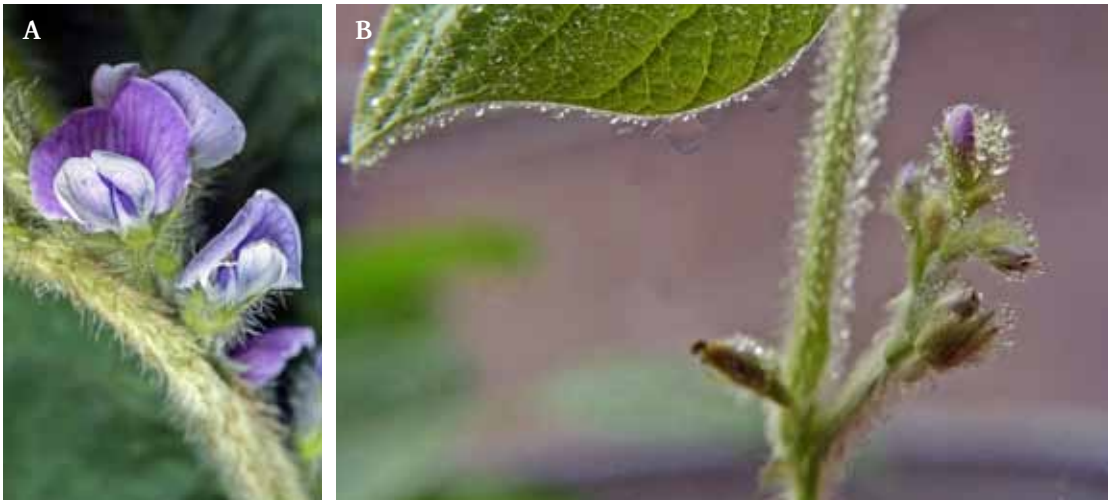


**Figure 11.** Soybean flowering: axillary and terminal inflorescence. A) Inflorescence in the axil of soybean; B) Terminal inflorescence.

# STRUCTURE OF A SOYBEAN FLOWER

Soybean produces a typical papilionaceous flower, with a tubular calix of five unequal sepal lobes, and a five-parted corolla, consisting of a) one posterior banner petal; b) two lateral wing petals; and c) two anterior keel petals in contact with each other but not fused.

When a bud in the axil of a trifoliolate leaf develops into an inflorescence, the stalk of that inflorescence remains stem-like, with typical stem anatomy, including epidermis, cortex, endodermis, vascular tissue, and considerable secondary growth from a vascular cambium (DZIKOWSKI, 1937). In the development of an inflorescence, the bract of each flower is homologous to a trifoliolate leaf, and the two bracteoles are homologous to the prophylls that normally develop at the base of every branch. After forming the primordia of the bracteoles, the apical meristem of the flower gives rise directly to the floral organs.

The 10 stamens collectively called the androecium occur in two groups, in which the filaments of nine of the stamens are fused and elevated as a single structure, whereas the posterior remains separate.

The single pistil is unicarpellate and has one to four campylotropous ovules alternating along the posterior suture (CARLSON and LESTER, 1987). The style is about half the length of the ovary and curves backward toward the free posterior stamen, terminating in a capitate stigma. Figures 12 and 13 show examples of flowers of the soybean family (Fabaceae), identifying its parts. Figure 14 shows a top transversal view of structures of the soybean flower and Figure 15 shows a schematic longitudinal view of a soybean flower.

Trichomes occur on the pistil and also cover the outer surface of the calyx tube, the bract and bracteoles. No trichomes are observed on the petals or stamens. The future flower is at first merely a knob-like primordium in the axil of the bract. The sepals are the first whorl of floral organs to be initiated. The anterior, abaxial sepal lobe arises first on the abaxial side of the flower primordium and is followed in rapid succession by the two lateral lobes, and finally, by the two posterior, adaxial lobes. Very early, the bases of these lobes broaden and fuse, and later becomes the calyx tube.

All organs of the flower develop rapidly except the petals, which do not elongate much until the anthers have well-developed microsporangia. The staminal tube, the free stamen, and the style elongate at the same pace. Thus, the anthers at maturity are clustered around the stigma. At this time, the petals grow very rapidly, soon surpassing the calyx, stamens, and pistil to become visible as the flower is in the bloom.

Photo: Decio Luiz Gazzoni

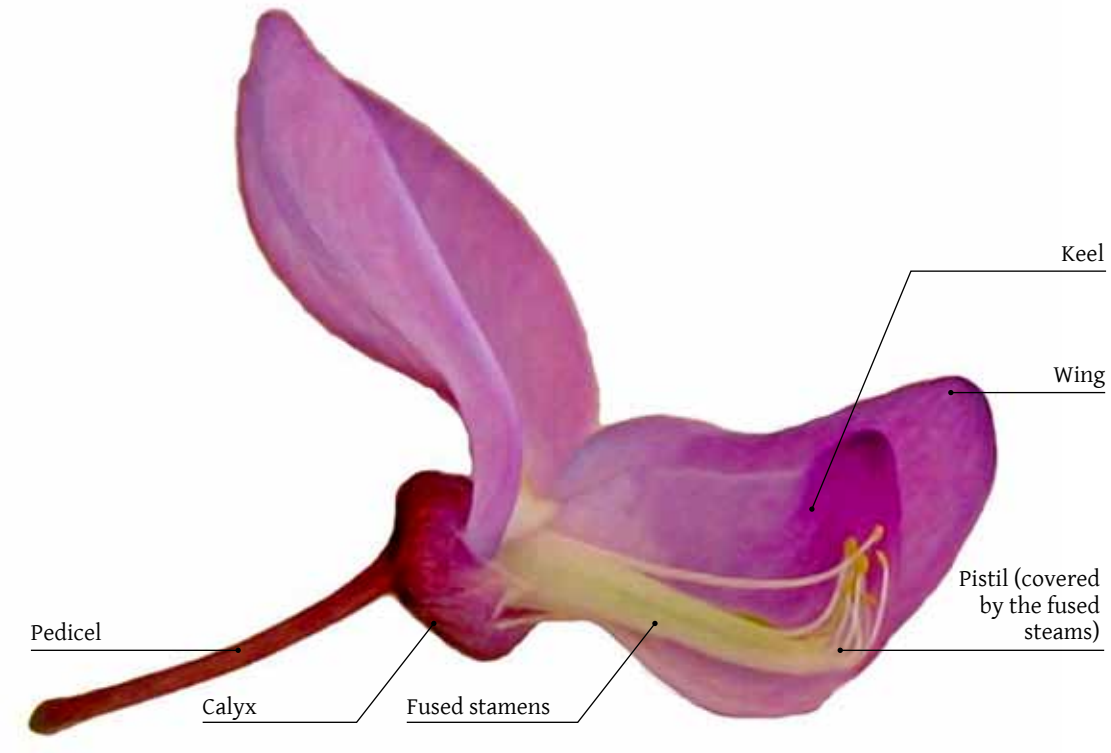


**Figure 12.** Structure of a typical Fabaceae flower.

Photos: Decio Luiz Gazzoni

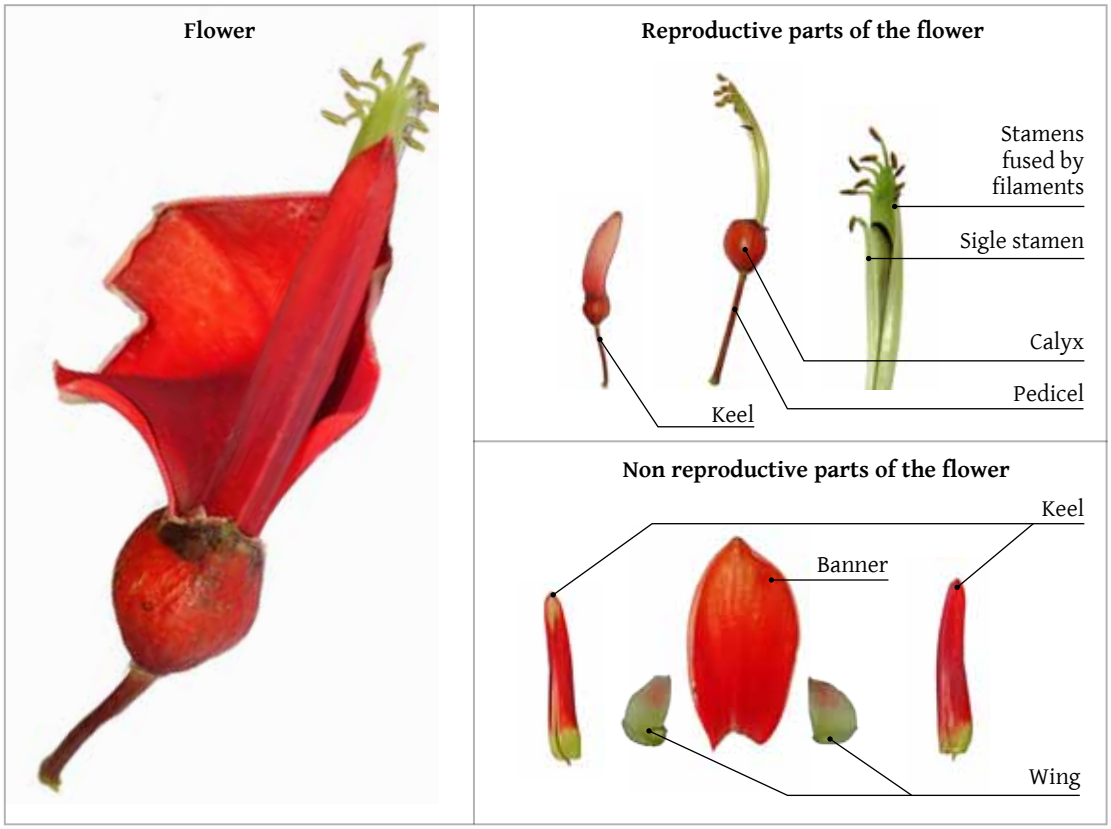


**Figure 13.** Flower of *Erythrina crista-galli* (family Fabaceae), showing its structure.

Photo: Decio Luiz Gazzoni

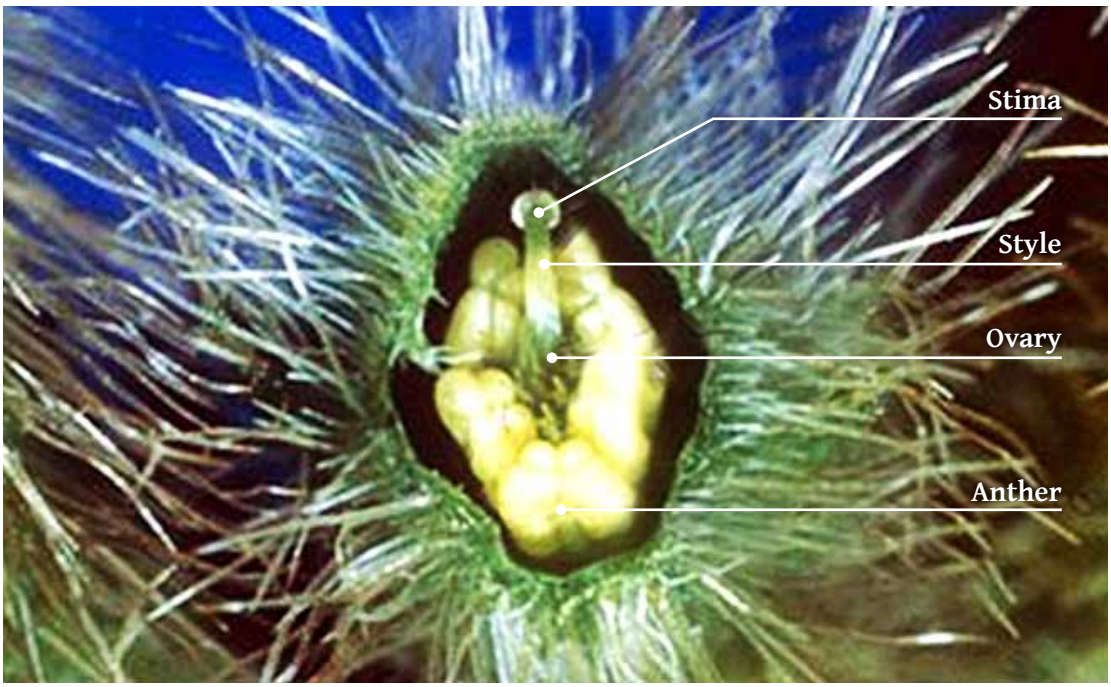


**Figure 14.** Top view of the main components of a soybean flower.

Illustration: Decio Luiz Gazzoni

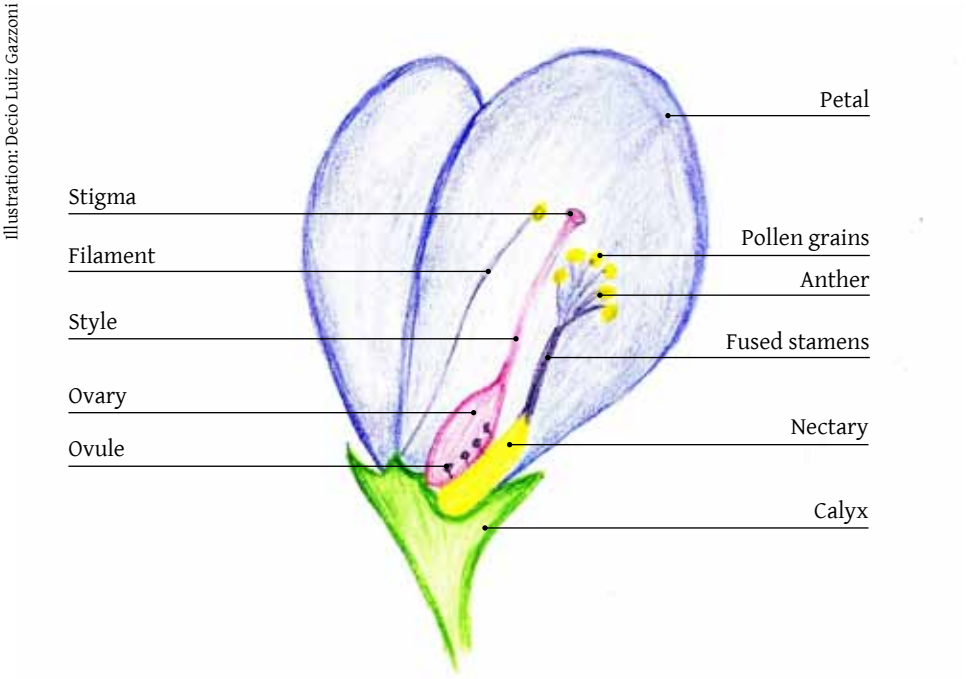


**Figure 15.** Schematic representation of the soybean flower, including the nectary.

Before the margins of the leaf-like pistil fuse, two to four ovule primordia are produced alternately, and develop simultaneously, on the inner surface of the margins of the placenta (GUARD, 1931; PAMPLIN, 1963). Each ovule becomes campylotropous, with its micropylar end directed upward toward the stigma.

The nectary is visible, about 10 days before anthesis, as a rim of tissue between the base of the pistil and the stamens. At the time of anthesis, the discoidal nectary is a fully formed cup about 0.2 to 0.4 mm in height encircling the base of the staminal sheath (CARLSON, 1973; ERICKSON; GARMENT, 1979).

The slightly oval nectary stomata are concentrated on each side of the adaxial indentation of the nectary, where it contacts the filament of the free ventral stamen. Most of the stomata are located over the rim and ventral interior surface of the nectar cup, occasionally in groups of two or three. On the abaxial side of the cup there are only a few stomata (WADDLE; LERSTEN, 1973; ERICKSON; GARMENT, 1979) noted that nectaries are largely vasculated by phloem branching from the staminal base.

Nectariferous tissue is, therefore, most closely associated with the stamens. It is not a random structure, but deliberate associated in order to force the insects to have close contact with the stamens to get the nectar. By the contact, the body of the pollinators is covered with pollen, carried to the stigma of the same flower (perfect flowers) or to the female flower on

monecious or dioecious flowers. Figure 16 shows soybean pollen grains under the microscope, while Figure 17 shows a honeybee visiting a flower, with the body covered by pollen grains, and highlighting the curbicula where pollen is deposited to be carried to the bee colony.

Photo: K. Raja Reddy /Mississippi State University


**Figure 16.** Pollen grains of soybean under the microscope.

Photo: Decio Luiz Gazzoni

**Figure 17.** Bee carrying a pollen ball in the "pollen basket", the corbicula, in the bee´s tibia.

Robacker et al. (1983) studied the behavior of soybean plants grown at various day and night air temperatures, soil temperatures and soil concentrations of N, P and K, to investigate effects of environmental conditions on flower characteristics, including flower production, color intensity, openness, size, nectar secretion and aroma emanation and on attractiveness of the plants to honeybees. Most flower characteristics increased as day air temperatures, at which plants were grown, increased from 20 °C to 24 °C and reached maximum values at 28 °C, before plateauing or declining at 32 °C, although flower size and nectar secretion continued to increase as growing temperature increased to 32 °C. Out of the two flower aroma components, emanation of one component increased while the other decreased, when growing temperatures raised. The hypothesis suggested by Robacker et al. (1983) is that the two aroma chemicals may communicate flower-readiness information to pollinators.

Flower production and flower openness responded linearly to night air temperature at which plants were grown, attaining highest values at higher (22 °C, 26 °C) vs. lower (14 °C, 18 °C) temperatures. Flower production also responded linearly to soil temperature, attaining highest values at higher (28 °C, 32 °C) vs. lower (16 °C, 20 °C) temperatures.

Of two levels of N (75 ppm and 175 ppm) and P (15 ppm and 30 ppm) fertilization tested, the higher level of N stimulated greater flower production, flower size and nectar secretion while the higher level of P decreased the same three flower characteristics. Conversely, lower N and higher P promoted higher fully flower openness.

