PROTOCOL TO DETECT AND ASSESS POLLINATION DEFICITS IN CROPS

FAO/IFAD PROJECT:

"DEVELOPMENT OF TOOLS AND METHODS FOR CONSERVATION AND MANAGEMENT OF POLLINATION SERVICES FOR SUSTAINABLE AGRICULTURE"

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BERNARD E. VAISSIÈRE (INRA, AVIGNON, FRANCE)
BRENO M. FREITAS (U. DE CEARÁ, FORTALEZA, BRAZIL)
BARBARA GEMMILL-HERREN (FAO, ROME, ITALY)

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Introduction

The following describes a protocol to be applied to focal crops at the farm scale level to (i) detect and assess pollination deficits in field situations in a standard and statistically testable way, and (ii) draw management conclusions from the proposed experiment for possible action to eliminate or at least reduce these deficits. It can also be used simply to assess pollinator density and diversity on a focal crop for comparison purposes among different sites.

Pollination is the transfer of pollen from the producing anthers to the receptive stigma and it is an essential preliminary step for the sexual reproduction of flowering plants. Pollination level can be precisely measured as the number of compatible and viable pollen grains that reach a stigma during the effective pollination period, and it is therefore directly related to yield for all crops in which the output is a product of sexual reproduction. Indeed, pollination management should be regarded as a production factor in its own right for all these crops as it can affect the agronomic and economic yields and their many components such as fruit set and seed set, fruit quality (e.g. size, aspect, sugar content, flavor and nutritional content), seed quality (e.g. germination rate, oil content), other characteristics such as earliness and uniformity of output (e.g. rape), market value and profitability, and finally the environmental and societal impacts of a crop (McGregor 1976; Free 1993).

Section 1. Definitions and conceptual framework

The following conceptual framework underlies the protocol; the definitions of terms often lead to the need for further definitions, in a logical sequence. The terms defined are underlined.

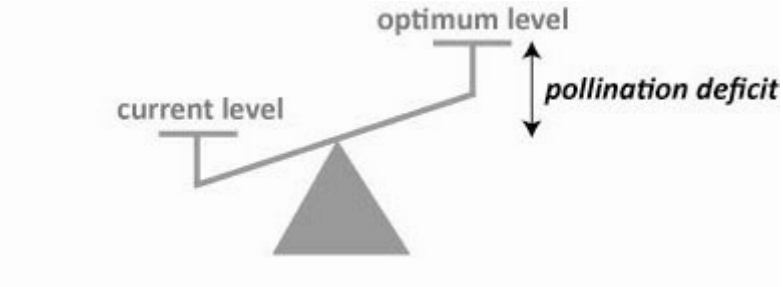

Figure 1. Pollination deficit in relation to optimum pollination level.

Optimum pollination: Pollination that leads to maximum sexual reproductive output given the current available resources. In the case of crops, this refers to the agricultural output that depends upon pollination, and it takes into account the production objectives in relation to the market. To define pollination deficits, we need to define (and understand) how we reach optimum pollination levels (Figure 1.)

Pollination deficit: Quantitative or qualitative inadequate pollen receipt which decreases the sexual reproductive output of plants (from Wilcock & Neiland (2002) who defined the concept of pollination failure).

Crop pollination deficit: Quantitative or qualitative inadequate pollen receipt that limits agricultural output.

Further defining this concept:

The inadequate pollen receipt may be quantitative, qualitative due to a deficient quality of the pollen grains deposited, or inadequate with respect to timing, that is occurring outside the period of effective pollination based on stigmatic receptivity and ovule senescence.

A quantitative pollination deficit is an insufficient number of conspecific pollen grains deposited onto the stigma during the effective pollination period (see below). It is often the result of an insufficient number of visits by pollinators. Coffee plants in Central America were evidently suffering from a quantitative pollination deficit before the introduction of Africanized honey bees since their yield increased significantly following the arrival of these bees (Roubik 2002).

