

VISIT PATH PATTERN OF THE HONEYBEE (*Apis mellifera* L.) ON THE SUNFLOWER CAPITULUM -CORRESPONDENCE WITH THE LOCATION OF SEEDLESS AND INCOMPLETELY DEVELOPED FRUITS

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SUMMARY

The occurrence of fruits with absent or poorly developed embryo, also defined as seedless or incompletely developed fruits (IDF), significantly reduces the sunflower yield. Failures in pollination, fertilization and physiological or morphological defects in the ovary and embryo, either post-pollination or post-fertilization genotype- and/or ambient-induced, are most common reasons for the development of this type of fruits.

A detailed study of the visit path pattern of diurnal pollinators in the crop, mainly honeybees (*Apis mellifera* L.), showed that there was a significant negative correlation ($r^2 = -0.61$; $p < 0.05$) between the areas covered by foraging paths (ACP), with the total IDF counted in the capitulum.

Almost a complete limitation of insect visitation (0-30% ACP) resulted in poor seed set and IDF percentage ranging from 10 to 17%. Partial limitation of the insect visitation (60 to 90% ACP) diminished the number of IDF to 5 to 9%. It is concluded that as much as 30% or more of the capitulum's area must be covered by bee visits to minimize the development of IDF.

Key words: *Apis mellifera*, foraging, *Helianthus annuus*, pollen, seed set, sunflower

INTRODUCTION

The sunflower [*Helianthus annuus* L., var. *macrocarpus* (DC.) (Ckl.)] is an important crop for the production of oil seed in temperate regions of Argentina. The total number of fruits (cypsela) per plant that fully develop seed (kernel) is one of

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its most important yield components for both seed and oil production (Connor and Hall, 1997).

At maturity, the head (capitulum) of self-sterile and self-fertile sunflower cultivars usually show a set of fruits with a different degree of pericarp and embryo development. In most of them, the embryo reaches its full size filling the internal cavity of the ovary. These fruits can be defined as fully developed (FDF) (Lindström *et al.*, 2006; 2007). Nevertheless many mature fruits often contain ovules that did not fully develop into seeds. In those fruits, seed growth processes stop at different moments, leaving the fruit with an incompletely developed pericarp and/or seed (seedless or incompletely developed fruits or IDF; Lindström *et al.*, 2004). So, even though IDF generally tend to be considered as “seedless” or “empty”, in some of them the embryo can reach a certain level of development (Alkio *et al.*, 2002; Alkio and Grimm, 2003; Lindström *et al.*, 2004). IDF can be randomly distributed all over the capitulum surface (Hernández *et al.*, 2002; Lindström *et al.*, 2004). Most of them are easily identified because as a consequence of the arrested embryo and pericarp development, the hull is usually compressed by neighboring fruits showing an abnormal external collapsed appearance (Figure 1). Others are most difficult to identify because the hull partially develops without any seed inside.

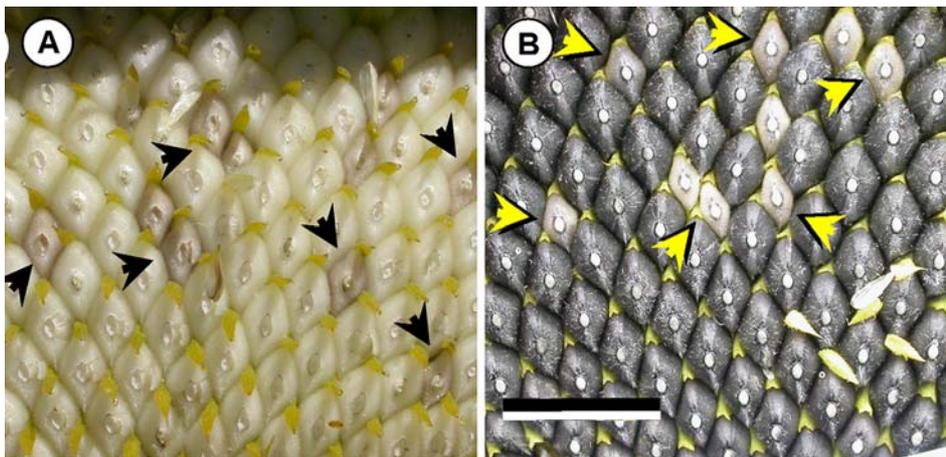


Figure 1: Location and external appearance of IDF in the peripheral region of the capitulum. (A) Four days after the first anthesis; (B) At physiological maturity (Schneiter and Miller, 1981), 48 days after the first anthesis. Arrows indicate the location of the pre-defined IDF observed in A; they are less developed than those surrounding them, with a darker coloration of the pericarp. In B, note the contrast of the pericarp color of the IDF (arrows) and the decrease in their development in comparison with those surrounding them. The scale for both figures is 1 cm.