Soybean plants attractiveness to honeybees varied positively with flower characteristics and also with environmental conditions. For plants grown at a day air temperature of 28 °C, night air temperatures of 22 °C or 26 °C, the higher level of N combined with the lower level of P were the most attractive to honeybees.

Severson and Erickson Junior (1984) investigated the nectar characteristics of 17 soybean cultivars in order to assess the potential for preferential foraging by honeybees. Nectar secretion occurred between ca. 9:00 and 15:00 h and individual flowers bloomed only one day. Mean nectar production per flower varied from 0.022 µl to 0.127 µl among cultivars, while total nectar carbohydrate content varied from 301 µg/µl to 1,354 µg/µl. Fructose, glucose, and sucrose content varied from 42 µg/µl to 314 µg/µl, 43 µg/µl to 262 µg/µl, and 97 µg/µl to 986 µg/µl, respectively. Total carbohydrate per flower varied from 16.0 µg to 134 µg. The ratios of fructose:glucose:sucrose among cultivars were distributed along a broad continuum, from those with low sucrose (ca. 1.2:1.0:1.4) to ones with high sucrose (ca. 1.2:1.0:6.7).

There were significant differences between purple and white flowered varieties for fructose and glucose content, nectar per flower, and total carbohydrate per flower

(SEVERSON, 1983). He observed that white flowered varieties had a more uniform carbohydrate content per flower throughout the day than did purple flowered varieties. There were no significant differences in total carbohydrate content but fructose and glucose content in white flowered varieties varied significantly with day, time, and temperature while sucrose content of purple flowered varieties varied significantly with time.

Severson (1983) examined nectar characteristics of soybean cvs. Centennial and Coker 237 grown in Marion, AR, during 1980 and 1981. Nectar secretion decreased with time and increased with temperature, while fructose, glucose, sucrose, and total carbohydrate content and carbohydrate per flower increased with time and temperature. He also observed that environmental factors promoting plant stress appear to influence nectar carbohydrate composition as water deficiency promoted a decrease in sucrose content and a 4-fold increase in fructose and glucose content.

Severson (1983) also analyzed soybean cvs. Bragg, Centennial, Coker 237, and Davis for honeybee attractiveness in cage studies. He found out that honeybee foraging was nonrandom as individual bees exhibited greater varietal fidelity to 'Centennial' and 'Davis' than to 'Bragg' and 'Coker 237'. Attractiveness to honeybees was not correlated with nectar characteristics as 'Coker 237' and 'Bragg' appeared to produce a more highly attractive nectar (i.e. nectar quantity and quality) than 'Centennial' or 'Davis'. Initial honeybee attraction to soybean flowers is theorized to be determined by floral volatile components with 'Centennial' and 'Davis' producing a more attractive volatile spectrum (i.e. quantity and/or quality) than the cultivars Bragg or Coker 237.

Soybean flowers have a period of viability for pollination and fecundation, which regulates bee foraging, highly related to the availability of pollen and nectar (MONSI, 1942). According to Free and Williams (1973), the sugar concentration of nectar in the flowers determines the frequency of visitors, while the volume determines the quantity of honeybee foragers that will visit. Under Brazilian conditions, the visit of honeybees to soybean flowers normally occurs between 9:00h and 15:00h every day. The peak of these activities and the time in which the flowers will remain open vary between the cultivar and the local environmental condition.

The contents of total sugar in nectar varied from 37 to 45%, and the sugar of soybean flowers increased and the volume decreased according to the time of the day and temperature (ERICKSON, 1984; DELAPLANE and MAYER, 2000). The intensity of foraging by honeybees is linked to the nectar volume and nutritional characteristics (HEINRICH, 1979; HAGLER, 1990) and particularly to the sugar profile (WALLER, 1972; ABROL and KAPIL, 1991; ABROL, 2012; BIELESKI and REDGWELL, 1980; BREWER et al., 1974).

The volume of nectar on each flower varied significantly between soybean cultivars (0.2 to 0.5 µL), also increasing with warmer temperatures, as observed by Sverson and Erikson Junior (1984). When soybean flowers were compared as two distinct groups, there were no apparent differences in nectar characteristics among white-flowered and purple-flowered cultivars. Time of the day was the primary factor affecting soybean nectar characteristics; nectar fructose, glucose, sucrose, and total carbohydrate content increased with time of day, while the volume of nectar per flower decreased. Day-to-day and temperature effects on nectar characteristics were minimal. Comparisons made within individual sampling periods suggest that there are differences in nectar characteristics among cultivars, which could encourage preferential foraging by honeybees.

# FLOWER, PODS AND SEEDS ABSCISSION ON SOYBEANS

Several investigators have reported that a soybean plant produces many more flowers than can develop into pods. From 20 to 80% of' the flowers are subjected to abscise depending on the genetic and environmental conditions (DALL'AGNOL, 1980 ; HANSEN; SHIBLES, 1978; HARDMAN, 1970; VAN SCHAIK; PROBST, 1958; WIEBOLD et al., 1981). Most cultivars with many flowers per node have a higher percentage of flower abscission than those with few flowers per node (ANDREWS, 1966).

Abscission is not restricted to flower and can happen from the triggering of flower development to the pods. Abscission can occur at the time of bud initiation, during the development of floral organs, at the time of fertilization, during the early proembryo stage, or at any stage of cotyledon development (WEBSTER and LEOPOLD, 1977). Flower abscission occurs most often from 1 to 7 days after the start of flowering (stage R1) (KATO and SAKAGUCHI, 1954; KATO et al., 1955; PAMPLIN, 1963; WILLIAMS, 1950), and pod abscission after flowering (CARLSON AND LERSTEN, 1987). Normally, the earliest and latest flowers are more often abscised, but even after pod set, some of the seeds or even entire pods can be aborted (ZHANG; SMITH, 1999).

Apparently, the plant produce more flower than the photosynthates reserves can support if thoroughly developed into pods and seeds, and a continuous balance is ongoing to fine-tune the plant capacity to generate the maximum amount of viable seeds to perpetuate the species. There are no reports of insects pests responsible for soybean flower abscission larger than the natural process normally produces. This hypothesis is much in line with the well-known soybean resilience to insect pests attacks and damages, even along the reproductive

stage. However, in spite of its resilience to insect attack, one of the most susceptible period to insect damage during the soybean development is the beginning of pod set. A single puncture of a bug can abort a seed or an entire pod, because the structures are very small and sensitive.

Hansen and Shibles (1978) found that, in two indeterminate cultivars, abscission was greatest on the lower stems, whereas pods were retained most often in the middle portions of the plants. In 11 determinate cultivars, in contrast, most harvestable pods were in the top third of the canopy and abscission increased in the lower portions (WEIBOLD et al., 1981).

Abernathy et al. (1977) reported that failure of fertilization is insignificant as a cause of floral abscission in soybean. Abscising flowers were mostly all fertilized and usually contained proembryos that had undergone two or three cell divisions.

In general, the earliest and latest flowers produced tend to abscise most often. Additionally, individual ovules or entire ovaries may abort. Kato and Sakaguchi (1954) noted that the basal ovule, which is the last one to be fertilized, would frequently abort. Also, the terminal ovule would often abort because of its poorer ability to compete for available water.

# STAMEN AND OVULE DEVELOPMENT

The first whorl of five stamen primordia arises shortly after the initiation of the petal primordia, and is quickly followed by the second stamen whorl. The sequence of development is the same for both whorls of stamens, except that it occurs later in the inner whorl. Each stamen primordium contains a more or less homogenous mass of cells surrounded by a protoderm layer. As the stamen develops, its apical portion forms a four-lobed anther and a short filament (Figure 18).

Each anther lobe consists of a central region of archesporial (primary sporogenous) cells bounded peripherally by four to six layers of cells derived by periclinal divisions of the protoderm. These outer layers mature later into epidermis, endothecium, parietal layers, and tapetum. Toward the center of the anther, the archesporium is bound by the centrally located connective tissue, in which the single stamen bundle occurs.

Palmer et al. (1978) and Johns and Palmer (1982) determined the average numbers of pollen grains from fertile plants *(Msl)* and the average numbers of coenocytic microspores

from genetic male sterile plants *(msl,msl)* in soybeans. Comparisons were made between the average numbers of pollen grains and the average numbers of coenocytic microspores with respect to environment where plants were grown, and to stamen position in the flower. Pollen production from fertile plants varied from 374 to 760 pollen grains per anther, among genetic lines and environments.

Photo: Decio Luiz Gazzoni

**Figure 18.** Open soybean flower: Detail of fused stamens involving the pistil, and the anthers covering the stigma, with pollen grains capping the top of the structure.

The ovule of soybean has two integuments (bitegumic), and both ovule and embryo sac are bent back on themselves (campylotropous). Megaspores form deep in the nucellus (crassinucellate) (PRAKASH and CHAN, 1976). As many as four ovules first appear as small masses of tissue on the placenta, at alternate sides of the posterior suture of the unicarpellate pistil.

The cells of an ovule primordium are all about the same size and covered by a single-layered protoderm. Within 1 – 2 days after ovule initiation, several hypodermal archesporial cells are distinguishable. These cells are larger than the neighboring ones and have more densely staining cytoplasm. Soon, one of the archesporial cells surpasses the others in size and becomes the functional megasporocyte. The neighboring cells of the archesporium become

less prominent and quickly resemble the rest of the cells of the young ovule. Periclinal divisions in the hypodermal region produce two parietal layers of nucellus between the elongate megasporocyte and the epidermis of the ovule.

At the time of fertilization, the nucellus still surrounds the embryo sac, but only the epidermis remains intact at the micropylar end, in direct contact with the outer integument (PAMPLIN, 1963). As the seed develops following fertilization, the nucellus ruptures at the micropylar end, exposing the embryo sac so that the suspensor of the embryo is now in direct contact with the epidermis of the outer integument. The chalazal end of the nucellus persists for several days, but continued development of the endosperm finally results in its complete obliteration by 14 days after fertilization (PAMPLIN, 1963).

# POLLINATION OF SOYBEAN FLOWERS

By the time of pollination, the diadelphous stamens have been elevated so that the anthers form a ring around the stigma. The pollen thus is shed directly on the stigma, resulting in a high percentage of self-fertilization (WILLIAMS, 1950). Natural crossing on soybeans may reach up to 2%. It has been noted that pollination may occur the day before full opening of the flower (DZIKOWSKI, 1936).

Fertilization starts when the pollen grain germinates and sends a pollen tube through the style and into the egg sac, through an opening called the micropyle. The growth of the pollen tube requires proteolytic enzymes that can digest the sporophyte tissue just ahead of the lengthening tube. The generative cell within the pollen grain undergoes mitosis to produce two sperm cells. These cells follow the tube into the egg sac where one sperm unites with the egg to form a zygote, while the other sperm joins the two nuclei in the central cell to form the triploid tissue called endosperm.

The wet stigma is overtopped by a proteinaceous film that originates from the cuticle. The film probably prevents desiccation of the abundant quantities of lipoid exudate present at the distal end of the stigma and confine the exudate to the stigmatic surface. It may also contain recognition factors to facilitate the fertilization (CHEUNG et al., 1995).

Erbar (2003) pointed out that the transmitting tissue of the stigma is made up of papillae with lateral protrusions that anastomose with each other. Papillae of this type occupy the distal end of the stigma and secrete most of the stigma exudate. Proximal to them are one to three whorls of free papillae lacking protrusions, which are also secretory.

According to Erbar (2003), there are numerous exudate-filled channels in the stigma and style, with pollen tubes growing in these channels, which provide nutrition and mechanical guidance. At the base of the stigma, in the transition zone between stigma and style, there is a gradual increase in the amount of exudate between cells, except in the center of the style. These cells comprise the stylar-transmitting tissue, secreting an exudate similar in appearance to that of the stigma.

Pollen usually germinates on the surface of the film overlying the stigma exudate. Germination can also occur among the lower whorls of papillae, but these tubes then grow into the stigma before entering the style.

Although many pollen grains are deposited on the stigma, and most of them germinate and grow into the stigma and upper style, the majority of the tubes atrophy and die before reaching the distal end of the ovary. Only a few pollen tubes reach the locule and compete for ovules to fertilize (HERRERO and HORMAZA, 1996).

After germinating, pollen tubes grow between the cells of the stylar-transmitting tissue. The ovarian transmitting tissue forms a secretory obturator on top of which the pollen tubes grow toward the ovules (ERBAR, 2003). Its exudate is pectinaceous, which perhaps controls the direction of pollen tube growth chemotactically (CHEUNG et al., 1995). During growth of the pollen tube toward the ovule, the generative cell divides and forms two male gametes, the sperm cells.

Finally, the pollen tube grows through the micropyle of the ovule, between nucellar epidermal cells, and enters the filiform apparatus of the degenerate synergid. Here the pollen tube tip bursts and releases the two sperm cells. One sperm fuses with the egg and forms the diploid zygote, the first cell of the embryo, while the other sperm fuses with the secondary nuclei, forming the primary endosperm nucleus (RAY et al., 1997). Rustamova (1964) noted that the time from pollination to fertilization varies from about 8 to 10 h. Thus, the day of full opening of the flower is likely the very day of fertilization or, perhaps, is one day after fertilization.

Soybeans are considered as cleistogamic, self-pollinating plants. The first studies indicated cross-pollination rate on soybeans to be as low as 0.04% in Wisconsin, with different varieties of soybean grown in adjacent rows, in different places (WOODWORTH, 1922), ranging from 0.70% and 0.18% in Virginia, in successive years (GARBER and ODLAND, 1926), and less than 1% in Iowa and Maryland (WEBER AND HANSON, 1961). The cross pollination on soybeans is mediated by pollinators, especially the honeybee (Figure 19 and 20).

Photo: Decio Luiz Gazzoni

**Figure 19.** *Apis mellifera* visiting a soybean flower.

Photo: Decio Luiz Gazzoni

**Figure 20.** Foraging bee covered by pollen grains.

According to Ahrent and Caviness (1994), based on a 2-yr average, cross-pollination varied from a low of 0.09% for Walters cultivar to a high of 1.63% for 'Brim'. The same authors mention that results show that cultivars differ significantly in the extent of cross-pollination and

as much as 2.5% outcrossing may occur in maturity groups IV, V, and VI cultivars (classification used in the US) in some environments, where adequate pollinators are present and other conditions are favorable.

The dispersion of pollen in soybean was reported by Abud et al. (2003), with the maximum dissemination of pollen of transgenic cultivars in neighbor lines falling in the range of 0.44% to 0.45%. When distances were greater, the frequency was reduced drastically. Considering one line between the two studied ones, the cross pollination ranged from 0.04 to 0.14%, decreasing to zero when 11 lines (6.5 m apart) separated the crossing lines. Chiari et al. (2011) found a range of cross-pollination rate from the minimum of 0.2% to peaks of 2.67% on soybeans caged without and with Africanized honeybees.

Details of the flower and ovule development of soybeans are shown on Table 2.

**Table 2.** Flower and ovule chronological development in soybean.

| Days before flowering | Morphological and anatomical features |
|---|---|
| 25 | Initiation of floral primordium in axil of bract. |
| 25 | Sepal differentiation. |
| 20-14 | Petal, stamen, and carpel initiation. |
| 14-10 | Ovule initiation; maturation of megasporocyte; meiosis; four megaspores present. |
| 10-7 | Anther initiation; male archesporial cells differentiate; meiosis; microsporogenesis. |
| 7-6 | Functional megaspore undergoes first mitotic division. |
| 6-2 | Second mitotic division results in four-nucleate embryo sac.<br>Third mitotic division results in eight-nucleate embryo sac.<br>Cell walls develop around antipodals and egg apparatus forming a seven-celled and eight-nucleate embryo sac.<br>Polar nuclei fuse. Antipodal cells begin to degenerate. Nucellus begins to disintegrate at micropylar end and on sides of embryo sac.<br>Single vascular bundle in ovule extends from chalaza through funiculus and joins with the carpellary bundle. |
| 1 | Embryo sac continues growth; antipodals disorganized and difficult to identify.<br>Synergids with filiform apparatus; one synergid degenerating.<br>Tapetum in anthers almost gone. Pollen grains mature; some are germinating.<br>Nectary surrounding ovary reaches maximum height. |
| 0 | Flower opens; usually day of fertilization; resting zygote; primary endosperm nucleus begins dividing. Nectary starts collapsing. |

Source: Carlson and Lersten (1987). The times are a compilation of data from several soybean cultivars studied by Carlson (1973), Kato et al. (1954), Murneek and Gomez (1936), Pamplin (1963), Prakash and Chan (1976). The sequence of development is essentially the same regardless of cultivar but the absolute times vary with environmental conditions and with cultivars. Modern cultivars surely have different schedules, which will also vary according to latitude, temperature and presence/absence of biotic and abiotic stresses.

# EMBRYO, ENDOSPERM AND SEED COAT DEVELOPMENT

After fertilization, the soybean plant becomes far more susceptible to insect pests, especially those feeding on pods and seeds. Once fertilized, the vacuole in the zygote becomes smaller and finally disappears entirely about the time of the first cell division, which occurs about 30 hours after pollination (PAMPLIN, 1963; RUSTAMOVA, 1964).

Soueges (1949) described soybean embryogeny from the first division of the zygote through the early cotyledon stages. The first division of the zygote is transverse. The apical cell, facing the central cell, will become the embryo. The basal cell, facing the micropyle, forms the suspensor, an ephemeral structure that may aid early embryo growth. Continued divisions of the derivatives of the apical cell produce the spherical proembryo, at about 3 days. The proembryo is approximately the same size as the somewhat conical suspensor (LINSKENS et al., 1977). A well-defined protoderm is present in the proembryo ca. 5 days after fertilization (Figure 21).