A quantitative pollination deficit could be an outcome of conditions such as:

- Ineffective/insufficient transport and deposition of pollen onto the stigmas;
- Insufficient pollen production (as the first flowers of strawberry, *Fragaria x ananassa* Duch., grown out of season under greenhouse);
- Lack of male flowers relative to female ones in dioecious crop species, such in orchards of kiwifruit *Actinidia deliciosa* A. Chev. C. F. Liang),
- Lack of staminate flowers relative to pistillate ones in monoecious crops, as can occur at the onset of flowering in very early plantings of zucchini) *Cucurbita pepo* L.;
- Lack of male-fertile flowers relative to male-sterile ones in hybrid seed production.

A qualitative pollination deficit is when sufficient conspecific pollen is deposited onto the stigma, but this pollen is not effective for fertilization. This reduced pollen quality may result from a low intrinsic viability and/or the genetic origin of the pollen in self-incompatible species for which the pollen must come from a plant genetically different from that of the receptive stigma for fertilization to occur.
A qualitative pollination deficit could be an outcome of conditions such as:

- Poor pollen viability, as in some fruit varieties and crops such as strawberry when grown under low light conditions early on under greenhouses;
- Lack of pollenizer flowers in self-incompatible crops.

The effective pollination period is the period during which the pollen deposited onto the stigma can result in fertilization. Pollen that is deposited either before or after this period will not be effective for fertilization and therefore for production (Sanzol & Herrero 2001).

The limitation of agricultural output may be quantitative (that is with respect to yields), or qualitative (with respect to fruit or seed characteristics), or inadequate with respect to timing (e.g., because of delayed or extended fruiting). Limitation of agricultural output has productivity aspects, but also sustainability aspects because a useful crop component of a sustainable farming system may be dropped because of poor pollination.

This protocol has been developed to address pollination in a way that is realistic for farmers, and so the yield is the primary focus. The fact that crop plants can compensate for pollen limitation with longer flowering periods and more flowers means that the whole plant, rather than individual flowers or even a sample of flowers, needs to be considered. Along the same line, fruit set and/or seed set can be resource-limited, and thereby the results obtained by increasing pollination levels on a subset of flowers on a plant may result in a larger fruit from

those flowers, but not greater overall production on a plant basis (Knight et al. 2005). Agricultural output should therefore always be based on a whole plant or larger scale (plot, field), and pollination treatments must be carried out on a similar scale, that is with the whole plant as smallest experimental unit.

Section 2. Protocol objective and structure

The protocol aims at applying methods following a standard experimental design to assess the degree to which pollination is a limiting factor in the production of a focal crop at the field scale. Comparing crop responses under pollination levels resulting from current practices with those from enhanced pollinator abundance or diversity will indicate the presence, and degree, of a pollination deficit.

The protocol is structured as a hypothesis that there is a relationship between the pollination level X, the independent variable, and a part or the whole of crop yield Y, the dependent variable, as reflected in the following equation and overview of parameters.

$$Y = F(X) + A$$

where :
Y is the total crop yield measured in agronomic or economic units ;
F(X) is the yield resulting from the pollination service measured in the same unit as Y;
and A is the yield resulting from autonomous self-pollination and wind pollination measured in the same unit as Y.

Pollination level is critical to the yield for all crops in which the output is a product of sexual reproduction. But, unless we know the precise relationship between the yield and the number and genetic diversity of pollen grains that reach the stigma during the effective pollination period, we cannot quantify directly the optimum level of pollination service needed to achieve maximum output. It then becomes necessary to use alternate variables as proxies to assess this level of pollination. Such proxies include **pollinator density (pollinator/floral unit) & pollinator diversity** assuming that the main pollinating species are known among the floral visitors.

Based upon the above, the protocole will now be described in 6 sections as follows :

General considerations for study field selection (see Section 3)

Treatments to modulate the pollination level and independent variables (see Section 4)
- Local pollinator supplementation;
- Landscape context / field location in relation to natural habitats

Layout of experimental sites (see Section 5)
- Establishing the experimental site
- Locating the experimental site within a study field

Pollinator dependent variables and data collection (see Section 6)
- Pollinator density
- Pollinator diversity
- Covariables

Production dependent variables and sampling units (see Section 7)

- Agronomic yield
- Economic yield

Statistical analyses (see Section 8)

Section 3. General considerations for study field selection

Whenever comparisons will be made between study fields, these should be located in environments that are as similar as possible (similar topography, soil, slope, exposure), and managed in a uniform way also (same seed source or same genetic material, same cropping system).