The fertilization histories of those fruits cannot be determined externally. Thus, visual inspection of developing and mature fruit contents, sometimes aided by a dissecting microscope, is the most common mean for distinguishing FDF from IDF.

The real causes of the origin of IDF are unknown but several proximate mechanisms that have been put forth to explain the low seed to ovule ratio in many species of the *Angiospermae* can be applied to sunflower. Their origin could be attributed to environmental, physiological and/or anatomical causes as well as to lack of pollination or to post pollination failures during the early development of embryos (Gillaspy *et al.*, 1993; Connor and Hall, 1997; Cantagallo *et al.*, 2004; Hernández and Bellés, 2005). Most of the times, poor seed set or unfilled fruits occur mainly due to inadequate pollination (Birch and van der Sandt, 1985), competition for resources between developing ovaries or vascular deficiencies at the ovary-receptacle interphase (Durrieu *et al.*, 1985; Hernández and Orioli, 1991; Hernández and Palmer, 1992; Alkio and Grimm, 2003).

Because the development of IDF is a cause of significant reductions in seed and oil production of sunflower, determining the reasons and the degree of this reduced fecundity, are important goals in studies of sunflower breeding.

Sunflower pollen is heavy and sticky. It cannot be carried by wind so pollen transport by insects is necessary for improved seed set (Birch and van der Sandt, 1985; Skinner, 1987; Medan *et al.*, 2003; DeGrandi-Hoffman and Chambers, 2006). Several studies have shown that the foraging activity of the honeybee (*Apis mellifera* L.) can increase seed set and yield (Parker, 1981a; Fell, 1986; Medan *et al.*, 2003; DeGrandi-Hoffman and Chambers, 2006).

The foraging pattern of the honeybee on the self-fertile sunflower capitulum has not been deeply studied and its relationship with seed set has not been totally established. Giurfa and Núñez (1993) observed in capitula of *Carduus acanthoides* that the foraging pattern is irregular and that the insect visits each flower once. Previously Parker (1981b) demonstrated the random paths followed by honeybees over a flowering sunflower head but he did not quantify them and the pattern of paths created by all daily visitors over a head was not further described.

The aim of the present work was to determine an association of the position of IDF within the capitulum with the paths of visits of one of its main daily pollinators. It is proposed that a fraction of the IDF is due to the absence of pollinators visits. Therefore, we studied the relationship between the paths of daily visits of honeybees on the capitulum and the pattern of incompletely developed fruits within capitula of cultivated sunflower.

MATERIALS AND METHODS

Plant material and cultural techniques

The experiment was carried out at the Agronomy Department-UNSur, Bahía Blanca, Argentina (lat. S 38° 45'; long. W 62° 11'). The soil at the field location was a Typic Ustipsamment (USDA, 1999). A low self-fertile experimental sunflower genotype, provided by Dow Agrosiences of Argentina, was sown starting the first week

of October, at three dates separated by 5-day intervals, in order to obtain plants at the beginning of flowering (first anthesis, according to Schneiter and Miller, 1981) during several consecutive days and to study them individually. Preliminary trials demonstrated that this sunflower cultivar produced more than 40% seedless fruits per capitulum when plants were caged to prevent insect interaction during flowering (L.F. Hernández, unpublished). It was also chosen because the external color of the fruit's pericarp, white at early stages of fruit development and black-striate at maturity, facilitates the identification of IDF (Figure 1). After seedling emergence, plant density was adjusted to 5.6 plants/m². The crop was managed according to conventional agronomical practices (Pereyra and Farizo, 1981). Weeds and pests were adequately controlled. Water was supplied by drip irrigation. Forty bee hives were located near the experimental plot was (500 m). This was an adequate population of honeybees, which ensured that visitation at flowering was highly intense. At mid-anthesis, about 4.0 honeybee visits were counted on average at 40 open capitula in 15 min.

Daily records of temperature and solar radiation were obtained from a meteorological station located 800 m from the experimental field (Figure 2).