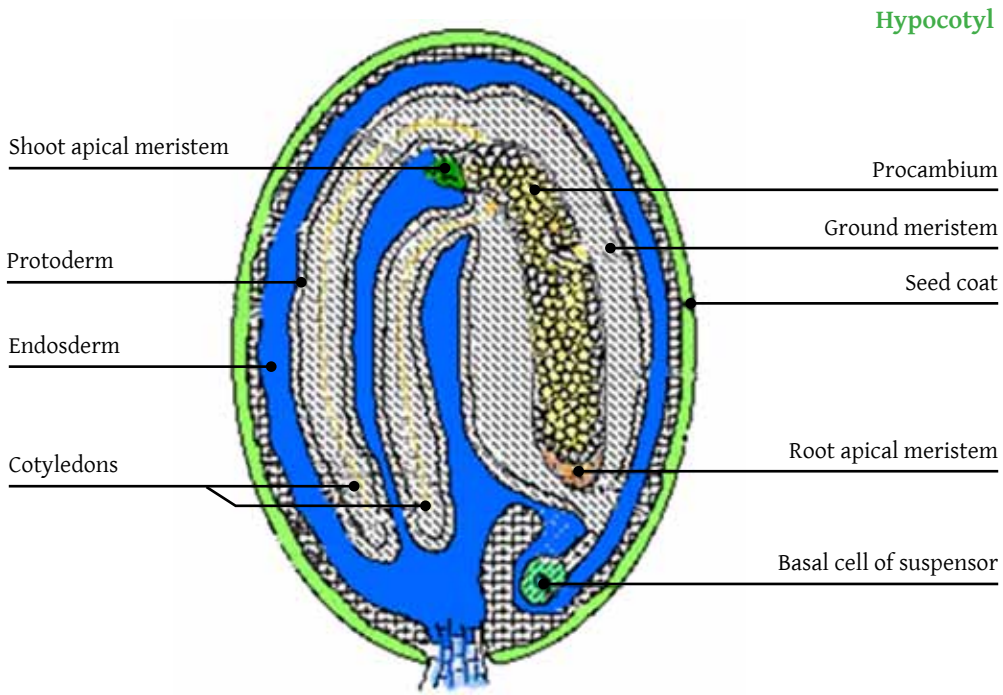


Illustration: Decio Luiz Gazzoni

**Figure 21.** Development of the embryo, seed and endosperm.

About 6 to 7 days after fertilization, localized divisions at opposite sides of the proembryo, just below the protoderm, initiate the cotyledons. Pamplin (1963) observed that the cotyle-

don at the chalazal side of the embryo seems to be initiated first, but it is quickly followed by initiation of the second cotyledon, which grows rapidly and soon reaches the same size as the first cotyledon. As the cotyledons continue to develop, there is a gradual rotation such that the embryo, with its cotyledons, moves 90°. Then the cotyledons assume the position they will have in the mature seed, with their inner surfaces forming a plane to the sides of the ovule.

Still according to Soueges (1949), at this stage the cotyledons appear circular in outline, but rapid growth along the margins, especially toward the chalazal end of the ovule. This results in a pronounced elongation of the cotyledons, giving them their typical reniforrn shape. Ten to 12 days after fertilization, the tissue systems of the hypocotyl are well blocked out and consist of protoderm, the ground meristem of the cortex, and procambium. The derivatives of the hypophysis have formed the initials of the root, which until the time of germination remain limited to a small area at the end of the hypocotyl, just above the point of attachment of the suspensor (PAMPLIN, 1963; LERSTEN, 1982, 1983).

The epicotyl is initiated simultaneously with the origin of the two cotyledons, as a residual meristem between them. Pamplin (1963) stated that it first appears as an elongated mound of deeply staining cells between the bases of the cotyledons. The outermost cell layer becomes the tunica. About 14 days after fertilization, the epicotyl forms the primordial of the two primary leaves at right angles to the point of attachment of the two cotyledons. The primary leaves continue to enlarge and by 30 days have reached their maximum dormant embryo size, and have assumed the conduplicate vernation characteristic of the plumule of the mature seed.

The first trifoliolate leaf primordium, differentiated about 30 days after fertilization - near the base of the two simple leaves -, remains reduced in size and does not resume development until the time of germination. The sequence of seed filling is shown on Figure 22.

Photos: Decio Luiz Gazzoni

**Figure 22.** Sequence of soybean seed development: smaller grains are much more sensitive to insect damage.

Parallel to the embryo development comes the endosperm development, as the primary endosperm nucleus divides almost immediately after the fertilization. By the time of zygote division, the endosperm already has several free nuclei (PAMPLIN, 1963; PRAKASH and CHAN, 1976). Divisions of the endosperm nuclei occur as simultaneous cycles for several days following fertilization. The nuclei and common cytoplasm of the endosperm are displaced toward the periphery of the embryo sac by the development of a large vacuole in the center of the mass of endosperm. The free nuclei of the sac-like endosperm are spaced uniformly within the cytoplasm.

By five days after fertilization, the endosperm begins to become cellular around the embryo at the micropylar end of the embryo sac and, by 8 days, the heart-shaped embryo is completely embedded in cellular endosperm (MENG-YUAN, 1963; PRAKASH and CHAN, 1976; TAKAO, 1962). Endosperm cell walls develop gradually toward the chalazal end of the embryo sac; by 14 days, they extend almost to the chalazal end of the ovule (TILTON et al., 1984a, b, c).

The inner integument consists of two to three cell layers at the time of fertilization. After that, periclinal division, especially in the chalazal end of the ovule, result in an increase in thickness of the inner integument, to about 10 cell layers (REMBERT JUNIOR, 1977). About 10 to 14 days after flowering, the inner layer of the inner integument becomes more densely staining and differentiates as an endothelium or integumentary tapetum, which presumably has a nutritive function (BUSS and LERSTEN, 1975).

The outer integument at fertilization is two to four cell layers thick except in the region of the micropyle and the hilum, where it is considerably thicker (PAMPLIN, 1963). After fertilization, periclinal divisions occur and the outer integument becomes approximately 12 to 15 cells layers thick (PRAKASH and CHAN, 1976). The epidermis of the outer integument consists of isodiametric cells at the time of fertilization. During growth and maturation of the seed, these cells elongate radially, especially near the hilum. The epidermal cells of the funiculus in the hilum region also elongate radially so that in the hilum there is a double layer of elongate thick-walled epidermal cells.

Details on the development of seed and pods on soybeans are presented on Table 3.

**Table 3.** Seed and pod chronological development in soybean.

| Days after flowering | Morphological and anatomical features |
|---|---|
| 0 | Resting zygote. Several divisions of primary endosperm nucleus. |
| 1 | Two-celled proembryo. Endosperm with about 20 free nuclei. |
| 2 | Four- to eight-celled proembryo. |
| 3 | Differentiation into proembryo proper and suspensor. Endosperm in peripheral layer with large central vacuole. |
| 4-5 | Spherical embryo with protoderm and large suspensor. Endosperm surrounding embryo is cellular but elsewhere it is mostly acellular and vacuolated. |
| 6-7 | Initiation of cotyledons. Endosperm mostly cellular. |
| 8-10 | Rotation of cotyledons begins. Procambium appears in cotyledons and embryo axis. All tissue systems of hypocotyl present. Root cap present over root initials. Endosperm all cellular. |
| 10-14 | Cotyledons have finished rotation and are in normal position, with inner surfaces of cotyledons parallel with sides of ovules. Cotyledons elongate toward chalazal end of ovule. Primary leaf primordia present. Endosperm occupies about half of seed cavity. Extensive vascularization of seed coat. |
| 14-20 | Continued growth of embryo and seed. Reduction of endosperm tissue by assimilation into cotyledons. |
| 20-30 | Primary leaves reach full size. Primordium of first trifoliolate leaf present. Cotyledons reach maximum size. Endosperm almost gone. |
| 30-50 | Continued accumulation of dry matter, and loss in fresh weight of seeds and pods. Maturation of pods. |
| 50-80 | Maturity time |

Source: Carlson and Lester (1987). The times are a compilation of data from several soybean cultivars studied by Bils and Howell (1963), Carlson (1973), Fukui and Gotoh (1962), Meng-yuan (1963), Kamata (1952), Kato et al. (1954), Ozaki et al. (1956), Pamplin (1963), Suetsugu et al. (1962). The sequence is the same for modern cultivars, but times will vary according to genetic background, latitude, environmental conditions and presence/absence of biotic/abiotic stresses.

# BEES AND PLANTS RELATIONS

## NECTAR, A KEY MEDIATOR

Nectar is an aqueous, sweet plant secretion, which mediates plant interactions with pollinators and defenders, sometimes protecting against robbers and microorganisms by secondary compounds and antimicrobial proteins, present on its composition (HEIL, 2011). Nectar contains water, sugars and amino acids to attract pollinators and defenders and is protected from nectar robbers and microorganisms by secondary compounds and antimicrobial proteins. Floral (FN) and extra floral (EFN) nectar secretion can be induced by jasmonic acid, it is often adjusted to consumer identity and consumption rate and depends on invertase activity. Invertases are likely to play at least three roles: the uploading of sucrose from the phloem, carbohydrate mobilization during active secretion and the post secretory adjustment of the sucrose:hexose ratio of nectar (LINDEN and LAWHEAD, 1975). However, according to Heil (2011), it remains to be studied how plants produce and secrete non-carbohydrate components, and more research is needed to understand how plants produce nectar, the most important mediator of their interactions with mutualistic animals.

Proctor et al. (1996) point that floral nectar must be understood as a key reward offered by plants to their pollinators, aiming to attract them and get their loyalty. Fahn (1979) describes nectar as a plant exudate, secreted by glandular tissues located on various floral parts. They are so important that its structure and features are largely considered in plant taxonomy and phylogeny.

Nectar can be secreted on virtually all plant organs (except roots), and the site of secretion usually coincides with its function, although nectaries that are functionally extra floral (EF) might be found inside the inflorescences, though not involved in pollination (ELIAS, 1983).

According to Brandenburg et al. (2009) and De La Barrera and Nobel (2004), many angiosperm and some gymnosperm species produce floral nectar (FN) to attract insect or vertebrate pollinators to achieve adequate fertilization and outcrossing. On the other side, ex-

tra floral nectar (EFN) attracts ants, parasitoids and generalist predators, and serves as an indirect defense against herbivores for more than 100 families of ferns, gymnosperms and angiosperms (KOPTUR, 1992; HEIL, 2008). Many plants produce nectar in diurnal rhythms, partially adapted to consumer activity (HEIL et al., 2000; KUO and PATE et al., 1985; TILMAN, 1978; CORBET and DELFOSSE, 1984).

Heil (2011) mentions that even though it is not known whether plants really can adjust the *de novo* nectar secretion, a reabsorption of FN has been shown unambiguously (NEPI and STPICZYNSKA, 2008). Using different methods, Pederson et al. (1958) and Ziegler and Lütge (1959) demonstrated the uptake and allocation to other plant organs, including labelling studies of artificially applied $^{14}C$-labelled sucrose or glutamine. The reabsorption of non-consumed FN is apparently common, though this phenomenon has yet to be demonstrated for EFN (NEPI and STPICZYNSKA, 2008).

The reabsorption of non-secreted nectar by the post-secretory floral nectary has been related to programmed cell death in the nectary tissue, in combination with a phloem that remains active and the resulting changes in source-sink relationships (GAFFAL et al., 2007; KUO and PATE et al., 1985). Under this scenario, extra floral nectaries are likely to lack the capacity to reabsorb nectar, because the regulation of EFN secretion is not dependent on ontogenetically programmed patterns.

# NECTAR COMPOSITION, DYNAMICS AND ROLE

Nectar is a sweet liquid but, as an average, nectar sugars represents less than 2% of the current net photosynthesis of a given time (PATE et al., 1985). Plants regulate nectar production according to the consumption rates (CORBET and DELFOSSE, 1984; BOLTEN et al., 1979; INOUYE et al., 1980; CORBET et al., 1979; GILL, 1988; PYKE, 1981; PEDERSON et al., 1958; ROBERTS, 1977) and reabsorbs the exceeding (PACINI, 2003).

Heil et al. (2000, 2007) and Nepi and Stpiczynska (2008) refer that it is still partially unclear where non-carbohydrate nectar components are synthesized, how its compounds enter the nectar, how plants adapt nectar secretion to consumption rates or consumer identity, and also how non-consumed nectar is reabsorbed. Despite Gonzales-Teuber and Heil (2009a, b) and Nicolson et al. (2007) have mentioned that little is known about nectar components other than sugars and amino acids, and also about synthesis of nectar components and the regulation of their secretion, important discoveries were recently made. Nectar proteins, called nectarins, were identified in tobacco (*Nicotiana* spp.) FN

with protection activity, as well as on Acacia EFN and pollination droplets of gymnosperms (CARTER and THORNBURG, 2004; GONZALES-TEUBER et al., 2009a, 2010; WAGNER et al., 2007; CARTER et al., 2007; WENZLER et al., 2008; WOLF et al., 1981).

On investigating nectar composition, Heil et al. (2005) and Kram et al. (2008) found some enzymes that play a central role in the post secretory tailoring of nectar chemistry. Kessler and Baldwin (2007) identified nectar odors eliciting pollinator behavior. Radhika et al. (2010) associated the hormone jasmonic acid (JA) with the modulation of the secretion of FN, while Heil et al. (2001), Heil (2004) and Heil et al. (2004) reached the same conclusion regarding EFN.

Three genes encoding for putative transcriptions factors have been established to be involved in nectary development: CRABS CLAW (CRC), and BLADE-ON-PETIOLE 1 and 2 (Bowman and Smyth, 1999; McKim et al., 2008). Afterwards, Ruhlman et al. (2010) discovered a gene encoding an apoplastic invertase in *Arabidopsis thaliana*, whose function is required for FN secretion. The first proteomes obtained from the nectars of various species were mentioned by Gonzales-Teuber et al. (2010), Park and Tornburg (2009), Peumans et al. (1997) and Hillwig et al. (2010).

Two alternative and nonexclusive scenarios were mentioned by Heil (2011), regarding the origins of carbohydrates, the major class of nectar components: (i) direct transport from the phloem; and (ii) accumulation of starch in the developing nectary and its hydrolysis during active secretion. Alternatively, some carbohydrates might even derive from *in situ* photosynthesis. The direct secretion of the products of the current assimilation process has been shown repeatedly for FN, using the girdling of flower shoots as well as darkening and defoliation experiments (GAFFAL et al., 2007; VON CZAMOWSKI, 1952). Radhika et al. (2008) used $^{13}C$ labelled $CO_2$ to demonstrate that EFN also contains sugars that have been assimilated during the last hours before secretion.

The second scenario is supported by studies of Horner et al. (2007) and Ren et al. (2007), showing that nectaries of ornamental tobacco and *Arabidopsis* accumulate large quantities of starch. The degradation of this starch into mono and disaccharides coincides with the onset of nectar secretion during anthesis. A breakdown of accumulated starch, as well as programmed cell death during and after secretion, have also been described for further, taxonomically unrelated species such as soybean (*Glycine max*) (HORNER et al., 2003) and common fox-glove (*Digitalis purpurea*) (GAFFAL, 2007; BAKER and BAKER, 1975). According to Pacini et al. (2003), many species possess amyloplasts in their nectary tissue that can become directly connected to the vacuole and consecutively emptied during the phase of most active FN secretion (GAFFAL et al., 2007).

According to the first scenario mentioned by Heil (2011), carbohydrates are uploaded as sucrose from the phloem to the secretory tissue where they are stored and/or processed (KRAM and CARTER, 2009; WENZLER et al., 2008). It is widely known that, during active secretion, sucrose is metabolized by cell wall invertases, producing hexose-rich nectars and creating the required source-sink relationships (FREY-WYSSLING et al., 1954; AGTHE, 1951; ZIMMERMANN, 1953). More recently it was established that genes coding for complete sucrose biosynthesis are up regulated in *A. thaliana* nectaries (KRAM et al., 2009), and the expression patterns of genes involved in starch metabolism allow a clear separation of an anabolic phase before anthesis and a catabolic phase during secretion in nectaries of ornamental tobacco (REN et al., 2007).

According to Zimmermann (1953) and Heil et al. (2005), sucrose can also be eliminated from nectar by post-secretory hydrolysis, which is mediated by invertases that are secreted into the nectar itself. Ruhlmann et al. (2010) discovered an apoplastic invertase required to create the sink status for active nectar secretion. A mutant line, which lacked this activity was referred by Ruhlmann et al. (2010) and Kram and Carter (2009). This enzyme was associated to reduced levels of starch accumulation within the nectary, demonstrating that apoplastic invertases might also play a central role in the uploading of sucrose from the phloem and its subsequent storage in the nectariferous tissue.

Nonetheless, Gaffal et al. (2007) and Ren et al. (2007) demonstrate that starch accumulation can only account for a part of the sugar that is secreted during the peak activity of floral nectaries. Moreover, Pacini et al. (2003) alerts that extra floral nectaries have not been reported to store starch and that all carbohydrates are likely to come directly from the phloem, and nectar formation and secretion depend on vesicle-based mechanisms. Matile (1956) and Heil et al. (2004) remember that floral nectaries are phylogenetically derived from extra floral nectaries, then direct transport from the phloem seems to represent the original mechanism, whereas starch accumulation could be an alternative strategy for the secretion of large amounts of sugar, during the peak activities of floral nectaries, as stated by De La Barrera and Nobel (2004)

Anyway, there are a lot of open questions regarding where non-carbohydrate nectar constituents are produced, where and how they are added to the prenectar and how they are secreted, according to Heil (2011). This author theorizes that, considering the abundance and chemical diversity of nectary proteins and the lack of reports of many of these nectarins from other tissues, synthesis in the nectary tissue seems probable.

In fact, the secretory cells of *Vigna unguiculata* EFNs contain protein-rich inclusions (KUO and PATE et al., 1985) and all Nectarin genes that correspond to nectar proteins in the FN of orna-

mental tobacco are expressed in the nectary tissue, as stated by Thomburg (2007) and Carter and Thornburg (2004), while some of them are under the control of a MYB305 transcription factor (LIU et al., 2009). Furthermore, tobacco nectarins contain signal peptides for secretion and can, therefore, only be secreted by the fusion of vesicles with the plasma membrane.

Nectar widely vary in its composition, depending on the plant species and environmental parameters. Several sugars dominate the total solutes in floral nectar, whose main components are disaccharides, like sucrose, and monosaccharides, as fructose and glucose. The proportion of the sugars in the nectar composition is deeply variable, especially according to the species (BAKER and BAKER, 1983a, b; FREEMAN et al., 1985; STILES and FREEMAN, 1993).