When it is not possible to find the full complement of fields indicated below that are located in similar environments (topography, soil, slope, exposure), and managed in a uniform way (same seed source or same genetic material ; same cropping system), it is possible to use a design in pairs in which the two fields within a pair should be as similar as possible while differences between pairs are allowed. Within a pair, there will always need to be one field that will serve as control while the other field will be treated so as to have potentially improved pollination. With such a design, the number of pairs to find will be equal to half of the total complements of fields. Even still, the two paired fields will need to be at least 2 km apart from each other- see below.

For long fields (> 450 m in length), comparisons can be made along a gradient between different areas within the field if it is possible to locate a “pollinator front” – either hives, or a natural area – on one side. It is the uniformity within a field that will be especially important in both the environment (uniform topography, soil, slope, exposure) and management (same seed source or same genetic material, same cropping system). However, in this case, there can be important differences in the environment and management between the different fields since each field will be considered as a block for the statistical analyses.

In all cases, individual fields should be separated from each other by a distance at least equal to 2 km and if possible greater than the maximum modal foraging distance of the managed pollinator species used (3 to 5 km for honey bees and bumble bees – Buchmann & Shipman 1991; Steffan-Dewenter & Tscharntke 2000; Goulson & Stout 2001). In the case of wild pollinators, the maximum foraging distance can range from 1.2 km for small bees (Beil et al. 2008) up to 6 km for large carpenter bees such as *Xylocopa flavorufa* (Pasquet et al. 2008).

When there is no ‘field’ as such, for example for cucurbit plants such as pumpkin, *Cucurbita moschata* (Duchesne ex Lam.), that are grown around houses in many rural areas all over the world, a study ‘field’ will be composed of a set of one or several patches, each patch including one or several plants of the focal crop. Still the selection of such a study ‘field’ will need to take into account all the requirements previously laid out, especially in terms of being set in a uniform environment and being similarly managed so that the pollinator treatment will be the main difference between the set of patches that will be compared. For example, one study ‘field’ may consist of patches of cucurbit plants around houses located far way from the closest beehives and/or patch of natural habitat, while the other study ‘field’ will consist of

cucurbit plants around houses with beehives nearby and/or close to a patch of natural habitat.

Section 4. Treatments to vary the level of pollination service, from current to potentially improved, and independent variable recording

Improved pollination can result from improved pollen transport, deposition and fertilization effectiveness. Hand pollination would be the obvious method to achieve full control of the amount, viability and origin of the pollen used for pollination. However, for most crops it is essentially impossible to undertake hand pollination at the whole plant scale. In order to achieve improved pollination, there are still many other possible approaches. A few of them are considered here in that they are simple, can be applied over a wide range of situations and are amenable to manipulation over a short time scale for experimental purposes. For each, we will examine below the pros and cons, and the implementation modalities. Those applying the protocol can select amongst these treatments to attain potentially improved pollination. These treatments are:

4.A Pollinator (bee) supplementation.

Most crops are pollinated by bees, especially honey bees (Klein et al. 2007; Rader et al. 2009). Eusocial bees, such as honey bees – whether western honey bees (*Apis mellifera* L.) or eastern honey bees (*Apis cerana* F.) – as well as bumble bees such as *Bombus terrestris*, and solitary gregarious species such as leafcutter bees (*Megachile rotundata*) and mason bees (*Osmia* spp.) have been domesticated and their nests can be moved around for crop pollination (Delaplane & Mayer 2000). It is therefore possible to supplement the local pollinator fauna by introducing colonies, nests or cocoons of these species. **Use of non native species should be strongly discouraged** as they could have severe negative impacts on the local pollinator fauna and, indeed, whole ecosystems (Hingston & McQuillan 1999, Goulson 2003, Kato & Kawakita 2004).