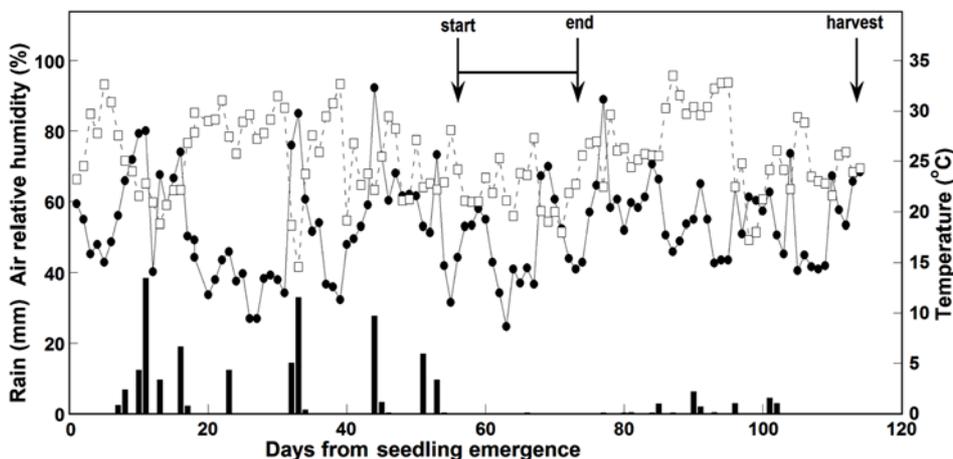


Figure 2 Mean daily temperature (O; °C) relative humidity (●; %) and rain (mm, black bars) for the experimental location. The segment between start and end arrows indicate the time when all the observation on capitula were made.

Plant selection and pollinator visits observations

Two plants displaced 4 to 5 days in time for each seeding date were randomly selected in the stand (n=6). Before the first anthesis, the selected plants were staked, orienting the side of the capitulum with flowers eastwards. At first anthesis, when the ray floret corollas allowed the visualization of the first circle of the disc flowers, the capitulum diameter was measured and four reference points were marked at the periphery using color pearl head pins. The capitulum of one plant at

a time was then continuously recorded using a digital camera. The filming process took 2 to 3 days, from first anthesis and until the first 4-6 rows of peripheral flowers finished opening. Daily and continuous observations of plants, from 8.00 a.m. to 5 p.m., allowed interrupting filming when visitors were absent (for instance, at midday). Honeybee foraging on a head was continuously watched until bees left that head. At dusk, the inflorescence was covered with mesh bags to avoid the action of night pollinators. This also ensured that nectar could accumulate and possible scent-marks evaporate (Giurfa and Nuñez, 1992; Gawleta *et al.*, 2005). After the study was completed, the observed plant was protected during the night until harvest and the procedure was repeated with another plant that has reached the stage of first anthesis. No study was conducted on the center of the capitulum because the poor seed set in this region or the occurrence of IDF can have multiple causes and the results are more likely to be masked by unknown reasons.

Data processing

Each digital file saved in MOV format was processed digitally for each observed plant (n=6) using VideoPoint vers. 2.5 (Lenox Softworks, Lenox, MA) software to define, in Cartesian coordinates, the foraging routes of all the diurnal pollinators. Only honeybees and sporadically carpenter bees (*Xylocopa* sp.) were observed, the latter also being filmed. The bee's thorax was the reference point of movement to digitalize the route followed by the insect during its visit (arrival-departure) to the capitulum surface. The program allowed to digitalize the routes followed simultaneously by more than one bee. The landmarks on the capitulum allowed the correct location and correspondence of the recorded paths on the capitulum at anthesis and at maturity (Figure 2).

Each image of the capitulum was fractionated in 60 sectors and the pixel density corresponding to the foraging routes in each sector was quantified (Figure 3).

The routes followed by the honeybee were graphically represented for each capitulum on an axis of Cartesian coordinates and the pixel density was analyzed using a plug-in specially designed to operate with the software Object-Image 2.21 (<http://simon.bio.uva.nl/object-image.html>; Vischer *et al.*, 1994) on a Macintosh platform. After calculating the area of each sector of the capitulum, the average pixel density was estimated for each sector and a covering map or "density map" (pixels % per sector) was drawn for each capitulum of each of the six observed plants (Figure 3).

The visitation density was analyzed considering the route followed by the bee when walking on the capitulum. Flying bees were not considered. As the tracing of the followed routes was not corrected by the width of the bee's thorax, which can reach an average value of 3.0 mm (Winston, 1991), it was not considered whether the bee, during its movement, could carry away pollen from adjacent anthers or shed pollen attached to its body. Nevertheless, the width of the route followed by the bee can be estimated as approximately the diameter of an open corolla.