Not only carbohydrates but also other compounds, such as amino acids, phenols, lipids and antioxidants are found on nectar composition, though normally in trace amounts (BAKER and BAKER, 1975; 1983a). The unique mix of a specific plant nectar leads to a particular taste and odor, which may be essential for maintaining certain pollinator groups (Southwick, 1990), especially their fidelity to specific plants. Some authors theorize that an interactive action might be present and pollinators influence the composition of the nectar like sugar ratios as well as flower and inflorescence morphology, shaped to their needs or preferences (BAKER and BAKER, 1990), on a co-evolutionary background.

To illustrate the point, hummingbirds prefer sucrose solutions instead of equivalent amount of monosaccharide ones (MARTÍNEZ DEL RÍO, 1990; STROMBERG and JOHNSEN, 1990), so hummingbird-pollinated flowers tend to produce nectar with predominance of sucrose, while bee-pollinated flowers would present higher contents of hexose (BAKER and BAKER, 1983a, b). However, in other instances nectar composition may be a conservative character due to phylogenetic constraints (GALETTO et al., 1998).

Nectar production may show different patterns according to the pollinators visiting the flowers (FEINSINGER, 1978; CRUDEN et al., 1983; GALETTO and BERNARDELLO, 1995). Baker and Baker (1983a, b) points out the possible co-evolutionary relationships between nectar traits and pollinator type. For instance, Hawk-moth-pollinated flowers produce abundant but less concentrated nectar, contrarily to bee-pollinated flowers that secrete highly concentrated nectar in lesser amounts, whereas hummingbird-pollinated flowers show intermediate values (PYKE and WASER, 1981; OPLER, 1983; BAKER and BAKER, 1983A; SUTHERLAND and VICKERY, 1993).

Thus, the knowledge of nectar production dynamics is paramount to understand the ditrophic plant-animal relationship. Galetto and Bernardello (2004) demonstrated that there is a specific rhythm for nectar secretion throughout the lifespan of a flower, turning it pos-

sible that the nectar production dynamics of a species can be determined. The strategy of the plant of offering nectar, the activity patterns, frequency and diversity of pollinators of a plant species, the rates of nectar consumption by animals, among others, could not be understood without deep knowledge of nectar characteristics and dynamics.

## NECTAR PRODUCTION AND THE ROLE OF ENZYMES

Several studies targeted the action of the apoplastic invertase on tissues surrounding the phloem, which create the flowing of sugars to non-photosynthetic tissues, as is the case of flowers. The apoplast is the free diffusional space outside the plasma membrane, interrupted by air spaces between plant cells and by the plant cuticle. This way, the apoplast is formed by the continuum of cell walls of adjacent cells as well as the extracellular spaces, forming a tissue level compartment. The apoplastic route facilitates the transport of water and solutes across a tissue or organ. This process is known as apoplastic transport. The apoplast is important for all the plant's interaction with its environment. The main carbon source (carbon dioxide) needs to be solubilized in the apoplast before it diffuses through the plasma membrane into the cytoplasm and is used by the chloroplasts during photosynthesis. The apoplast is also a site for cell-to-cell communication.

Ruhlman et al. (2010) observed that the production of nectar is closely linked to the presence of the enzyme apoplastic invertase, therefore, larger nectar production was dependent on an increase in enzyme activity in addition to the photosynthetic capacity of the plant itself. Then we can infer that increasing the volume of nectar promoted by its withdrawal is largely linked to an increased activity of the invertase enzyme. These same authors observed that in modified Arabdopsis, silencing the gene encoding one of the isoforms of the predominant enzyme in the reproductive organs prevented the production of nectar.

The rationale of this process was proposed by Cheng and Chourey (1999). Invertase promotes the hydrolysis of sucrose to the hexoses glucose and fructose and can be located in the cell wall (apoplastic), vacuole (vacuolar) or cytoplasm (cytoplasmic). The cell wall invertase is important when unloading the phloem apoplastic follows a path facilitating the passage of sucrose to the drains tissues. The authors' studies concluded that the importance of apoplastic invertase is not restricted to the breakdown of the sucrose molecule, since the injection of glucose and fructose has the same effect on the passage of photosynthates to the phloem tissue drains. In this case, the authors attribute to the enzyme a possible broader role as signaling or regulatory factor.

Further evidence of the importance of apoplastic invertase in phloem unloading process that follows this path is the abundance of transcripts in these tissues, according to Jin et al.

(2009) and its almost non-existence in tissues with symplastic unloading (RUAN and PATRICK, 1995), on tomato tissues. In *Vicia faba*, tissues surrounding the seed formation, which have no connections of plasmodesmata, also show high expression of the enzyme (WEBER et al., 1996). Furthermore, suppression of the expression of the apoplastic invertase-coding gene reduces the production of rice grains; when the invertase concentration increases, yield increase is observed, thus confirming the crucial role of the enzyme in the development of non-photosynthetic organs (WANG et al., 2008).

# NECTAR SECRETION

Most authors agree that nectar represents a kind of "secreted phloem sap" for most species (DE LA BARRERA and NOBEL, 2004; FAHN, 1988; LÜTGE, 1961; CARTER et al., 1999; FREY-WISSLING, 1954; AGTHE, 1951). Meanwhile, although nectaries connection to phloem or xylem is the rule, in a large number of species the plant vascular traces do not reach the epidermis. Wist and Davis (2006) reported that 50% of Asteraceae lack direct vascular connections in their floral nectaries. A taxonomically broader review also found that nectaries of more than one-third of all plant species lack any direct vascularization (FAHN, 1988). According to Davis et al. (1988) and Elias et al. (1975), only a minority of the nectaries receive direct vascular supply from xylem and phloem and, even in those ones, the branches reaching the parenchyma or the epidermis are frequently origineted in the phloem.

A detailed pathway has not yet been elucidated to describe how carbohydrates and other nectar components are uploaded from the phloem to the nectariferous tissue, metabolized and secreted to the exterior. Both symplastic pathway and transport through the apoplasm are mentioned. Most likely, prenectar is transported in vesicles that move through the symplast via plasmodesmata and are secreted via exocytosis (KRAM and CARTER, 2009). Nectar carbohydrates can be prestored in the nectariferous tissue, a step that at least seems to be involved in the secretion of nectars that are more concentrated than the phloem.

The most commonly accepted evidence suggests that prenectar is transported in vesicles, where its composition can be changed, and afterwards secreted by exocytosis, as described by Kram and Carter (2009). Carbohydrates pre-storage might be involved, with invertases playing a double role in the uploading of pre-nectar from the phloem, and in the mobilization of carbohydrates during active secretion (RUHLMANN et al., 2010; VON CZAMOWSKI, 1952).

According to Heil (2011), there are five major evidences supporting the model described above:

• Nectar secretion via trichomes excludes an apoplastic transport for the respective species, owing to apoplastic barriers in the external cells walls in the stalk and intermediate cells of the trichomes (FAHN, 1988; KUO and PATE et al., 1985);

• Vesicles are common in nectariferous tissues (FAHN, 1988; KUO and PATE et al., 1985);

• Secreted nectars are characterized by a wide range of concentrations and sucrose: hexose ratios (BAKER and BAKER, 1975; BAKER and BAKER, 1982) and, therefore, cannot be produced only by a passive flow or transport mechanisms exclusively driven by sucrose-cleaving enzymes;

• Non-carbohydrates such as lipids and proteins are likely to be synthesized in the nectariferous tissue and then must enter the prenectar before its secretion (NICOLSON et al., 2007; KRAM et al., 2008);

• Nectar secretion depends on several fast-acting control mechanisms, which cannot result solely from passive source sink relationships.

# BEE GENERAL ORIENTATION

Generally speaking, when approaching blossoms, honeybees are firstly attracted by their color and/or the form. These cues are then used for successful revisits. According to Hsu and Young (2012), the bees receives visual cues through two types of parallel channels located behind the retina. The first channel is used for colors; the other is a monochrome channel directed to the orientation and edge of an item in their visual field. In the integration process of these channels, the priority and interaction between them are significant, as these chromatic and achromatic signals coexist naturally.

Hsu and Young (2012) trained bees to detect form and color, afterwards tested for its ability to differentiate combinations of opposite patterns. The bees choose the correct color but the wrong form pattern in the above experiment as well as for other manipulations. The effect of the color training for the blue reward pattern differed from that of the green reward pattern. The color pattern choices tended to be more correct if blue was the target during the training process, indicating that the chromatic signal was the main cue in pattern discrimination. The authors concluded that color tended to be the decisive factor in a conflicting situation. In addition, the color blue was preferred over green, indicating that color preference was involved in bees' visual recognition.

Honeybee visitation to a flower can be considered a two stage process. At first involves orientation from a larger distance and then, secondarily, close-range orientation during which the bee alights and probes for nectar. Von Frisch (1967) established that bees are

guided to particular flowers by floral aroma, color, and shape. Hansen et al. (1964), Clement (1965), Free (1993), and LeLeji (1973) observed foraging preferences associated with floral color. Butler (1951) concluded that honeybees were attracted over distances of several feet by the sight of a flower (i.e. color and shape) but at last were induced to enter the flower by its aroma. Floral aroma, color and shape all therefore appear to influence initial honeybee visitation and provide foraging landmarks, which honeybees utilize to optimize foraging on a specific plant species.

However, reaching a flower of a given plant is not enough, sustained fidelity to a given species requires foraging reinforcement. This way, flowers must offer pollen or nectar in quantities exceeding the foraging threshold of individual honeybees. This foraging threshold is determined by the minimal floral reward acceptable to foraging honeybees and the abundance and reward quality of other plant species competing for honeybee visitation. It is a matter of efficiency and minimal energy consumption for the maximum recollection of pollen and nectar.

A balance exists wherein individual flowers provide the minimum food rewards to attract bees and induce them to visit and subsequently pollinate other flowers of the same species, while bees forage to maximize their energy returns and visit a minimum number of flowers (HEINRICH and RAVEN, 1972; ROBACKER and AMBROSE, 1981).

Fidelity is not always the best behavior. The acuity of floral fidelity exhibited by individual honeybees can also prove detrimental to cross-pollination efforts. If enough variance in floral characteristics exists within a plant population, some members of that population may be reproductively isolated. The fidelity of foraging bees to one kind of flower has been proposed as a means of sympatric speciation by ethological isolation (GRANT, 1949). This becomes particularly apparent in agronomic practices when anticipated crosses between two varieties of a given species do not occur due to pollinator discrimination between varieties. As an example, Hansen et al. (1964) stated that active selection by foraging honeybees was detrimental in alfalfa (*Medicago sativa* L.) breeding programs.

Honeybee fidelity/discrimination between varieties or lines of numerous agronomic crops has been reported including: birdsfoot trefoil (*Lotus corniculatus* L.) (DeGrandi and Collison, 1980); Brussels sprouts (*Brassica oleracea* L.) (Faulkner, 1974); carrots (*Daucus carota* L.) (Erickson et al., 1979); cotton (*Gossypium*_spp.) (Moffett and Stith, 1972); onions (*Allium cepa* L.) (LEDERHOUSE et al., 1972); soybeans (*Glycine max* (L.) Merr.) (KETTLE and TAYLOR, 1979); sunflowers (*Helianthus annuus* (D.C.) Ckll.) (TEPEDINO and PARKER, 1982).

Plant factors related to bees' attraction, orientation and discrimination are inherited. Genetic variation and heritability of floral nectar quantity and carbohydrate concentration

has been demonstrated among alfalfa clones (WALLER et al., 1974; TEUBER and BARNES, 1979) and birdsfoot trefoil cultivars (MURRELL, SHUEL and TOMES, 1982; MURRELL, TOMES, and SHUEL, 1982). Loper (1976) observed an increased emanation of individual aroma compounds from flowers of $F_1$ alfalfa plants. Therefore, selection for varietal compatibility in floral characteristics of agronomic crops requiring honeybee-mediated cross-pollination appears possible.

# NECTAR AND ATTRACTION OF POLLINATORS

Pollinators visit flowers looking for pollen and nectar, as food components for the colony. The concentration and abundance of nectar in flowers has been shown to affect honeybee foraging activity (BUTLER, 1945; CORBET, 1978; KAUFFELD and SORENSEN, 1971; PEDERSEN, 1953; VANSELL, 1934). From the plant strategy standpoint, nectar must attract mutualists and beneficials and repeals nonmutualists, robbers or undesirable visitors, and these functions must be performed simultaneously and depends on the nectar chemistry.

Nectar is a complex of carbohydrates, basically a solution of fructose, glucose, or sucrose in water with minute amounts of many other plant compounds like other carbohydrates, amino acids, proteins, mineral ions, organic acids, vitamins, lipids, antioxidants, glycosides, alkaloids, and flavonoids (WALLER et al., 1972; BAKER and BAKER, 1977; LUTTGE, 1977; SCOGIN, 1979; GILLIAM et al., 1981). Content of carbohydrate in the nectar may vary from 4 to 60%, depending on species and on environmental conditions (SHUEL, 1975; KLEINSCHMIDT et al., 1978).

Although nectar sugars are 100-1000 fold more concentrated than amino acids, the laters can significantly affect the attractiveness of nectar. While birds and bats can obtain nitrogen from other sources, many adult insects feed only on liquids. Therefore, insect-pollinated flowers should possess more amino acids in their nectar than vertebrate-pollinated flowers. In this sense, high amino acid concentrations have been reported for FNs from flowers adapted to butterflies (BAKER and BAKER, 1982), flies (POTTER and BERTIN, 1988) or bees (PETANIDOU et al., 2006). Similarly, ants prefer nectars rich in amino acids, and as well as many insect pollinators, ants can show strong preferences for specific, usually essential amino acids (BLÜTGEN and FIEDLER, 2004; CARTER et al., 2006; GONZÁLES-TEUBER and HEIL, 2009).

Based on a survey of almost 900 plant species, Percival (1975) indicated that three general patterns of nectar carbohydrate composition exist: sucrose dominant; equal amounts of su-

crose, fructose, and glucose; dominant fructose and glucose. It has been proposed that nectar quantity and quality may play an important role in determining the search of foraging honeybees (WADDINGTON and HOLDEN, 1979), although it is not clear whether bees alter their foraging strategies considering the hourly and daily fluctuations in nectar characteristics of individual species.

Frey-Wyssling (1955) supported the theory that nectar secretion is related to the phloem supply to floral nectaries because the number of cells between phloem sieve tubes and the surface of floral secretory tissue cannot exceed a certain amount (in most cases about ten cells) for abundant nectar secretion to occur. However, nectar composition differs considerably from that of phloem sap (ZIEGLER et al., 1959) and has been proposed by Luttge (1977) to be a function of specific active transport mechanisms.

In this context, the primary enzyme would be the apoplastic invertase located in the tissues surrounding the phloem unloading and that allows sucrose to non-photosynthetic organs that follow an apoplastic pathway after the phloem, such as the reproductive organs. Ruhlman et al. (2010) observed that the production of nectar is closely linked to the presence of the enzyme apoplastic invertase; therefore, a greater nectar production was dependent on an increase in enzyme activity in addition to the photosynthetic capacity of the plant itself. These authors observed that in Arabdopsis, in the absence of genes encoding isoforms of the enzyme, predominantly in reproductive organs, prevented the production of nectar.

Invertase promotes the hydrolysis of sucrose to the hexoses glucose and fructose and can be located in the cell wall (apoplastic), vacuole (vacuolar) or cytoplasm (cytoplasmic). The cell wall invertase is important when unloading the phloem follows an apoplastic path facilitating the passage of sucrose to the drains tissues. Some studies even conclude that the importance of apoplastic invertase is not restricted to the breakdown of the sucrose molecule, since the injection of glucose and fructose has the same effect on the passage of photosynthates to the phloem tissue drains. In this case, the authors attribute to the enzyme a possible broader role as signaling or regulatory (CHENG and CHOUREY, 1999).

Further evidence of the importance of apoplastic invertase in phloem unloading process that follows this path is the abundance of transcripts in these tissues (JIN et al., 2009) and its almost non-existence in tissues with symplastic unloading (RUAN and PATRICK, 1995), both observed from tomato tissues. In *Vicia faba*, tissues surrounding the seed formation, which have no connections of plasmodesmata, also show high expression of the enzyme (WEBER et al., 1996).

Honeybees collect sucrose solutions in the 30-50% range in preference to higher or lower concentrations (WALLER, 1972; WOODROW, 1968). Jamieson and Austin (1956) found that

honeybees can discriminate between sucrose concentrations differing by only 5%. They showed that bees can distinguish between 50% and 45%, but not between 50% and 47.5% or between 47.5% and 45% sucrose.

Carbohydrates and free amino acids in the nectar are paramount for attraction, but because animals differ in their nutritive preferences, the composition of the nectar determines the spectrum of its consumers. For example, hummingbirds, butterflies, moths and long-tongued bees usually prefer sucrose-rich FNs, as do most ant species that feed on EFN, whereas short-tongued bees and flies prefer FN rich in hexoses (GONZALES-TEUBER and HEIL, 2009A; NEPI and STPICZYNSKA, 2008; NEPI et al., 2009; BLÜTGEN and FIEDLER, 2004). However, some nectarivorous birds and ants lack the enzyme invertase, being not able to process sucrose, thus preferring sucrose-free nectars, according to Heil et al. (2005) and Martínez del Río (1990).

Previous studies reached quite different conclusions. Whitehead and Larsen (1976) determined that the maximum response of honeybee galeal chemoreceptors occurs with sucrose concentrations of about 1.5 molar (50% w/w) and glucose or fructose concentrations of about 3.0 molar (50% w/w). Honeybee carbohydrate preferences, based on nectar contents of plant species preferred by bees, indicated that honeybees might prefer nectar with relatively equal quantities of fructose, glucose, and sucrose (FURGALA et al., 1958; KROPACOVA, 1965). However, Wykes (1952) examined the gustatory response of honeybees to sugar solutions varying in composition but having the same total sugar concentration, showing that honeybees prefer solutions of sugars in this descending order: sucrose, glucose, maltose, and fructose. Bachman and Waller (1977) and Waller (1972) showed that honeybees prefer sugar solutions in which sucrose is both the main constituent and is near a concentration of 50%.