Pros and cons:

Applicable regardless of the location of the crop;
Applicable regardless of the crop production process (e.g. greenhouse, open field);
Builds on what is already known about the effective pollinators of the crop

Pollination depends upon pollinator species introduced;
Limitation to managed pollinators;
Unclear relationship between stocking rate of introduced bees and forager density on focal crop (it is usually a good idea to record pollinator density and diversity at least once just before pollinator introduction);
Effect of pollinator addition is usually not additive in relation to existing pollinator populations;
Possible negative effects of high pollinator density;
Use of non-native species could have detrimental impacts on native species

Implementation modalities and independent variable recording

description of implementation action	numbers required

Introduce managed pollinators in or nearby half of the study fields at onset of effective flowering (flowering that will produce crops). The stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) should be the same in all treated fields. Its value should be set based on the reproductive biology of the crop and the literature (e.g. usually 1 to 10 honey bee colonies per ha of focal crop ; McGregor, 1976; Delaplane & Mayer 2000) Record the stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) in each study field.	5 fields with and 5 fields without pollinators introduced.
In large fields with length > 450 m long, introduce pollinators along a single side perpendicular to its length to get a gradient of pollinator density (Vaissière et al. 1984). Record the stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) and the distance to the closest introduced pollinator unit at each experimental site (i.e. each location of measurement - see below) in each study field.	5 fields > 450 m long with pollinators introduced on a single side to get a gradient of pollinator density from near to far from side with introduced pollinators (usually one experimental site for recordings can be set at each 150 m distance of the pollinator front).

4.B Landscape context

Pollinator abundance and diversity vary with landscape context, in such a way that wild bee populations are generally greater close to natural habitat and in areas with a high cover of natural habitat (Chacoff & Aizen 2006, Ricketts et al. 2008). Thus the distance of the focal field to an area of natural habitats or the relative surface occupied by natural habitats within a 2 km radius around the study field can be used to create differing levels of pollination service, especially since recent results suggest that a guild of pollinators is often more effective than a single species (Klein et al. 2003; Hoehn 2008).

Pros and cons:

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Realistic variations of pollinator abundance & diversity;
Takes into account all pollinator fauna and can therefore be especially useful when the pollinating species are unknown;
Useful for crops for which pollination is achieved only or mainly by unmanaged pollinators (e.g. for crops pollinated by beetles such as oil palm Elaeis guineensis Jacq. and atemoya or custard apple, Annona squamosa L. x A. cherimola Mill. ; cocoa, Theobroma cacao L., pollinated by midges; and papaya, Carica papaya L., pollinated by moths);
Consistent with farming policy in some areas

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Potential correlated factors that affect yield and its components can confound results (e.g. fields along river bottom may all benefit from better soil conditions);
Requires landscape heterogeneity to locate fields in contrasting situation;
Repeatability may be limited over the years due to year-to-year fluctuations in pollinator populations.

Implementation modalities and independent variable recording

description of implementation action	numbers required
In a uniform area (similar topography, soil, slope, exposure), locate fields in landscape of predominantly intensive agriculture and fields in landscape dominated by natural habitats. Natural habitats must be assessed locally but will be similar to CORINE level 1 habitat classifications (see land cover.pdf downloadable at http://www.eea.europa.eu/publications/COR0-landcover) on the general level of forest, natural grassland, brush, etc. Record the proportion of natural habitat around each study field within a 1 km radius.	5 fields in landscape of predominantly intensive agriculture, and 5 fields in landscape dominated by natural habitats.
Locate fields close to (≤ 200 m) and far from (> 1 km) the closest patch of natural habitat. The patches of natural habitat should be as large as possible so as to provide as	5 fields close to (≤ 200 m) and 5 fields far from (> 1 km) the closest patch of natural habitat

diverse a pollinator fauna as possible. For small bees, area should be ≥ 0.5 ha; for large bees, a larger patch is needed. Record distance to closest patch of semi-natural habitat in each study field.	
Locate long fields (> 450 m long) with a single side perpendicular to its length adjacent to a patch of natural habitat, so as to have a gradient of distances from the edge of this patch across the field. Record distance to edge of natural habitat at each experimental site (i.e. each location of measurement – see below) in each study field.	5 fields > 450 m long to have a gradient of pollinator density from near to far from edge with natural habitat

4.C Introduced pollinators & landscape context together

This treatment to secure a range of pollination services combines the introduction of managed pollinators together with naturally occuring variation in pollinator populations due to landscape diversity. Recent results suggest that the combination of the two approaches can be more effective than either one alone. For example, Greenleaf & Kremen (2006) showed that wild bees that were more abundant and diverse near wild habitat enhanced honey bee pollination effectiveness on sunflower (*Helianthus annuus* L.) for hybrid seed production. Using this experimental design could produce some interesting results in disaggregating the respective contributions of managed versus wild pollinators to crop yields.