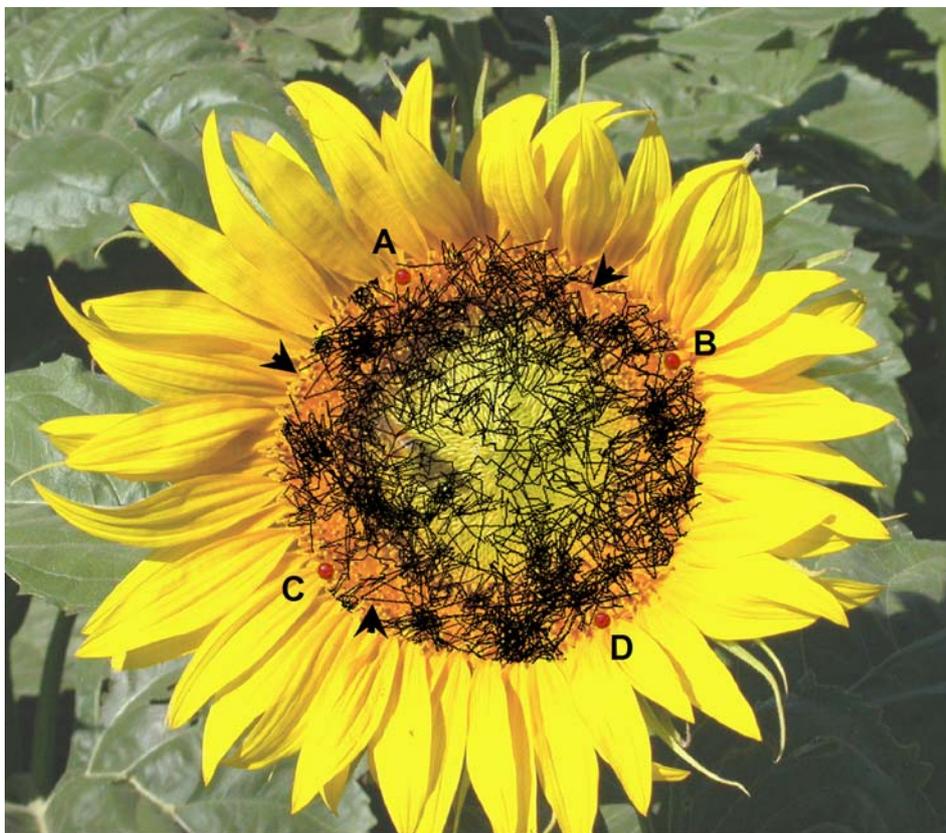


Figure 3: Pattern of routes of daily visits of honeybees (*Apis mellifera* L.) and, in less proportion, carpenter bees (*Xylocopa* sp.) during two consecutive days after the first anthesis, on the capitulum of one of the 6 plants studied in this work. The tracing of the image of 2 pixel width was accomplished after processing the digital images with the software VideoPoint. Arrows show some of the unvisited regions. The red circles noted with letters A, B, C and D correspond to the reference points defined on the capitulum and marked with color pearl head pins. The routes followed towards the central region of the capitulum were not considered in this study because these flowers were not open at the time of the analysis.

At harvest, the location of the IDF on each mature capitulum was defined for each plant properly identified, using the reference landmarks (Figure 1). The IDF proportion was calculated by dividing each capitulum into sectors. The IDF proportion was then compared with the intensity of visitations per each sector or area covered by paths (ACP; Figures 4 and 5).

When the obtained data were subjected to an analysis of linear and non-linear correlation, the differences between the treatments and sampling date means were compared by the LSD test.

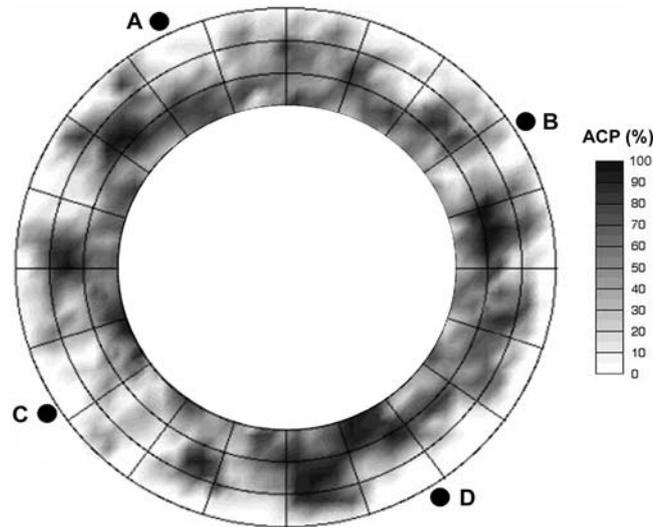


Figure 4: Areas covered by pollinator paths (ACP%) per sector ($n=60$) in which each capitulum of the 6 studied plants was divided to conduct the analysis. ACP (% of total area of each sector) values, obtained according to the methodology described in the text, correspond, for this figure, to the capitulum of Figure 3. The black points noted with letters A, B, C and D correspond to the reference points defined in each capitulum (see Figure 3).