Besides carbohydrates and amino acids, which are present in high proportion in nectar, other compounds are involved in the nectar capacity of attraction. Volatile organic compounds (VOCs), like benzyl acetone, have been related to pollinator attraction, and scented petals have been known for centuries. In line with this information, nectar odors are considered a relevant signal for pollinators (RAGUSO, 2004). On the other side, gelsemine and iridoid glycosides exhibit repellent properties (HEIL, 2011).

Weiss (2001) demonstrated that butterflies and moths prefer artificial flowers containing scented nectar in contrast with ones containing pure sugar solutions, while Röse et al. (2006) mentioned that parasitoid wasps localize cotton (*Gossypium hirsutum*) EFN using only its odors, the same for mites that use nectar odors to distinguish between host and non-host plants, according to Reyneman et al. (1991).

# NECTAR, AROMA AND FIDELITY OF POLLINATORS

The importance of olfaction in recruitment of forager honeybees has been well documented (VON FRISCH, 1967; JOHNSON and WENNER, 1970). Honeybees have large numbers of antennal placoid sensilla, which are the principal chemoreceptors for floral aromas (LACHER, 1964). Antennal amputation has indicated that the acuity of scent perception in honeybees varies with the number of intact sense organs on the antennal segments (RIBBANDS, 1955). Indeed, it has been suggested that olfaction plays a more important role in forager recruitment than the dance maneuvers observed in colonies (JOHNSON and WENNER, 1966; JOHNSON, 1967; WENNER, 1967; WELLS and WENNER, 1973).

Gonzáles-Teuber and Heil (2009a, b) referred that the origin of FN scent is linked to the volatiles released by the petals, which are absorbed and rereleased by the nectar. However, a wide array of VOCs occur in the nectar of wild tobacco (*Nicotiana attenuata*), and many of these compounds have not been detected in other flower parts, suggesting that in certain species nectar emits its own scent, as referred by Kessler and Baldwin (2007). Like other nectar compounds, these volatiles serve to both attract pollinators and protect from nectar robbers such as ants (KESSLER and BALDWIN, 2007; JANZEN, 1977).

Kolterman (1969) concluded that scent was more important in conditioning honeybees than color, form, or time of day. Manning (1957) observed that honeybee discrimination was greater with a change of scent than with a change of flower pattern or shape. Boren et al. (1962) and Pedersen (1967) suggested that odor was partially responsible for differential foraging by honeybees on selected clones of alfalfa, while Kriston (1969) found that honeybees could be conditioned to scents resembling floral aromas more quickly than to nonfloral aromas.

Contrarily to soybeans, production of floral volatiles in alfalfa has been extensively studied so, in the absence of specific data for soybeans, it is important to verify what happens with alfalfa, which belongs to the same botanical family Fabaceae. Emanation of volatiles from alfalfa flowers follows a daily cyclic pattern, which is controlled by photoperiodically induced rhythms (LOPER and LAPIOLI, 1971). Honeybee selection among flowers from seven clonal lines of alfalfa, presented in bouquets for three consecutive days, was consistent from day to day (LOPER and WALLER, 1970). Flower aroma differences were indicated as a possible basis for this selection. Loper et al. (1974) observed that honeybee selection of 28 alfalfa clones appeared to depend on quantity and quality of floral volatiles. The terpene ocimene has been identified as the major volatile component of alfalfa flowers (LOPER et al., 1971). Myrcene, limonene, and linalool have also been identified as components of alfalfa flower volatiles (Loper, 1972) and olfactory discrimination by honeybees has been demonstrated with these compounds (WALLER et al., 1972, 1973, 1974).

An endogenous rhythm in volatile production was also demonstrated with *Cestrum nocturnum* (Solanaceae) flowers (OVERLAND, 1960). An increase in the emanation of individual aroma compounds from flowers of some $F_1$ alfalfa plants, over that observed in parent plants, suggests that total flower aroma and floral aroma character can be genetically influenced (LOPER, 1976; CHIALVA et al., 1982). It may therefore be possible to alter the floral character of alfalfa or other plant species to increase their attractiveness to foraging honeybees (BUTTERY et al., 1982).

Flower volatile production in a number of other plant species has been studied. Examination of over 150 species of orchid demonstrated the existence of a complex volatile spectrum involving approximately 50 different compounds (DODSON and HILLS, 1966; HILLS et al., 1968). Floral volatile production in orchids is species specific and is instrumental in pollination as their principal pollinators, the euglossine bees, discriminate between orchid species by olfaction (DODSON et al., 1969; NEILAND and WILCOCK, 1998). Complex volatile spectra have been reported for flowers from banana shrub (*Michellia figo* Spreng) (TODA et al., 1982), *Castanopsis caspidata* Schottky (YAMAGUCHI et al., 1979), clovers (*Trifolium* spp.) (HONKANEN et al., 1969), and elder (*Sambucus nigra* L.) (VELISEK et al., 1981).

# NECTAR AND PROTECTION

Carbohydrates, amino acids and volatiles serve mainly in the attraction and nutrition of legitimate nectar visitors; however, nectars also contain other compounds, for example, proteins and several classes of secondary metabolites (ZLATKIS et al., 1973). Mentions to nectar proteins date to the first half of last century, as described by Buxbaum (1927) and Lütge (1961). These proteins can be source of organic nitrogen, but there are other important functions associated with them.

For instance, the nectarins in the FN of ornamental tobacco *(Nicotiana langsdorffii x Nicotiana sanderae*) are likely to protect FN from microbial infestation through the Nectar Redox Cycle (CARTER and THURNBURG, 2004; CARTER et al., 2007; PARK and THORNBURG, 2009; CARTER et al., 2006). Anyway, FN proteomes seem to be small, as only five proteins have been found in the FN of ornamental tobacco, eight in the FN of *Jacaranda mimosifolia* and ten in the FN of *Rhododendron irroratum* (KRAM et al., 2008), but exceptions exist as mentioned by Gonzáles-Teuber (2009a) in *Acacia* myrmecophytes mutualism, where more than 50 different proteins were identified. It is worth mentioning that this species houses ant colonies for their indirect defence. Heil (2011) describes the majority of the nectarins as pathogenesis-related proteins, such as chitinases, glucanases and thaumatin-like proteins.

Kram (2008) suggested a role in antimicrobial defence for the GDSL lipases (hydrolytic enzymes with multifunctional properties) in the FN of the blue jacaranda (*Jacaranda mimosifolia*). As nectars are commonly infested by microorganisms, most nectarins seem to be involved in protecting nectar against them, particularly yeasts, whose metabolic activities change nectar composition (HERRERA et al., 2008, 2009). Nevertheless, although the presence of some nectar-infecting microorganisms or nectar robbers might have beneficial effects on the plant (LARA and ORNELLAS, 2002), Herrera et al. (2008) propose that most plants benefit from keeping the nectar as sterile as possible, maintaining control on its chemical composition.

Nectaries open stomata can be a point on entry of plant pathogens, as early mentioned by Ivanoff and Keith (1941) and Keith and Ivanoff (1941). More recently, Buban et al. (2003) and Farkas et al. (2007) describe the nectary as the primary site of infection by *Erwina amylovora*, causal agent of the bacterial fire blight of apples and pears. To protect the plant, nectars contain antimicrobial nectarins to protect the nectary tissue from infection. It has also been observed that some nectars are toxic, due to some compounds toxic to robbers and pests, but sometimes also to beneficials, like pollinators. The toxicity of the nectar is due to secondary metabolites, non-proteins amino acids, phenolics and alkaloids (BAKER, 1977; ADLER, 2000).

# EFFECTS OF NECTAR AND POLLEN REMOVAL

Nectar removal by floral visitors may have a pronounced effect on the total amount secreted by a flower. Although in some species nectar removal does not modify its production pattern (GALETTO and BEMARDELLO, 1993, 1995; GALETTO et al., 2000), in others the total amount of sugar in the nectar may increase (PYKE, 1981; GALETTO and BEMARDELLO, 1995; CASTELLANOS et al., 2002) or decrease (GALETTO and BEMARDELLO, 1992; BEMARDELLO et al., 1994; GALETTO et al., 1997). Predictions for these patterns are not straightforward because they may be related to pollinators, environmental factors, plant resource allocation, or other factors (GALETTO and BERNARDELLO, 2004).

Looking at the several characteristics of nectar production and dynamics, and also its interaction with pollinator, several authors investigated the effect of nectar removal, the impact over plant resources allocation (to nectar or seed production) and, at last, over cross pollination. Ornelas and Lara (2009) suggested a possible theory to link cross pollination level, nectar removal and increased yield. Studying different levels of nectar replenishment and pollen receipt by the stigma, they suggested that pollination intensity and nectar replenishment interact in their effects and affect seed production. Their study on *Penste-*

*mon roseus* involved seven levels of manual nectar removal as follows: 1) once at the end of the flower life; 2) once a day for two days during the staminate phase; 3) once a day for two days during the pistillate phase; 4) once a day during four consecutive days; 5) twice a day for two days during the staminate phase; 6) twice a day for two days during the pistillate phase; 7) twice a day during four consecutive days. Pollen manipulation involved the following treatments of anthers placed onto virgin stigmas: 1) low frequency – one anther from one donor; 2) medium frequency – two anthers from two donors; 3) high frequency - four anthers from two donors.

By manipulating rates of nectar replenishment and patterns of pollen receipt, these authors found evidence for a trade-off between the plant investing resources in nectar or in seeds, particularly at intermediate levels of nectar replenishment. The maximal seed production was reached when flowers received intermediate levels of pollen addition and nectar removal. However, when the frequency of nectar removal was increased, seed production decreased to levels similar to naturally pollinated flowers. These results suggest that *P. roseus* seed production is pollen limited either on self or cross-pollinated plants. Nevertheless, the magnitude of pollen limitation was more pronounced when plants paid the cost of attracting additional pollination (nectar replenishment costs).

The highest seed mass occurred after four nectar removals (once a day over 4 days) as well as after nectar removal at the end of the flowers' lives, supporting the idea of resource allocation to seed maturation under low nectar-removal rates. Ornelas and Lara (2009) stated that these results are congruent with recent metaanalyses and recent theoretical models, which showed that pollen limitation may reduce seed set, as measured by the response to supplemental pollen should be common in wild plants, and that the magnitude of pollen limitation may be greater when plants are more attractive to pollinators.

When nectar removal was increased there was a detrimental effect in terms of seed production (ORNELAS and LARA, 2009). An increase in pollen deposition resulted in more seed production to a point where nectar replenishment became presumably costly for the maturation of additional seeds. The control of no nectar removal and different pollination intensities helped to untangle the effect of nectar removal and the effect of pollination intensity. These authors also found that seed production did not increase linearly with increased pollination intensity, when no nectar was removed. This finding confirmed the independent effect of pollination intensity.

By manipulating rates of nectar replenishment and patterns of pollen receipt, Wang et al. (2008) also found evidence for a trade-off between the plant investing resources in nectar or

in seeds, particularly at intermediate levels of nectar replenishment. The maximal potential seed set was reached when flowers received intermediate levels of pollen addition and nectar removal; however, when the frequency of nectar removal was increased seed production decreased to levels similar to naturally pollinated flowers. These results suggest that *P. roseus* seed production is pollen limited in both populations, and that the magnitude of pollen limitation was more pronounced when plants paid the cost of attracting additional pollination (nectar replenishment costs).

Although some animal-pollinated flowers respond positively to nectar removal by producing additional nectar, this extra secretion is not always expensive (ORDANO and ORNELAS, 2005). The expenses of nectar production are negligible in terms of both floral tissue investment (HARDER and BARRETT 1992; LEISS et al. 2004), vegetative growth (GOLUBOV et al. 2004) or in seed production (ORDANO and ORNELAS 2005; ORNELAS et al. 2007), in spite that it can also be reasonably high in terms of devoted energy, photosynthate assimilation (PLEASANTS and CHAPLIN 1983; SOUTHWICK 1984) or seed production (PYKE 1991; ORDANO and ORNELAS 2005).

Pyke (1991) demonstrated the costs of nectar production in wild plants of *Blandfordia nobilis*, which were hand-pollinated to ensure optimal pollination. His results showed that availability of resources and not the level of pollination limit the number of seeds per plant.

Some studies have shown a peak in seed production at intermediate levels of pollinator visitation, with a decrease in seed production at higher visitation levels (BÚRQUEZ et al., 1987; BÚRQUEZ and CORBETT, 1991; YOUNG, 1988; HERRE, 1990) that may result from the removal of previously deposited pollen on the stigma (GORI, 1983). In addition, pollen deposition is variable from one flower to another (STEPHENSON 1981; BURD, 1995). These results might indicate saturation of bee population foraging in the field, collecting more nectar and more pollen than advisable, forcing the plant to invest resources to produce more nectar, or preventing maximum potential of fecundation. This kind of result is contrarily to the idea that more bees foraging on soybean would result in more cross-pollination, more efficiency on fecundation, at last higher yields.

# NECTARIES

Nectaries can be extremely diverse with respect to their localizations, structures and even their secretion mechanisms, according to Elias (1983), Fahn (1988) and Pate et al. (1985). In some species there is no externally visible structure for a nectary (FREY-WYSSLING and

HÄUSERMANN, 1960) and its presence can be identified only when nectar appears on the plant surface, or they can form anatomically distinct and sometimes highly conspicuous structures with a complex ultrastructure (HEIL, 2011).

Nectaries can be connected to the phloem, the xylem, both or have no direct vascular connection, as stated by Fahn (1988) and Wist and Davis (2006). The nectar exits through modified stomata that remain permanently open or through specialized trichomes (FAHN, 1988; WIST and DAVIS, 2006; VASSYLIEV, 2010; FRANCESCHI and GIAQUINTA, 1983). Such differences can even occur within the same plant and functional types of nectaries. For example, stipular extra-floral nectaries of cowpea (*Vigna unguiculata*) form an area of widely spaced secretory trichomes that lacks any direct connection to the vascular system, according to Kuo and Pate (1985). They also mention that extra floral nectaries on the inflorescence stalk consist of a region with secretory, cone-shaped tissues that are connected to the phloem and release EFN through permanent1y open stomata.

The nectary and glandular trichome secretions may be spatially and functionally timed and, therefore, may jointly contribute to the final nectar composition. Broersma et al. (1972), Levin (1973) and Rivera (1996) refers glandular floral and vegetative trichomes present on some species, which could be sites of production of anti- microbial compounds.

## SOYBEAN NECTARIES AND PRODUCTION OF NECTAR

In recent years, several studies targeted to elucidate the development and the functional features of nectaries (DURKEE et al., 1981; DURKEE, 1983; DAFNI et al., 1988; FAHN, 1988; BEARDSELL et al., 1989; FIGUEIREDO and PAIS, 1992; ZER and FAHN, 1992; RABINOWITCH et al., 1993; BELMONTE et al., 1994; STPICZYNSKA, 1995; NEPI et al., 1996; O'BRIEN et al., 1996; GAFFAL et al., 1998). While some investigation focus on the mature stage when nectar is present or on the whole composition of the nectar (PERCIVAL, 1961; BAKER and BAKER, 1981; RABINOWITCH et al., 1993; ECROYD et al., 1995; DAVIS, 1997; CARTER et al., 1999; CARTER and THORNBURG, 2000; THORNBURG et al., 2003), others concentrate on nectar sugars content, like glucose, fructose, and sucrose, largely responsible for its functionality and characteristics (BUTLER et al., 1972; BAKER and BAKER, 1981; ROSHCHINA and ROSHCHINA, 1993).

In spite that the family Fabaceae comprises several cultivated plants of economic importance, there have been relatively few (ANCIBOR, 1969; WADDLE and LERSTEN, 1973; GULYÁS and KINCSEK, 1982) studies dealing with the development, anatomy, and ultrastructure of

legume nectaries. Species involved on more recent studies are *Lotus corniculatus* (MURRELL et al., 1982; TEUBER et al., 1980), *Phaseolus vulgaris* (WEBSTER et al., 1982), *Pisum sativum* (RAZEM and DAVIS, 1999), *Trifolium pratense* (PICKLUM, 1954; ERIKSSON 1977) and *Vicia faba* (WADDLE and LERSTEN, 1973; DAVIS et al., 1988; DAVIS and GUNNING, 1991, 1992, 1993; STPICZYNSKA, 1995). Authors that have investigated soybeans nectaries, its structure and functionality are Purseglove (1968), Carlson (1973), Waddle and Lersten (1973), McGregor (1976), Erickson and Garment (1979), Carlson and Lersten (1987), Crozier and Thomas (1993) and Horner et al. (2003).

The study from Horner et al. (2003) details changes occurring inside the cells and tissues of the nectary, the subtending floral receptacle, the vasculature that innervates the nectary, and the adjacent gynoecium glandular trichomes. They demonstrated that soybean nectaries have a different ultrastructure and method of secretion (holocrine), not previously reported for any other legume. The soybean nectary undergoes programmed cell death and ceases to exist much beyond the pollination stage. These data serve as a foundation for observing nectaries in the wild annual soybean *Glycine soja* and the wild perennial *Glycine* species where cross-pollination and nectar output are greater than the cultivated *G. max* (BROWN et al., 1986; SCHOEN and BROWN, 1991; FUJITA et al., 1997). The structure of the floral nectaries on soybeans develop between the bases of the central gynoecium and lateral stamen ring. They have a discoid shape, being formed immediately before flower opening.

A nectary consists of thin-walled parenchyma cells, showing dense cytoplasm and a nucleus, Golgi bodies and vesicles, mitochondria, plastids, endoplasmic reticulum, many ribosomes, and one or more vacuoles. The fingers of phloem penetrate the nectary at its base. They are composed of sieve tubes and companion cells, both with very small wall ingrowths and thought to provide sugars (HORNER et al., 2003), being originated from 10 vascular bundles with xylem, which innervate the stamen ring peripheral to the nectary. The flower receptacle, just below the base of the nectary, gynoecium, stamen ring, and more peripheral petals and sepals, displays the main vascular bundle and its branches that innervate all of these flower organs. Surrounding these basal vascular bundles there is a connective tissue that contains many calcium oxalate crystals.

Besides sugars, other substances are likely to be found on nectar (GRIEBEL and HESS, 1940; VOGEL, 1969; BAKER and BAKER, 1973, 1975, 1983; DEINZER et al., 1977; RODRIGUEZ-ARCE and DIAZ, 1992; ROSHCHINA and ROSHCHINA, 1993; ECROYD et al., 1995; FERRERES et al., 1996; CABRAS et al., 1999; PETANIDOU et al., 2000; KAPYLA, 1978). Variation in the ratio of xylem and phloem in vasculature of the nectary seems to affect carbohydrate composition (FREI, 1955; FREY-WYSSLING, 1955; ESAU, 1965; CONRAD and PALMER, 1976). Attractiveness

is the main characteristic of the nectar, but some of its compounds are known to protect against microbial attack or against insects eating the developing fruit and its included seeds (CARTER et al., 1999; CARTER and THORNBURG, 2000; THORNBURG et al., 2003).

Homer et al. (2003) mention three stages of soybean nectary development: preactive, active, and postactive. During both preactive and active stages, nectaries are composed of a single-layered epidermis, containing many open stomata, with guard cells having thickened walls, starch-engorged plastids, and other organelles. During the active and postactive stages, additional crystals of various types (prismatic, needle-like clusters, and cubical) are found within some of the nectary cells, in intercellular spaces, and on the outside surface near the base of the collapsed nectary. The composition of these crystals is not known. The receptacle calcium oxalate crystals do not disappear during nectary development and degeneration (HORNER et al., 2000; ILARSLAN et al., 1997; 2001).

Horner et al. (2003) presented a detailed description of the three stages of nectary development on soybeans, associated to the soybean flower (as shown on Figure 23), which are described below.

Photos: Decio Luiz Gazzoni

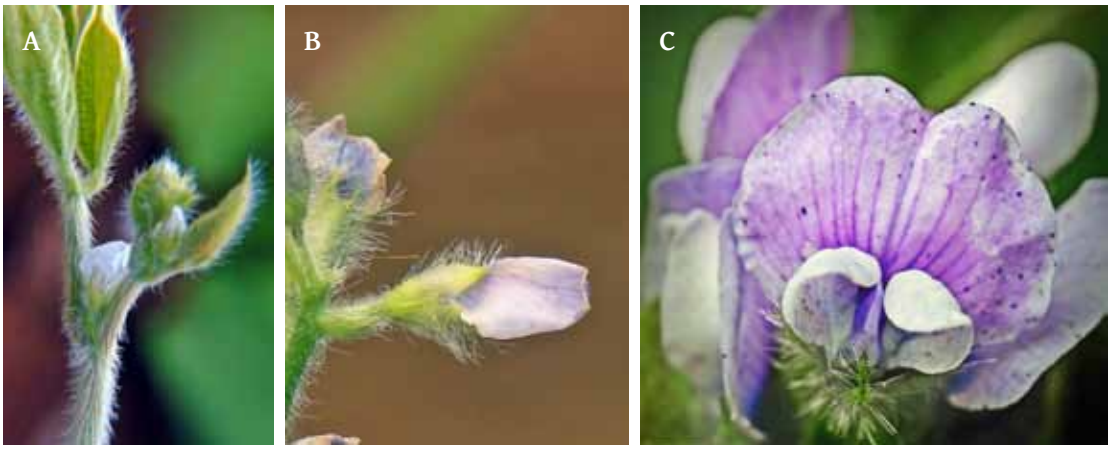


**Figure 23.** External view of the soybean flower, according to the stages of nectary development. A) Pre-active; B) Active; C) Post-active.

## PREACTIVE STAGE

During this stage, the nectary is formed between the bases of the stamen ring and the gynoecium. At the very beginning of the preactive nectary stage, there are no trichomes on the young gynoecium. Thin-walled, single-cell, elongated non-glandular trichomes develop soon after, followed by two- to three-celled, elongate trichomes with very thick walls, and then short five- to seven-celled glandular trichomes. In addition, early in nectary development, small globular bodies appear in the space between the young nectary and the gynoe-

cium. These bodies vary somewhat in diameter, and their origin is unknown. They disappear as the preactive nectary enlarges.

In sections through the nectary, crystals are observed within the basal region of the nectary, but most often, they occur within a layer two to three cells thick below the base of the nectary. This layer extends from the shared base between the nectary and the stamen ring, and the shared base between the nectary and gynoecium. Afterwards, a circular mound of special parenchyma cells is observed, covered by a single layer of epidermis with distinct stomata occurring all over the nectary surface. They consist of two guard cells and a pore, with the slope toward the gynoecium displaying more stomata. A distinct, continuous electron-dense cuticle covers all cells.

Nectary parenchyma cells are thin walled with one or more large vacuoles, as are the non-stomatal epidermal cells. Plasmodesmata occur between adjacent special parenchyma cells, and between these cells and epidermal cells. The thick-walled guard cells contain prominent plastids filled with starch and other organelles, but typically lack plasmodesmata.

## ACTIVE STAGE

During this stage, while the flower is still closed, the nectary has enlarged to its maximum size. At the beginning of this stage, special parenchyma around the phloem fingers become highly vacuolated. Vacuoles are filled with non-water-soluble material and ribosome-like particles. In many of these cells, as well as in the non-stomata epidermal cells, straight tubes with files of ribosome-like particles occur in the cytoplasm, or traverse plasmodesmata, and cytoplasmic bridges are observed associated with the vacuoles. Before and during the time the tonoplast fragments and the vacuole contents mix with the cytoplasmic organelles, bundles of tubules are often seen pressed to the outside of the vacuole tonoplast and in the cytoplasm. These cells then collapse, releasing their contents through the pores of the guard cells and onto the nectary surface. This holocrine secretion is different from that reported for other legume taxa and most other non-legume taxa and suggests apoptosis. At the end of the process, the remaining nectary special parenchyma cells follow the same fate, along with the epidermal cells, so that the entire nectary collapses, leaving only some of the guard cells intact.

There are two types of elongate non-glandular trichomes, along with one type of short five- to seven-celled glandular trichome on the gynoecium adjacent to the nectary. These later trichomes seem to be developed and become functional during the active and postactive stages, following nectary collapse. This observation suggests that the nectar may consist of a variety of compounds originating from both the nectary and the glandular trichomes.

The cytoplasmic side of the walls of the sieve elements and companion cells, and to a lesser extent the adjacent special parenchyma, display small wall in-growths that appear to completely cover the inside of the walls, but are not as pronounced as wall in-growths of transfer walls described for other taxa.

The special parenchyma cells undergo several changes before collapsing and release their contents. Special parenchyma cells farther away from the phloem fingers contain dense peripheral cytoplasm consisting of a nucleus, mitochondria, plastids, Golgi bodies with vesicles, arrays of rough endoplasmic reticulum (RER), smooth endoplasmic reticulum, unassociated ribosomes, rather large vesicles or small vacuoles with fibrillar material in them, and a larger central vacuole with several smaller surrounding vacuoles.

As each parenchyma and epidermal cell vacuole enlarges, the narrowing peripheral cytoplasm is pressed to the cell wall. Gaps appear in the tonoplast in various places, forming entrances for the cytoplasm to mix with the vacuole contents. Later, the vacuole disappears as an entity, and the mixed contents are bounded by just the plasmalemma and the cell wall. Many special parenchyma cells at this stage of development appear partially or completely collapsed, some with mixed cytoplasm and vacuolar contents.

When the collapse of the nectary nears completion, all of the parenchyma cells and the phloem cells are collapsed and are indistinguishable from each other, except for the epidermal cells and the guard cells. The non-stomata epidermal cells go through the same stages of degeneration as the special parenchyma, and they eventually collapse. As mentioned earlier, before collapse, the vacuoles display characteristic narrow bridges of cytoplasm, cytoplasmic straight tubes with ribosome-like particles, and tubular bundles. In addition, arrays of small needle-like crystals, of unknown composition, occur in the vacuoles of some of the degenerating epidermal cells, and some of the special parenchyma cells, before collapse. Crystalline material of unknown composition is also observed in the inter-cellular spaces beneath the guard cells.

## POSTACTIVE STAGE

In just-opened flowers, the nectaries are collapsed, with the exception of the guard cells, which remain intact beyond the last stage sampled. The nectary is transformed into a degenerated and collapsed mound, partially covered with a residue of electron-dense material with small cubical-like crystals, which might be derived from nectar and other secretions.

# TRICHOMES AND NECTARIES

Gynoecium non-secretory and secretory trichomes are associated with nectary development. During the final period of preactive nectary development, three types of trichomes appears on the epidermis of the gynoecium. The first type is elongated, thin-walled, non- glandular, unicellular trichome, containing a large vacuole with peripheral cytoplasm and a very large nucleus (HORNER et al., 2003).

The second type of elongated, non-glandular trichome consists of one or two basal cells with thickened walls and a long terminal cell with a much thicker wall than the unicellular trichome. The outer trichome wall also displays papillae. These trichomes contain relatively large nuclei with large nucleoli and occur in the uppermost part of the gynoecium toward the stigma (HORNER et al., 2003).

The third type of trichome develops later than the two elongated, non-glandular trichomes, is smaller and shorter, consisting of 5-7 cells in linear array, as described by Horner et al. (2003). These latter trichomes are dispersed among the longer trichomes from the base to the upper part of the gynoecium. The nucleus of each cell is much smaller than the nucleus in the two types of non-glandular, elongated trichomes, and it is centrally located in each cell.

# SOYBEAN YIELD, BEES AND ENTOMOPHILOUS POLLINATION

In spite of being a cleistogamic, self-pollinating plant, not only the honey (domestic) bee (*Apis mellifera*) but also other pollinating insects are found on soybeans. Monasterolo et al. (2015) studied visitors on soybean flowers and the effects of pollinator visits on soybean reproductive success, on a fragmented Chaco forest landscape in Argentina. Visitation rates were assessed in relation to distance from the forest, comparing to soybean bee assemblages with those on wild flowers in the nearby forest fragments. The authors also carried out an exclosure experiment in order to assess the contribution of insect visits to soybean reproductive success, as well as they also analyzed the relationship between visitor body size and the distance from the forest to the visited flower.

Five species of bees, belonging to two families, were observed visiting soybean flowers, which were also well represented in the forest. *A. mellifera* was the most abundant species, found on soybean flowers at all distances from the forest. Instead, wild visitors displayed a turnover of species throughout those distances. The smaller species were restricted to areas closer to the forest, while the larger ones were often found toward the interior of the crop.

Total visitation rates were significantly and negatively affected by distance to the forest (MONASTEROLO et al., 2015). All plant productivity variables measured in the exclosure experiments were significantly improved in exposed flowers, duplicating the values observed without the visit of pollinators, highlighting the forest role as pollinator reservoir for soybeans.

## BEES AND CROSS POLLINATION ON SOYBEANS

Beekeepers have often reported that honeybees produce significant amounts of soybean honey (HAMBLETON, 1936; MILUM, *1940;* JOHNSON, 1944; PELLETT, 1947; DAVIS, 1952; JAYCOX, 1970A; PELLET, 1976), indicating active foraging and nectar and pollen collection on

soybean flowers. Also, van der Linden (1981) reported that 61 out of 63 samples of honey produced in Iowa contained soybean pollen, indicating that they were derived at least in part (5-10%) from soybeans. Nevertheless, on previous chapter, it was quite well discussed that soybeans are autogamous, cleistogamous and self-pollinating plants. However, a close look to the associated literature shows a controversy, as there are emerging evidences that soybeans could benefit from insect pollination. Major benefit is the yield increase when insect pollination is present, as earlier speculated by Robacker et al. (1983) and Free (1993).

At first, it is not fairly acceptable that increasing soybean yields would depend on cross-pollination, considering that, on cleistogamic plants, when the flower opens it is normally fertilized. Natural cross-pollination in soybeans has been estimated to be low, ranging from about 0.03% (CAVINESS, 1966) to 3.62% (BEARD and KNOWLES, 1971). Estimates by Woodhouse and Taylor (1913), Woodworth (1922), Garber and Odland (1926), Cutler (1934), and Weber and Hanson (1961) also fall between these extremes.

Hence, it is quite surprising that increased outcrossing levels have been reported in some soybean varieties in response to honeybee visitation. Gordienko (1960) reported outcrossing rates of 28% and 44% for two varieties of soybeans caged with honeybees. Honeybee pollination of male-sterile soybeans in caged plots accounted for a 477% increase in seed production over that observed without bees (KOELLING et al., 1981). Increases in outcrossing levels attributed to honeybee visitation have been reported for open-field grown male-fertile (CUTLER, 1934; BEARD and KNOWLES, 1971; ABRAMS et al., 1978; SADANAGA and GRINDELAND, 1981) and male-sterile (BRIM and YOUNG, 1971; SADANAGA and GRINDELAND, 1981) varieties.

One possible though partial explanation for this phenomenon is the expression of the mutant gene $p_2$, that caused the outcrossing level in soybeans to be increased from less than 1% to 4-15%, with the higher rates occurring in the proximity of honeybee colonies (BERNARD and JAYCOX, 1969). According to the authors, the gene $p_2$, which causes minute pubescence (puberulence) in soybeans, also reduces pollen vigor. By reducing the flower´s ability to pollinate itself, this trait increased the rate of natural outcrossing from its normal level of below 1% to about 10% in tests in 1967 and 1968. Bee colonies in proximity caused slightly higher percentages. Other observations point out to increased cross-pollination on commercial soybean fields, close to bee´s colonies (ABRAMS et al., 1978).

To fully discuss the relationship between pollination and soybean yield (or any other plant) one should take into consideration the energy cost for attracting the pollinators. Pyke (1991) stated the importance to understand the adaptive nature of floral nectar production and the associated costs and benefits in terms of growth and/or reproduction (PYKE, 1981; PYKE

and WASSER, 1981). Nectar production may use up to 37% of a plant's available energy (PLEASANTS and CHAPLIN, 1983; SOUTHWICK, 1984) but might not affect growth or reproduction. On the opposite, Pyke (1991) reported that removal of nectar from flowers of Christmas bells (*Blandfordia nobilis*) increased the plant's net nectar production but reduced its ability to produce seeds. This is a demonstration that nectar production entails a cost to a plant in terms of growth and/or reproduction, and that both the gains and costs associated with nectar production may be estimated in the same 'currency' (reserves).

As a plant's nectar production increases, there should therefore be a trade-off between pollinator-mediated increases in numbers of fertilized seeds and decreases in seed number due to the costs of producing the nectar. In the case of self-pollinating plants, with low cost in terms of nectar production, attracting pollinators for open pollination might result in trading off costs with seed production.

Ornelas and Lara (2009) suggested a similar theory to link cross pollination level, nectar removal and increased yield. Studying different levels of nectar replenishment and pollen receipt by the stigma, they suggested that pollination intensity and nectar replenishment interact in their effects on seed production. Their study on *Penstemon roseus* involved seven levels of manual nectar removal and three pollination intensities. In spite the authors indicated an interaction between the intensity of nectar removal and pollination, relating it to increasing yields, this phenomenon varies among species, especially considering the degree of dependence of a given species to the entomophilous pollination. The increasing volume of nectar produced by the plant after nectar removal (both artificial or by flower visitors) might be linked to several metabolic pathways, including the sugar metabolism on the plant.

The potential level of outcrossing attainable by honeybee visitation may not yet be understood because floral color has been used in most if not all instances as the genetic indicator of cross- pollination. Since purple floral color is dominant to white (WOODWORTH, 1923), seeds obtained from white flowered plants that subsequently produce purple hypocotyls and flowers indicate that cross-pollination has occurred. However, honeybee discrimination between varieties relative to floral color and/or other characteristics has largely been ignored by plant breeders, and may represent a potential *bias* on the interpretation of the results.

Erickson (1975b) reported that, among soybean varieties, floral characteristics related to honeybee attractiveness (flower size, color, abundance, cleistogamy, aroma, nectar production, and blossom sequence) vary along a continuum between extreme limits for most of these characteristics. Therefore, honeybee discrimination between varieties could well have as significant effects on floral fidelity of foraging honeybees as interspecific differences. Outcrossing levels have been shown to be slightly higher when white and purple

flowered varieties are inter-mixed in the same row in opposition to separate row plantings (BEARD and KNOWLES, 1971).

Soybean flowers are not always attractive to honeybees, leading to the hypothesis that environmental conditions during growth and flowering of plants affect the development of flower characteristics (ROBACKER et al. 1983). In general, plants that grow in higher temperatures produce more nectar and are more attractive than those grown in cooler climates, up to a maximum of 29° C. Erickson et al. (1978) also observed that it was necessary to consider that the attraction of the soybean to honeybees was not the same for all varieties. Jaycox (1970b) found that number of bees varied from 680 to 810/ha, depending on the soybean cultivar, which represents roughly 1% of the population found on alfalfa (PEDERSEN, 1962), or 20% of the population observed on white clovers (WEAVER, 1965).

It is worth mentioning that, although the potentiality for nectar secretion (meaning attractiveness to bees) is hereditary, it is largely subject to climatic and edaphic aspects. Another factor that may influence the attractiveness of soybeans to bees is the distribution of flowers on the plants. The flowers of soybeans are not grouped into large heads and racemes as in clover and alfalfa, but instead are located at the leaf nodes of the plants. They are usually beneath the foliage on indeterminate cultivars. Heinrich and Raven (1972) point out that inflorescences enable a large pollinator, such as a bee, to meet its energy demand from a group of flowers that would be unsuitable individual1y. The amount of energy expended in walking between flowers can be 100 times less than an equivalent period of flight.

Even though honeybees do walk between flowers at soybean nodes and possibly between closely spaced nodes, the number of soybean flowers per plant is low. Therefore, more flying would be required to visit a given number of flowers on soybeans than on crops such as alfalfa or clover, justifying the highest number of bees visiting these crops, as contrasted with soybeans.