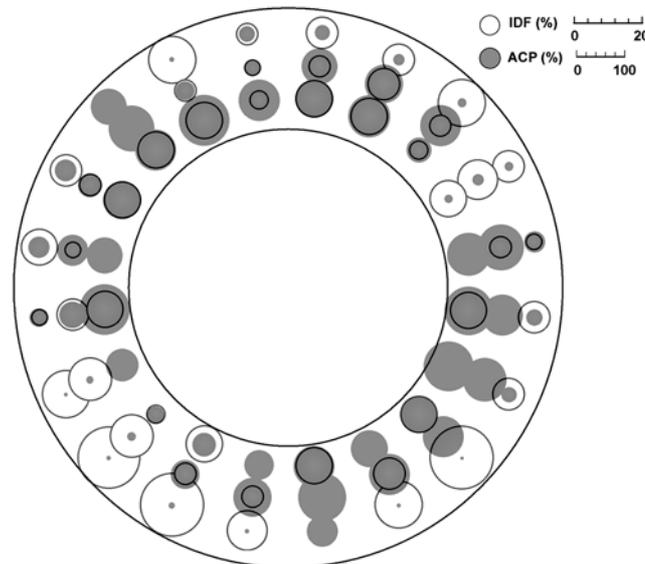


Figure 5: Emerging relationship between the area covered by visit paths (●; ACP%) and the IDF% (○) produced in each sector for the capitulum of Figure 3, numerically elaborated from data generated from Figure 4. The scales indicate the length of the diameter of each circle with the percentage magnitude of each variable.

RESULTS

The main floral visitors in our observations belonged to the order *Hymenoptera* (100% of total visits). Only two types of *Hymenoptera* visited the flowers during the day: *Apis mellifera* L., which made most of the visits (98%), and *Xylocopa* sp. (2%). The complete pattern generated by the routes followed on the anthesis ring (the distance defined between the row of open flowers at first anthesis and that produced the day after first anthesis) during two consecutive visiting days for one of the six studied plants is shown in Figure 3. The density of visits per capitulum sector calculated according to the described methodology is presented in Figure 4. The relationship between the percentage of areas covered by paths (ACP%) and the percentage of IDF per sector in the same plant is presented in Figure 5.

It can be seen that the path density is not homogeneous, showing zones with high and very low densities and some unvisited zones (Figures 3 and 4). In the same plant, the absence of visits or a low density of visits in a sector caused an increase in the percentage of IDF (Figure 5).

The results from the analysis of the 6 plants in which the capitula were separated in 60 sectors according to Figure 3, giving the relationship between the area covered by foraging paths and the percentage of IDF in each sector, are shown in Figure 6.

An inverse relationship was observed in the area covered by foraging paths (ACP%) and IDF% ranging from 0% to 30% of the area covered. Above this value, between 30% and 100% ACP, there was no correlation between the two parameters (Figure 6).

However, three segments of the relationship between the area covered by paths ACP% and the IDF% could be determined. Thus, between 0 and 30% ACP, an inverse relationship was observed ($r^2 = 0.61$; $n = 91$; $p < 0.05$) which could be fitted in a quadratic equation (Figure 6). Above this value, between 30 and 100% ACP, IDF magnitudes were presented in two broadly fluctuating levels. One of them fluctuated from 30 to 100% ACP and another from 56 to 100% ACP (Figure 6).

DISCUSSION

The sunflower is considered a cross-pollinated crop, originally self-sterile (Charlet *et al.*, 1997). Most cultivated varieties are self-fertile which means that florets can set seed when they are pollinated with pollen from the same flower head. Nevertheless some cultivated varieties are partially self-sterile and require pollen from another plant. Within-head selfing usually results in lower seed set, smaller seeds, lower oil content, and a lower germination rate compared to open crossing (McGregor, 1976). Thus, cross-pollination is advantageous except in highly self-fertile varieties.