It is important to consider that the soybean flower stigma becomes receptive one or two days before anthesis, while the anthers release the pollen before the flower opens, which are essential conditions for auto pollination (FEHR 1980; DELAPLANE and MAYER 2000). According to Yoshimura (2011), wind pollination is negligible due to the restricted range of soybean pollen dispersal and its short life.

Milfont (2012) refers that auto pollination and use of pesticides in soybean, including the period when flowers are present, created the sense that soybeans do not need (or benefit from) insect pollination.

Some authors commented that the low number of pods as related to the previous number of flowers (normally around 10-15%), might be attributed to a pollination deficit, thus reducing the soybean yield (MCGREGOR, 1976; FREE, 1993; DELAPLANE and MAYER, 2000). In contrast, Abernathy et al. (1977) reported that failure of fertilization is insignificant as a cause of floral abscission in soybean. Abscising flowers were mostly all fertilized and usually contained proembryos that had undergone two or three cell divisions.

# BEES AND SOYBEAN YIELD

Despite the above-mentioned regarding normally low levels of cross-pollination, soybeans are sometimes referred as partially dependent on insect pollination (KLEIN et al. 2003; GALLAI et al., 2009; ISSA, 1984). Lautenbach et al. (2012) reported benefits of insect pollination on soybean, in Brazil, Argentina, India, China and USA. Robacker et al. (1982, 1983) refer that investigations have shown higher soybean yields when bees were introduced in the field for pollination purposes, despite restrictions on the methodology used on those studies.

Soybean yields have been shown to be influenced by honeybee visitation (VILA, 1988; VILA et al., 1992). Yields of three varieties grown in Indiana were increased about 17.2% up to 32 m of honeybee colonies (ABRAMS et al., 1978) and the rate of yield increases declined rapidly beyond a 32 m radius from the colonies. In Wisconsin, two cultivars, Corsoy and Hark, yielded 14.8% and 16.4% increases, respectively, in cages with honeybees over those without (ERICKSON, 1975a, c). However, there were no significant increases in the yield of 'Chippewa 64'. In cage trials with 'Pickett 71' in Arkansas and Missouri, 15% more beans were produced in cages with bees than in cages without bees (ERICKSON et al., 1978) (Tables 4 and 5). In the same study, yields in open-field trials with 'Forrest' and 'Lee 68' were significantly higher at distances of up to 100 m from apiaries.

**Table 4.** Treatment Yield Means (g) for Thrashed Samples of 3.05 m of Row.

| Year/Cultivar | With bees | Without bees | Open Plots | Bees/No bees (%) | N |
|---|---|---|---|---|---|
| 1971 | | | | | |
| Chippe | 588 | 627 | 630 | -6.6% | 6 |
| Corsoy | 762 | 669 | 676 | 13.9%** | 6 |
| 1972* | | | | | |
| Hark | 783 | 744 | 797 | 5.2% | 9 |
| 1973 | | | | | |
| Hark | 500 | 430 | 480 | 16.3%** | 9 |

* Dry spring, poor germination, nonuniform stand. ** Significant at the 0.05 level.
Source: Erickson, 1976.

**Table 5.** Differences on yield components of soybeans due to presence or absence of bees.

| Treatment | Total beans (n) | Filled pods (n) | Thrashed beans (n) | Empty pods (%) |
|---|---|---|---|---|
| Bees | 782*** | 332*** | 664* | 15.7** |
| No bees | 643 | 276 | 577 | 18.6 |
| Open field | 889 | 386 | 813 | 10.7 |

*, **,*** Bees/no bees comparison significantly different at the 0.2, 0.1, and 0.05 levels, respectively.
Source: Erickson, 1976.

In Kansas, yield in soybeans cv. Forrest grown in cages containing bees were 20% greater than in cages without bees (KETTLE and TAYLOR, 1979). Yield increases in two varieties tested over 3 years in Delaware ranged from 2.2% to 16.0% in cages with honeybees versus cages without bees (MASON, 1979). Sheppard et al. (1979) found out that honeybee populations that appear adequate for hybrid seed production have been achieved by stocking soybean fields with one hive per 0.72 hectare.

Jung (2014) found no differences on the yield among field open soybeans or caged soybeans (with and without honeybees inside the cage), but caged experiments conducted by Erickson et al. (1978) in Arkansas and Missouri, resulted in up to 16% increase on the yields when honeybees were introduced in the cages.

In North Parana, Southern Brazil, Chiari et al. (2005, 2008) evaluated the effect of the honeybee pollination on production and quality of soybean seeds, concluding that seed production was higher in covered areas with honeybee colonies (51%) and uncovered areas (58%) than in covered areas without honeybee colonies. The pod number in covered treatment with honeybees was 61% higher as compared with the covered treatment without honeybees. However, the average weight of 100 seeds was larger in the area covered without honeybees.

The study of Chiari et al. (2008), established that the soybean yields in the covered area with honeybees (2.757 kg/ha) and in the uncovered area (2.828 kg/ha) were higher than in the covered area without honeybees (2.000 kg/ha). The number of pods/plant was greater in the covered area with honeybees (38) and in the uncovered area (32) as compared to the covered area without honeybees (21), but no difference was found for the seed weight or its germination.

In Ceará, Northeastern Brazil, Milfont et al. (2013) refer increments in soybean yield of 18.1%, comparing open area with free access to wild pollinators, plus introduction of honeybees, as compared to caged pollinate-free soybeans. When honeybees were not introduced in the

open soybean area, the yield increased 6.3% over the caged area. The increase in yield when honeybees were introduced in the area, compared to caged soybeans, was attributed to 10% increase in the number of pods, 3% increase in the number of pods with two seeds and 5% in the number of pods with three seeds.

In conclusion, there are conflicting results in the revised literature. While some authors found 10-50% increase in soybean yield, when insect pollination conditions are adequate, other refer that cross-pollination is in the range of 0.5-2% and soybean flowers are fertilized when open. A net of studies involving different years, genetic material, latitude and environmental condition should be set up to clarify the controversy.

# POLLINATORS FORAGING ON SOYBEANS

Pollinators are attracted to a specific flower – like soybean flowers – by nectar and pollen, and attractiveness is apparently proportional to its amount and nutritional quality.

There are few studies regarding the diversity and seasonal abundance of pollinators visiting soybean flowers, around the world. A total of 29 species of wild bees in four families of the order Hymenoptera (Apidae, Anthphoridae, Megachilidae, Halictidae) were collected on soybeans, in three regions of the United States by Rust et al. (1980). Twenty-two species were taken in Delaware from 14 varieties of soybeans. Soybean pollen was recovered from six of these species. Seven species were collected in Wisconsin and 10 in Missouri. *Melissodes bimaculata* (Lepeletier) and *Halictus confusus* Smith were found in all three regions. *Megachile rotundata* (F.), *Megachile mendica* Cresson, and *Dialictus testaceus* (Robertson) were the most abundant pollen carriers. The highest density of wild bees was 0.36 individuals/m on `Essex'. The highest single species density was 0.24 individuals/m2 for *Ceratina calcarata* Robertson on 'Columbus'.

The foraging bees collected by Rust et al. (1980) were: 1) Family Apidae: *Bombus impatiens* Cresson; *Bombus vagans* Smith. 2) Family Anthoporidae: *Ceratina calcarata* Robertson; *Melissodes* bimaculata (Lepeletier); 3) Family Megachilidae: *Megachile rotundata* (F.); *Megachile mendica* Cresson; 4) Family Halictidae: *Agapostemon virescens* (F.); *Augochlorella striata* (Provancher); *Halictus confusus* (Smith); *Lasioglossum coriaecum* (Smith); *Dialictus testaceus* (Robertson); *D. tegularis* (Robertson); *D. illinoenis* (Robertson); *D. obscurus* (Robertson); *D. pilosus* (Smith); *D. imitatus* (Smith); *D. zephyrus* (Smith); *D. versatus* (Robertson); *D. atlanticus* Mitchell.

Alves et al. (2010) studied the influence of Africanized honeybees foraging upon the sugar concentration in the nectar of soybean either caged with honeybee colony inside, semi-covered area for free insect visitation, uncovered area, and covered area without insect visitation. The covered area with Africanized honeybee colony presented higher sugar concentration than the covered area without insect visitation or the semi covered, but the sucrose

concentration on the open area was higher than the other treatments. The content of glucose was not affected by the treatments, while fructose concentration was lower in covered area without bees as compared to the other treatments.

Barella (2009) mentioned that *Apis mellifera* was the dominant species foraging on soybeans (57%) in Barra do Bugre-MT, while Meliponini species represented 29% of total insects (not necessarily pollinators) visiting soybean flowers.

Soybean blossoms have functional nectaries. Each flower of most cultivars produces only slightly less nectar than alfalfa in northern regions of the USA, and sugar concentrations in soybean nectars are 5-10% higher than those of alfalfa, when growing conditions are favorable (ERICKSON, 1984b).

In the central United States, soybean nectar production and bee visitation occur between 9:00 am and 3:00 pm, each day. Soybean nectar volume per flower - greatest in warmer climates - varies significantly among cultivars ranging from none to 0.2 µL / flower, with some flowers having as much as 0.5 µL, noting that the honey stomach of worker honeybee holds 35 µL - 50 µL (ERICKSON, 1984a). This author examined soybean nectar and reported a mean nectar sugar content of 37 to 45%. In Missouri and Arkansas, the total carbohydrate content in soybean nectar varied from 301 µg/µL to 1,354 µg/µL of nectar and from 15 µg to 134 µg/flower. He observed that floral sugar concentration increased, but volume decreased according to the time of day and temperature. Nectar sugar ratios (i.e. fructose:glicose:sucrose content) differ among soybean cultivars as well as with time of day within a cultivar.

Erickson (1984a) found no differences in carbohydrate content between purple and white flowered cultivars, but registered that earlier in Wisconsin, nectar production appeared to be most consistent in volume and carbohydrate content among white flowered cultivars, hence they were judged more attractive for bees than purple ones. However, a later work conducted in Missouri definitely dispelled this notion (ERICKSON, 1984).

Honeybee collection of soybean pollen is highly variable as is a cultivar's ability to produce quantities of pollen. Little soybean pollen may be gathered by bees in some areas. However, soybean pollen may comprise over 50% of the total quantity of pollens gathered by many bee colonies, on the studied areas, according to Erickson (1984). Soybean pollen pellets taken from the corbiculae of foraging bees are easily recognized by their grey-brown color, small size and compaction. A possible theory explaining the high soybean pollen share on bee´s collection is the absence of flowering meliferous plants coinciding with soybean blooming, partly due to the extensive crop cultivation along large areas.

Erickson (1984) questioned whether there would be a misunderstanding of cleistogamy and the fact that soybean blossoms are open for only a single day. He mentioned that studies in controlled environment found that only 33% of `Mitchell' soybean flowers examined were completely self-pollinated 3.5 h after the onset of photophase (artificial dawn), but 58% were self-pollinated 6.5 h after the photophase began. These results suggest that early in the day soybeans exercise a cross-pollination strategy, which is followed by a self-pollination strategy later in the day. He proposed that the timing of these strategies might vary with the cultivar's relative abundance of pollen and with other factors as well. Nevertheless, he was not completely sure of his theory and recommended that follow-up field studies were needed to examine this aspect of floral development under field conditions. The search of literature after 1984 did not find any study to test Erickson hypothesis.

# SOYBEANS AND POLLINATORS RELATIONS

Erickson (1976) stated that contrary to popular concepts and much of the scientific literature on soybeans, bees readily forage on soybeans to gather both nectar and pollen, but highlighted that little is known about the level of preference bees exhibit for soybean pollen or its nutritional quality. Bees gather large quantities of soybean nectar in many areas of the United States and under certain conditions in preference to nectar from other sources. Soybean nectar is of high quality and occurs in substantial quantity depending upon the cultivar and environmental circumstances, especially soil conditions that predispose high rates of nectar secretion in soybeans (ERICKSON, 1976). Juliano (1976) and Moreti et al. (1998) called the attention for the importance of entomophilous pollination on soybeans.

In general, plants compete well for the attention of bees with nectar sugar concentrations above 25%. The quality of soybean nectar (30 to 50% dissolved solids) appears to be slightly above average content of other plants, according to Erickson (1975a, b). The rate of nectar secretion in plants is controlled by a complex of interacting climatic and edaphic factors as well as inheritance (PERCIVAL, 1975).

According to Erickson (1975a), the flowers of certain soybean cultivars opened only partially or not at all in southern Wisconsin, but it was not finally determined whether the ability of a soybean cultivar to resist cleistogamy is an indicator of its ability to secrete nectar under less than optimum climatic conditions. Cultivars that were semicleistoflorous usually continued to secrete a small amount of nectar at lower temperatures, but nectar was obtained mainly from few cultivars that were cleistogamous. The author concluded that it is not likely that bees will visit these closed flowers.

Attractiveness to bees appeared to be heritable in soybeans as it is in most insect-pollinated plants and susceptibility to temperature-induced cleistogamy may provide a way to screen for some aspects of attractiveness of soybean cultivars to honeybees, particularly nectar and aroma production (ERICKSON, 1975b). The author pointed out that the significance of the late flowering allele $e_3$ in relation to nectar production should be studied further.

On the study of Chiari et al. (2013), conducted in Londrina-PR, the anthesis period of the soybean flower was 8h04min longer in the covered area without a honeybee colony than in the covered area with a honeybee colony, and in the area of free insect visitation. The average of the analyzed stigma receptivity was 87.35% and viability of soybean pollen was 89.82%. The flower abscission rate was 71.10% in covered areas without honeybee colonies, which was greater by 50.78% and 55.12%, respectively, than the covered area with a honeybee colony and area of free insect visitation. In this study, *A. mellifera* was the insect that most frequently (97.02%) visited the soybean flowers. The time that *A. mellifera* spent to collect nectar was greater in the covered area with a honeybee colony than in the area of free insect visitation.

Gazzoni (personal information) found no differences on honeybee population on soybean fields, related to the distance of the sampling point to the bee colony, up to 200m. Nevertheless, Erickson (1976) observed more filled pods and trashed beans on soybeans harvested closer (up to 50m) of the honeybee colony (Table 6).

**Table 6.** Effect of distance from the apiary on some soybean yield components.

| Distance from apiary (m) | Total beans (g) | Filled pods | Thrashed beans (g) | Empty pods (%) |
|---|---|---|---|---|
| 15 (**) | 860 | 367 | 810 | 8.5 |
| 20 | 933 | 401 | 885 | 11.0 |
| 50 (*) | 742 | 324 | 698 | 9.2 |
| 100 | 614 | 265 | 580 | 7.8 |
| 250 | 722 | 316 | 639 | 7.2 |
| 350/500 | 706 | 302 | 649 | 8.4 |

*, ** Statistically different from numbers below at 0.1 and 0.05 levels, respectively.
Source: Erickson, 1976.

Erickson (1976) informs that the average time foragers spent for nectar collection was 2.74 second/flower and 4.37 second/flower for the pollen collection. The author refers that *A. mellifera* visited, on average, 7.14 flowers/min, collecting nectar, and 3.75 flowers/min for pollen collection. The total sugar concentration in the honeybee stomach content was 41.19% in the covered area with a honeybee colony, greater than the 38.22% observed for the area of free insect visitation.

Jung (2014) found that honeybees concentrate its foraging on soybeans in Rio Grande do Sul (southern Brazil) farms between 9 am and 2 pm, and almost no visits to soybean flowers occurs after 3 pm. These results agree with the ones found by Gazzoni (personal information) for soybean farms on North of Parana.

The attractiveness of a bee to a plant is largely linked to the concentration of sugars in nectar, which can vary widely (2-3 to 75-77%) on the secreted nectar. Bees prefer the most concentrated nectar because they spend less time and less work to dehydrate to turning it into honey. According to Erickson (1975a), the mean quantity of dissolved solids in the nectar recovered from bees foraging on soybeans was 37% (range 18-55%; n = 30). The concentration of solids was lowest in the morning and increased as the day progressed. Concurrent samples were obtained from bees foraging on alfalfa for comparison purposes. In spite of being a known preferred flower, alfalfa nectar had lower solid soluble concentrations (Table 7).

**Table 7.** Soluble solids on the nectar of soybeans and alfalfa, by time of the day.

| Time | Soybeans | | Alfalfa | |
|---|---|---|---|---|
| | Mean (%) | Range (%) | Mean (%) | Range (%) |
| 10 AM | 34 | 18-43 | 28 | 22-34 |
| 12 noon | 37 | 31-52 | 25 | 22-30 |
| 02 PM | 40 | 23-55 | 30 | 21-41 |

Source: Erickson, 1975a

Robacker et al. (1983) investigated the effects of environmental conditions on flower characteristics, including flower production, color intensity, openness, size, nectar secretion and aroma emanation and on attractiveness of the plants to honeybees. Most flower characteristics increased the attractiveness as day air temperatures at which plants were grown increased from 20 °C to 24 °C, and reached maximum values at 28 °C before plateauing or declining at 32 °C, although flower size and nectar secretion continued to increase as growing temperature raised beyond 32 °C.

Of the two flower aroma components, emanation of one component increased while the other decreased with the elevation of the growing temperatures (Robacker et al., 1983). The suggested hypothesis is that the two aroma chemicals may communicate flower-readiness information to pollinators. Flower production and flower openness responded linearly to night air temperature at which plants were grown, attaining highest values at higher (22 °C, 26° C) vs. lower (14 °C, 18 °C) temperatures. Flower production also responded linearly to soil temperature, attaining highest values at higher (28 °C - 32 °C) vs. lower (16 °C - 20 °C) temperatures.

Of the two levels each of N (75 ppm and 175 ppm) and P (15 ppm and 30 ppm) tested, the higher level of N stimulated greater flower production, flower size and nectar secretion while the higher level of P decreased the same three flower characteristics, according to Robacker et

al. (1983). Conversely, lower N and higher P promoted flower openness. Honeybee attractiveness of plants varied positively with flower characteristics such that plants grown at a day air temperature of 28° C, night air temperatures of 22 °C and 26°C, the higher level of N of the lower level of P were the most attractive to honeybees.

Roumet and Magnier (1993) measured the gene flow by bee cross-pollination on soybeans, using a male sterile genetic material. The aptitude of leafcutter bees to pollinate male sterile soybean plants (*ms2* gene) in caged plots was evaluated in four experiments from both quantitative and qualitative points of view. The plant seed set was satisfactory, as on average it represented 60% of the male fertile isogenic seed set (range= 44-69%). The lower yield of male sterile plants was linked to a smaller number of fertile reproductive nodes, according to Chen et al. (1987) and Stelly and Palmer (1982). An efficient pollen flow was observed over the flowering period with both morphological and electrophoretic markers. Insect behavior was not influenced by flower color. Differences between flowering duration of pollen donors appear to be the major factor inducing unbalanced populations.

Nectar characteristics of 17 soybean cultivars grown in Hayti, MO, were examined in order to assess the potential for preferential foraging by honeybees (SEVERSON and ERICKSON, 1984). Nectar secretion occurred between ca. 9:00 and 15:00 h Central Daylight Time; the individual flowers bloomed only one day. Mean nectar production per flower varied from 0.022 µL to 0.127 µL among cultivars, while total nectar carbohydrate content varied from 301 µg/µL to 1,354 µg/µL. Fructose, glucose, and sucrose content varied from 42 µg/µl to 314 µg/µl, 43 µg/µl to 262 µg/µl, and 97 µg/µl to 986 µg/µ1, respectively. Total carbohydrate per flower varied from 16.0 µg to 134 µg.

According to these authors, the ratios of fructose:glucose:sucrose among cultivars were distributed along a broad continuum, from those with low sucrose (ca. 1.2:1.0:1.4) to ones with high sucrose (ca. 1.2:1.0:6.7). When compared as two distinct groups, there were no apparent differences in nectar characteristics among white-flowered and purple-flowered cultivars.

Time of day was the primary factor affecting soybean nectar characteristics. Nectar fructose, glucose, sucrose, and total carbohydrate content all increased with time of day, while the volume of nectar per flower decreased. Day-to-day and temperature effects on nectar characteristics were minimal. Comparisons made within individual sampling periods suggest that there are differences in nectar characteristics among cultivars, which could encourage preferential foraging by honeybees (SEVERSON and ERICKSON, 1984).

Recent concerns regarding within-crop transgene flow stimulated researchers to update natural crosspollination rates in conventional sowings of modern soybean cultivars. Ray

et al. (2003) conducted studies in 2001 and 2002 using two soybean cultivars, Pace (white-flowered) and DP3588 (purple-flowered), selected for their equivalent flowering dates. The experiments utilized the dominance of purple flower color over white flower color to identify natural cross-pollinations. In the first experiment, 12 rows of 'Pace' (white-flowered) flanked on each side by four rows of 'DP3588' (purple flowered) were sown in the spring of 2001. Seeds were harvested by row from each of the 'Pace' rows and examined for natural cross-pollinations in the next generation. In total, 73,512 potential hybrid plants were examined and natural cross-pollination rates ranged from 0.41% at 0.9 m from the pollen source to 0.03% at 5.4 m from the pollen source. These values were consistent with values previously reported in the literature.

In a second experiment, seeds of 'Pace' and 'DP3588' were alternately sown 15.2 cm apart within a row, in the same season of the previous study. At maturity, 167 Pace plants (white-flowered) were harvested and a total of 19,151 progeny were evaluated for natural cross-pollinations, expressed in the next generation. The progeny of 56 (33.5%) of the 167 parent plants showed no evidence of natural crosspollination. The progeny of the remaining 111 plants exhibited natural cross-pollination rates ranging from 0.65 to 6.32% and averaged 1.8%. The maximum rates reported here are considerably higher than most previously reported rates, according to Ray et al. (2003).

Relating to soybean pest resistance, there is a concern involving the potential negative impact of the soybean proteinase inhibitor – a trait for soybean resistance to insects - and the foraging strategy of honeybees, as referred by Sagili et al. (2005). Laboratory studies reported disruption of the digestive physiology and learning behavior in individual honeybees treated with Bowman-Birk inhibitor (BBI), a serine proteinase inhibitor expressed in some GM plants (DECHAUME-MONCHARMONT et al., 2005). These authors investigated possible behavioral effects of this transgene on honeybees at the colony level, maintained in laboratory conditions. A choice experiment, based on 150 free-flying individuals was set up, which performed over 7,700 visits on the flowers. The mean number of visits per hour, the mean time spent on the feeder and the interval between consecutive visits were not significantly different when the feeding sucrose solution was mixed with BBI at 100 $\mu g \cdot mL^{-1}$, a dose close to the expression level *in planta.*

Soybean is not considered an anemophilous plant, ones that have cross-pollination mediated by the wind. Yoshimura (2011) investigated the subject, using Durham pollen samplers located up to 20 m from the field edge. In addition, the dispersal distance was assessed in a wind tunnel under constant airflow and then it was compared with the anticipated distances based on the pollen diameter. In the field, the maximum pollen density per day observed was 1.235 grains $cm^{-2}$ $day^{-1}$ at three observation points within 2.5 m from the field. Inside the field,

the mean density did not reach the rate of 1 grain $cm^{-2}$ $day^{-1}$ during 19 flowering days. The results of the wind tunnel experiment also showed that the plants had almost no airborne release of pollen and the dispersal distance was shorter than theoretical value due to clustered dispersal. This study showed little airborne pollen in and around the soybean field and the dispersal is restricted to a small area. Therefore, wind-mediated pollination appears to be negligible, reinforcing that all cross-pollination on soybeans involves animal pollinators, especially bees.

# SOYBEAN PEST CONTROL AND THE IMPACT ON POLLINATORS

There are several insect pests attacking soybeans during its life cycle, since sowing to almost harvest. Major pests are defoliators, pod sucking and feeders. The critical period for negative impacts of chemical pest control on pollinators occurs when flowers are present on soybean plants. This period may range from 15 to 30 days, depending on several aspects, but especially if soybean cultivars are determinate or indeterminate. On determinate types, the R2 stage is characterized by full bloom, and the uppermost node develops flowers, so vertical plant growth stops. At R3 stage (pod set or beginning pod) the blooming period is over and almost no flowers are present on the plants. The blooming period for a determinate cultivar averages 15 days, the maximum being around 20 days.

In the case of indeterminate cultivars, blooming continues after R3, sometimes up to R5 (beginning seed), extending for ca. 30 days. This is a very important and crucial difference as far as impact of pest control on pollinators is involved, because, on determinate varieties, the blooming period does not overlap with pod formation and filling. Therefore, it is not necessary to control pod sucking or pod feeding pests while flowers are open in the plants. Contrarily, on indeterminate varieties – which are the most commonly cultivated in Brazil – pods and flowers are present on the same time on the plants. Therefore, while flowers attracts pollinators, pods might (or not) be attacked by pests, needing control practices (Figure 24).

In the majority of the soybean cultivars grown in Brazil, the vegetative part of the cycle (VE to Vn) represents roughly one third of the whole cycle. Nevertheless, this general rule depends on the cultivar, the latitude and altitude, climatic conditions (rain and daily and night temperature) and on soil pH and fertility (FERREIRA et al., 1983). To illustrate, higher temperatures induces shorter soybean cycles, and the first flower appears earlier than under normal temperatures. The same is valid for hydric or nutritional stresses (low N, P and K levels on the soil).

The golden rule for minimizing negative impact of pest control actions on pollinators is strictly following the Soybean Insect Pest Management recommendations (GAZZONI et al., 1980; 1981; 1982; 1999). Foliage feeders can be important pests along the whole cycle, though dominant species may be different according to locations, environmental conditions or the presence of natural enemies (predators, parasites, pest diseases), and even related to unsuccessful chemical pest control schemes. This include the attack of defoliator pests during blooming, on both determinate and indeterminate cultivars (GAZZONI and OLIVEIRA, 1979a, b; 1983). In this case is quite easy to manage the impact on pollinators, by following simple rules like observing damage and actions levels, monitoring the major pests and its population building, as well as avoiding pesticide application during preferred bees foraging times (from 9 am to 3 pm). Biological insecticides or minimum rates of pesticides, which are less deleterious to pollinators, are paramount for mitigating adverse impact. It is important to reinforce that it is useless to control pod feeders pests (either stinkbugs or caterpillars) prior to the presence of pods in the plants, and when they are at least 0.5cm long (GAZZONI, 1980; 1994).

Situation is more complicated to manage on indeterminate cultivars, because of the joint presence of flowers and pods on the plants. In this case, if stinkbugs or pod feeding caterpillars reach action levels, a pesticide control of these pests is recommended. In this case the golden rule is selecting, among recommended pesticides for the pests to be controlled, ones that have minimum impact on pollinators. In addition, the pesticide application should be performed avoiding the bees foraging preferred time, reducing the chance of pesticides direct reaching the pollinators body. Figure 24 shows a graph representation of the soybean cycle, highlighting the blooming period, and its association with possibility of pest attack.

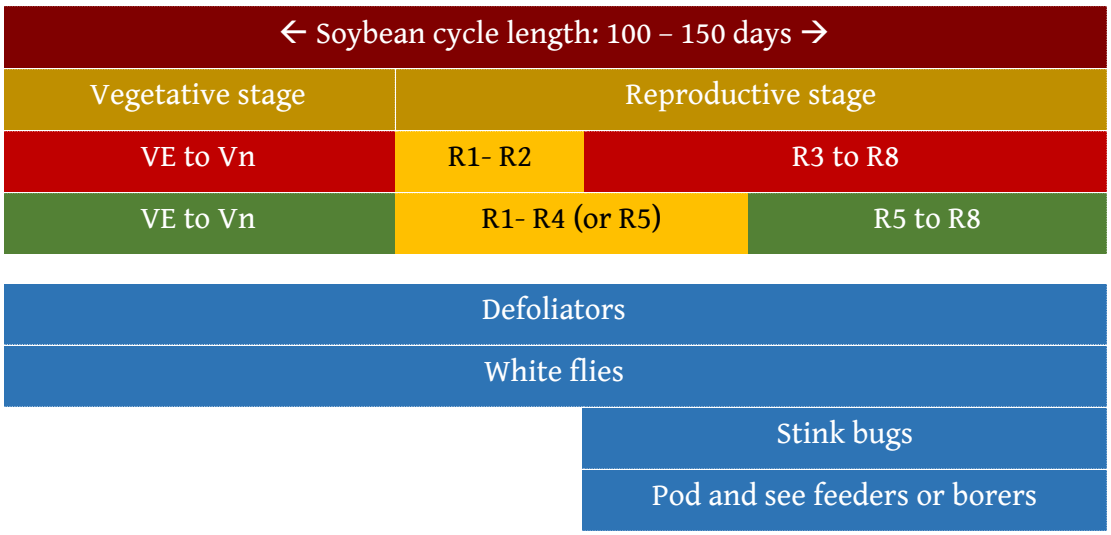


**Figure 24.** Soybean cycle, highlighting blooming period and suitability to pest attack.

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# GLOSSARY

**Abaxial**: Facing away from the axis of an organ or organism; the abaxial surface of a leaf is the underside or side facing away from the stem.

**Abscission:** Abortion of flowers or pods

**Adaxial:** Denoting the upper surface, opposite of abaxial.

**Androecium:** It is male reproductive whorl. The filament and the anther are two parts of androecium.

**Anther:** The plant organ that produces the male gamete.

**Anthesis:** The period during which a flower is fully open and functional.

**Apoptosis:** The controlled death of a cell.

**Archesporial:** The primitive cell, or group of cells, that give rise to the cells from which spores are derived.

**Bracteoles:** A little bract borne on the flower stalk above the bract and below the calyx.

**Campylotropous:**Of an ovule, orientated transversely, i.e. with its axis at right angles to its stalk, and with a curved embryo sac.

**Capitate stigma:** Enlarged or swollen at tip, gathered into a mass at apex, as compound stigma, a knoblike stigma terminating a style.

**Chalazal:** The region of an ovule that is opposite to the micropyle, where the integuments and nucellus are joined.

**Chasmogamy:** The pollination takes place in open flowers.

**Cleistogamic:** Of or relating to a flower that is self-pollinated in the bud, and may open or not after pollination.

**Crassinucellate:** Of an ovule with one or more layers of cells outside the embryo sac but distinct from the epidermis of the ovule.

**Diadelphous stamens:** Having the filaments of a flower united into two groups.

**Diaspore:** A plant dispersal unit consisting of a seed or spore plus any additional tissues that assist dispersal.

**Dioecious:** Having the male and female reproductive organs, especially flowers, on different individuals.

**Discoid**: Disk-shaped.

**Entomophilous:** Relative to insects

**Etiolated:** A plant developing with deficit or even without chlorophyll by preventing its exposure to sunlight.

**Filament:** The stalk of the stamen in a flower. It bears the anther and consists mainly of conducting tissue.

**Hypocotyl:** Part of the axis of a plant embryo or seedling between the point of insertion of the cotyledon and the top of the radicle. In some etiolated seedlings, the hypocotyl is greatly extended.

**Hypophysis:** The uppermost cell of the suspensor, which differentiates to form part of the root cap. The hypophysis will give rise to the radicle and the root cap; the cells of the suspensor will degenerate as the embryo matures.

**Keel petal:** The two bottom petals, below the wings, in flowers of the subfamily Faboideae of the plant family Fabaceae; sometimes joined to form a structure whose shape resembles the keel of a boat.

**Locule:** Any of the chambers of an ovary or anther.

**Megaspores:** In angiosperms, one of four haploid cells formed from a megasporocyte during meiosis. Three of the four megaspores degenerate. The remaining megaspore divides to produce the female gametophyte, which produces egg cells.

**Megasporocyte:** A diploid megaspore mother cell in an ovule that forms haploid megaspores by meiotic division.

**Micropyle:** A pore in the egg membrane of an insect oocyte, which allows sperm to enter and fertilize the ovule.

**Microsporangia:** Male spore development begins with the microsporangia and the embedded microspore mother cell. The diploid cell is found in a pollen sac of the anther, undergoes meiosis and produces 4 microspores (haploid). Then, each microspore undergoes mitosis to produce micro gametophytes (pollen grains, each one with two cells)

**Monecious:** Having the stamens and the pistils in separate flowers on the same plant.

**Nectary:** A gland that secretes nectar. Nectaries are usually located at the bases of insect-pollinated flowers, where they serve as an insect attractant.

**Nucellus:** The mass of tissue in the ovule of a plant that contains the embryo sac. Following fertilization, it may be absorbed by the developing embryo or persist to form a perisperm.

**Perianth:** Part of the flower composed by the calyx and the corolla.

**Periclinal:** Parallel to the surface of an organ or part. In periclinal cell division, the plane of division is parallel to the surface of the plant body.

**Pistil:** The female reproductive organ of plants.

**Plasmodesmata (singular: plasmodesma):** Microscopic channels which traverse the cell walls of plant cells and some algal cells, enabling transport and communication between them. Plasmodesmata evolved independently in several lineages and species that have these structures.

**Plumule:** The young shoot of a plant embryo above the cotyledons, consisting of the epicotyl and often of immature leaves.

**Primordium:** Derived from Latin primordium: the first, the beginning.

**Prophylls:** A plant structure resembling a leaf (as a bracteole) or consisting of a modified or rudimentary leaf (as a foliar primordium).

**Protoderm:** A plant tissue formed by the apical meristem of shoots and roots that subsequently gives rise to the epidermis.

**Puberulent:** Of a leaf, stem, etc. slightly downy with very short hairs; minutely pubescent

**Ribosome:** A complex molecular machine found within all living cells that serves as the site of biological protein synthesis (translation). Ribosomes link amino acids together in the order specified by messenger RNA (mRNA) molecules.

**Sporangia:** An enclosure in which spores are formed. It can be composed of a single cell or can be multicellular. All plants, fungi, and many other lineages form sporangia at some point in their life cycle. Sporangia can produce spores by mitosis, but in nearly all land plants and many fungi, sporangia are the site of meiosis and produce genetically distinct haploid spores.

**Sporogenous:** An elongated, spirally thickened, water-attracting cell in the capsule of a liverwort, derived from sporogenous tissue and assisting in spore dispersal.

**Sporopollenin:** A major component of the tough outer (exine) walls of plant spores and pollen grains

**Stamen:** The male reproductive organ of plants.

**Staminal sheath:** A protection coating of the stamens.

**Staminal tube:** Filaments of the stamens united so as to form a tube.

**Style:** An elongated part of a carpel, or group of fused carpels, between the ovary and the stigma.

**Suspensor:** The chain of cells that anchors a plant embryo in the surrounding gametophyte tissue. In flowering plants, the suspensor attaches the embryo to the embryo sac and extends to push the embryo into the endosperm.

**Synergid:** One of two small cells lying near the egg in the mature embryo sac of a flowering plant.

**Tonoplast:** The cytoplasmic membrane surrounding the vacuole, separating the vacuolar contents from the cytoplasm in a cell.

**Tunica:** The outer layer or layers of cells of the meristem at a shoot tip, which produces the epidermis and cells beneath it.

**Unicarpellate:** Having a single carpel (=pistil) which is the female part of the flower. Consists of the stigma, style and ovary.

**Zygote**: The result of the fusion of the sperm with the egg (haploid reproductive cells, or gametes). The zygote is a diploid cell that will start the cellular division to develop into a seed.

Embrapa
Soja

Apoio

A.B.E.L.H.A
Associação Brasileira de Estudos das Abelhas

The Brazilian Bee Studies Association – A.B.E.L.H.A. is a nonprofit civil association with no political or ideological connotation, established in 2014. Its mission is collecting, producing and disseminating science-based information, and the collaboration of a network of partners, aimed at the conservation of bees and other pollinators in Brazil, promoting their role in biodiversity and the harmonious and sustainable coexistence with different agricultural crops.

In addition to consolidating a knowledge platform on bees and other pollinators and becoming a source of consultation and awareness agent for the society, the association also aims to work in partnership with all stakeholders on the topic, such as producers, government, regulatory and supervisory bodies and researchers to suggest and encourage practices for use and conservation of pollinators.

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