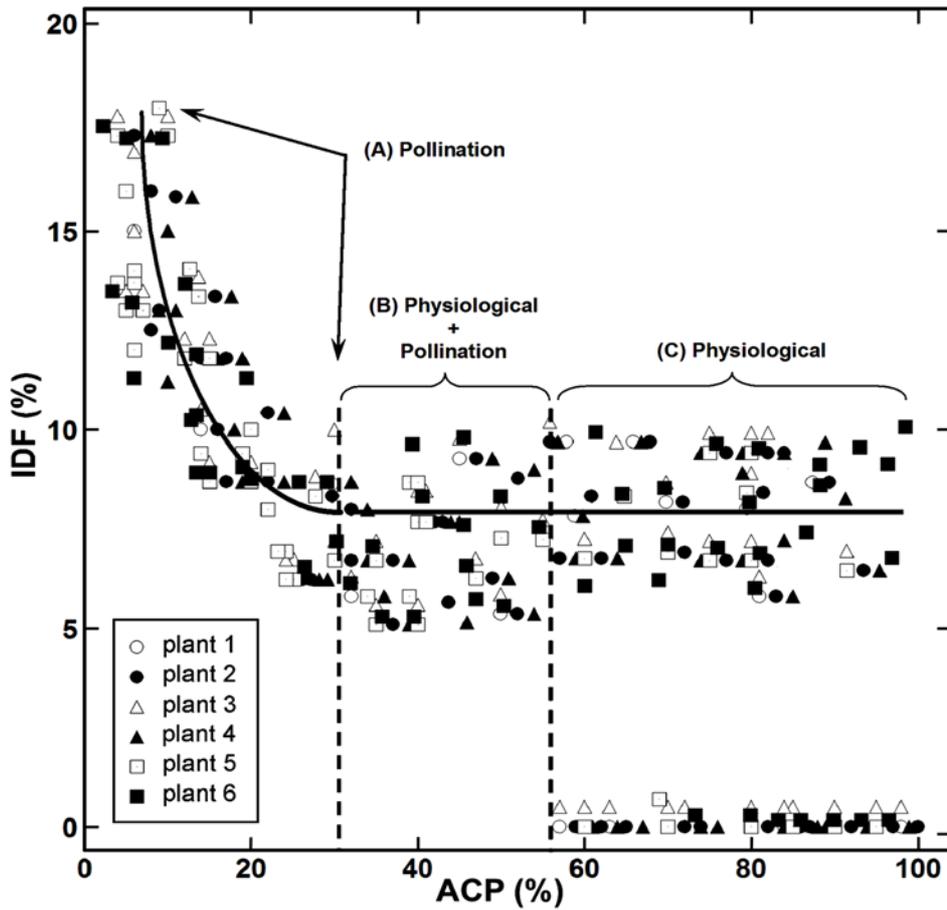


Figure 6: Relationship between the area covered by paths (ACP%) and the total percentage of incompletely developed fruits (IDF%) observed in each sector (n=60) in which each capitulum of the 6 sunflower plants was divided for analysis. The sum of observations of IDF% is separated in ranges from 0% to 30%, 30% to 60% and 60% to 100% of ACP to define three types of causes acting in the development of IDF: (A) mainly pollination failures, associated with the lack of pollinator visits (0-30% ACP; $IDF\% = 31.48 \times ACP^{0.43}$; $r^2 = -0.61$; $n=91$; $p < 0.05$), (B) physiological plus pollination failures and (C) mainly physiological causes.

Fruit set failures can result from a number of events preventing the development of the flower into a mature fruit (Stephenson, 1981; Stephenson, 1992). Lack of sufficient pollen loads on the stigma to fertilize all the flowers (Wilson and Schemeske, 1980; Snow, 1982; Gross and Werner, 1983; Schemeske and Pautler, 1984; Weins, 1984; Zimmerman and Pyke, 1988) and physiological and/or anatomical alterations and source limitations to provide for seed development (Wilson and Schemeske, 1980; Stephenson, 1981; Wyatt, 1981; Lee and Bazzaz, 1982; Weins,

1984; Zimmerman and Pyke, 1988; Burd, 1994; Connor and Hall, 1997) have been most commonly blamed as causes for a low seed set.

In sunflower, even though the competition for resources among the ovaries within the developing head can cause embryo abortion or failures in seed set (Stephenson, 1981; Bawa and Webb, 1984; Lee, 1988; Thomson, 1989), it is viable pollen that has to be adequately transported to the stigma to accomplish pollen tube growth in the pistil and fertilize the ovules (Connor and Hall, 1997).

According to the “non-uniform pollination hypothesis” (Thomson, 1985, 1989; Berry and Calvo, 1991; Brunet and Charlesworth, 1995) the observed patterns of IDF in the mature capitula may be attributable to variation in pollen receipt over the inflorescence flowering period. Specifically, the relatively low seed set in central areas of the sunflower capitulum has usually been attributable to insufficient pollen quantity or pollinators visits. Why are some florets left unvisited in the peripheral regions of the capitulum cannot be answered by the information obtained in this work. Recently, Giurfa (2004) demonstrated that the honeybee can discriminate color and regarding this, it has been noticed (L.F. Hernández, unpublished) that some floret corollas in different capitulum locations of recently open florets show comparatively different color intensity compared with their neighbors. In addition to visual cues, bees also perceive and use olfactory cues in floral choice (von Frisch, 1919; Laska *et al.*, 1999; Giurfa and Nuñez, 1992). It is also known that honeybees avoid probing flowers that have been recently depleted by conspecifics, presumably repelled by odors (foraging scent marks) deposited by the previous visitor (Giurfa and Nuñez, 1992; Gawleta *et al.*, 2005). Once its nectar load has been depleted the bee does not return to the same flower. Probably if this is the case, the reason to leave some disc florets unvisited (Figures 3-4) could be related with its proximity to already visited neighbor florets.

In this work, climatic conditions during capitulum maturation were optimal. No rain occurred during the observation period, which could lead to pollination failures by pollen lixiviation and air temperature was always near or below 30°C (Figure 2), a threshold level known to affect sunflower pollination (DeGrandi-Hoffman and Chambers, 2006).

Sunflower genotypes vary in their attractiveness to honeybees. Short corolla length, unpigmented stigmas, many stomata on the nectary, and high sucrose content of the nectar are preferred by honeybees. If a flower is never visited, it could be an indication that the floret *per se* is responsible for the lack of attractiveness by some intrinsic difference that make it special and “unvisitable” compared with the surrounding ones. Sammataro *et al.* (1984) found intragenotypic differences in the quality, quantity and anatomy of nectaries (Sammataro *et al.*, 1985). Perhaps inter-plant differences could also exist.

The availability of resources can vary in both space and time for an individual flower, due to local competition for resources (Stephenson, 1981; Wyatt, 1981). Hence, within a single plant, resources may be limited for some flowers but not for

others. Nevertheless we do not think this is the case for the external flowers of the sunflower capitulum. It has been observed that at early anthesis stages, recently opened flowers are not deprived from assimilate supply (Hernández and Orioli, 1991; Alkio *et al.*, 2002; Alkio and Grimm, 2003).

Finally, according to the “architectural effects hypothesis”, the pattern of seed production can also be provoked in some plants by intrinsic factors limiting the ripening of ovules located in some positions (Diggle, 1995). The proximate causes of these architectural effects are still unknown (Diggle, 1997), although cumulating evidence is showing that it can have important effect on the observed pattern of seed production (Medrano *et al.*, 2000 and references therein).

Regarding the completely hollow fruits that develop pericarp but not seed, it should be remembered that parthenocarpy, the formation of seedless fruit, occurs naturally in many species (George *et al.*, 1984) and can be artificially induced by hormone application, mainly auxins and gibberellins (Schwabe and Mills, 1981; García-Martínez and Hedden, 1997). In natural parthenocarpy, it has been suggested that the expression of parthenocarpic genes can affect the pattern of hormone production, transport and/or metabolism leading to hormone levels in the ovary capable of promoting growth even in the absence of pollination and fertilization (Nitsch, 1970; Gillaspay *et al.*, 1993).

The analysis of Figure 6 revealed, at first glance and considering the conditions under which the experiment and the observations were conducted, three cause and effect intervals. Assuming that no contribution from other pollinators occurred in the period from anthesis to fertilization, three factors could be defined which play a role in the generation of IDF. From 0% to 30% of ACP, the negative correlation found between ACP% and IDF% ($r^2 = -0.61$) suggests that within this range, the lack of pollen had a high incidence causing an absence of pollinator visits to that region (Figure 6); from 30% ACP and above, this value, *i.e.*, the stable level of IDF fluctuating from 5 to 10%, suggests that we have to consider two variables.

On the one hand, the absence of pollen could be maintained at 60% in those regions due to a low occurrence of bee visits, but on the other hand, factors related to the floral biology (physiological factors) of the studied genotype might be in action (Figure 6). Above 60% of ACP, the absence of IDF (0%) in several sectors (Figure 6) and again the occurrence of sectors with a fluctuating level of IDF% ranging from 5 to 10% of the total value would explain that the IDF generated in that region was produced due to physiological causes, which were neither detected nor studied in this present work but are probably characteristic for the low self-compatibility of the genotype used. Given the present information, it would be expected that in sunflower genotypes with high self-compatibility, the IDF fraction, although fluctuating, could descend to levels under 5% per sector.

CONCLUSIONS

In this work, fruit-set in sunflower was found to be positively correlated with the proportion of flowers that hosted honeybees when its value of bee visits was lower than 30%.

The correlation between the percentage of IDF per sector and the ACP (%) over 30% was weak (Figure 6), probably because it was masked with other variables, which would act to generate IDF.

Another weakness is the fact that the ACPs were sampled only after the bees settled during the day, without quantifying the behavior of other pollinators during the night.

Nevertheless, the positive relationship between the density of foraging routes and the development of IDF in several areas of the capitulum demonstrate the important role of day-sheltering bees as sunflower pollinators. Unvisited areas are positively correlated with the presence of seedless or incompletely developed fruits at maturity. This also suggests that some flowers from those areas are inclined to show absence or delay in pollination with respect to the adjacent flowers.

Due to night covering, we could assume that nocturnal pollinators would substitute the lack of visited sites by areas that bees did not visit during the day. Nevertheless if a deficiency in the nectary number or floret functionality occurred, these sites would not be visited at night either.

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**PATRÓN DEL CAMINO VISITAS DE ABEJAS MELÍFERAS
(*Apis mellifera* L.) EN EL CAPÍTULO DE GIRASOL.
CORRESPONDENCIA CON LA UBICACIÓN DE FRUTOS
VACÍOS O DE DESARROLLO INCOMPLETO**

RESUMEN

La aparición de frutos con el embrión ausente o parcialmente desarrollado, definidos también como frutos vacíos o de desarrollo incompleto (FDI), impacta negativamente en el rendimiento del girasol. Entre las razones frecuentemente responsables de la generación de este tipo de frutos, se encuentran las fallas en la polinización y la fertilización y defectos morfológicos o fisiológicos en el ovario y el embrión tanto genotípicos o producidos por condiciones del ambientales, presentados luego de la polinización o la fecundación.

Un estudio detallado del patrón del camino visitas de polinizadores diurnos en el cultivo, principalmente abejas melíferas (*Apis mellifera* L.), mostró que existe una correlación negativa ($r^2 = -0.61$; $p < 0.05$) entre el área cubierta por los caminos de visitas (ACV) con el total de FDI visibles en el capítulo.

En sectores del capítulo con una baja presencia de visitas (0-30% ACV) se observó un total de FDI de entre 10 y 17%. En sectores del capítulo con ausencia parcial de visitas, (60-100% ACV) el número total de IDF osciló entre 5 y 9%. Se concluye que al menos un 30 % de la superficie del capítulo debe ser cubierta por visitas de abejas para minimizar la generación de FDI.

**PATRON DES CHEMINS DE VISITES D'ABEILLES
MELLIFÈRES (*Apis mellifera* L.) SUR LES CAPITULES DU
TOURNESOL. RAPPORT AVEC LE PLACEMENT DES
FRUITS VIDES OU DE DÉVELOPPEMENT INCOMPLET.**

RÉSUMÉ

La présence des fruits sans embryon ou avec des embryons partiellement développés, définis aussi comme fruits vides ou de développement incomplet (FDI), provoque un impact négatif sur le rendement du tournesol.

Les motifs les plus fréquents de la genèse de ces fruits sont l'échec de la pollinisation et la fertilisation, les défauts morphologiques ou physiologiques de l'ovaire et de l'embryon, du à causes génétiques ou à des conditions du milieu présentes après la pollinisation et la fertilisation.

Une étude détaillée sur le patron des chemins de visites des pollinisateurs à la culture du tournesol durant la journée, notamment des abeilles mellifères (*Apis mellifera* L.), a montré une corrélation négative ($r^2 = -0.61$; $p < 0.05$) entre l'aire sillonnée par les chemins de visites (ACV) et le total de FID visibles dans le capitule.

L'absence des visites des abeilles (0-30% ACV) a provoqué le surcroît des FID qui ont varié entre 10 et 17%. Les secteurs du capitule avec une quantité des visites entre 60-100%, ont présenté un nombre total de FDI entre 5-9%. On peut conclure que au moins le 30% de la surface du capitule doit être visité pour minimisée l'apparition du FDI.