This dual approach will have the same pros and cons as the two treatments described in A and B above. However, it is especially important to remember the minimum distance between treated and untreated fields when planning the experimental design here so as to combine but not to confound the effects of both approaches. For example, if managed pollinators are introduced along one edge of a field, even a large one, while natural habitat is present along an adjacent or the opposite edge, it will not be possible to draw a conclusion as to which pollinator population led to the observed result. Also, if one wants to draw management conclusions from the proposed experiment, then the use of a factorial design is recommended, that is fields close and far from natural habitats should be combined with fields with and without pollinator introduction with 5 fields for each treatment combination (which gives a total of 20 fields). It may be very hard, indeed, to find such a large number of fields separated by the required isolation distance of 2 km as a minimum and yet located in environments that are similar (topography, soil, slope, exposure) and managed in a uniform way (same seed source or same genetic material ; same cropping system). In this case, one could locate five quartet of fields, that is five sets of 4 fields (one for each treatment combination) and the 4 fields within a quartet should be as similar as possible while differences between quartets of fields are allowed (each quartet will then be treated as a block for statistical analyses).

Implementation modalities and independent variable recording

description of implementation action	numbers required
Locate fields in intensive agricultural area located > 1 km from closest patch of natural habitat without supplementation by managed pollinators and fields adjacent to patch of natural habitat (≤ 200 m) and introduce managed pollinators along side of field close to natural habitat. Record distance to closest patch of semi-natural habitat and stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) for each study field.	5 study fields of each kind (total of 10 fields)
Select 10 fields in intensive agricultural area located > 1 km from closest patch of natural habitat and 10 fields nearby (≤ 200 m) natural habitat or in landscape dominated by natural habitats. Supplement half of each of these with managed pollinators along edge closest to natural habitat. Record distance to closest patch of natural habitat and stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) for each study field.	Factorial design (5 study fields for each combination of treatment => 20 study fields)
Locate 5 long fields (> 450 m long) with a single side perpendicular to its length adjacent to a patch of natural habitat, so as to have a gradient of distances from the edge of this patch across the field. Supplement these fields with managed pollinators along side close to natural habitat. Record the stocking rate of introduced pollinators (number of colonies or of bee nests or cocoons per unit area of study field) in each	5 fields > 450 m long

field. In addition, record at each experimental site (i.e. each location of measurement – see below) in each study field, the distance to the pollinator front.	

Section 5. Layout of experimental sites

Once the pollination treatment has been selected and the study fields have been located in agreement with the farmers, an experimental site will be established in each field for data collection (in long fields with a gradient of distances to the pollinator front, several sites will be established in each field). For fields that are large enough and planted with an herbaceous crop, the experimental site will cover a nominal area of 50 m x 25 m aligned along the rows and set in a representative area of each field following a basic design (Figures 2 and 3). For crops planted in rows, it is best to lay this experimental site along the rows to make it easier to set the plots for data collection (Figures 4). For fields large enough that are broadcast-sown, the layout of Figure 2 can also be used with the long axis of the site aligned with the longest axis of the field.

For fields > 450 m long for which the goal is to obtain a gradient of pollinator density, the experimental sites should be set perdendicular to the length of the field and at fixed distances from the edge with the pollinator front with 150 m increments (e.g. 25, 175 and 325 m from edge).

For fields that are not large enough or when the shape of the field does not allow for the establishment of such an experimental site – for example in the case of a long field planted on a terrace along mountain side – then the whole field will be used as an experimental site (Figure 5).

On the other hand, for very large fields, the experimental site should be set halfway between the geometric center of the field and its edge so as to represent an ‘average’ situation assuming a linear gradient of pollinators between the edge and the center of the field (Figure 3).

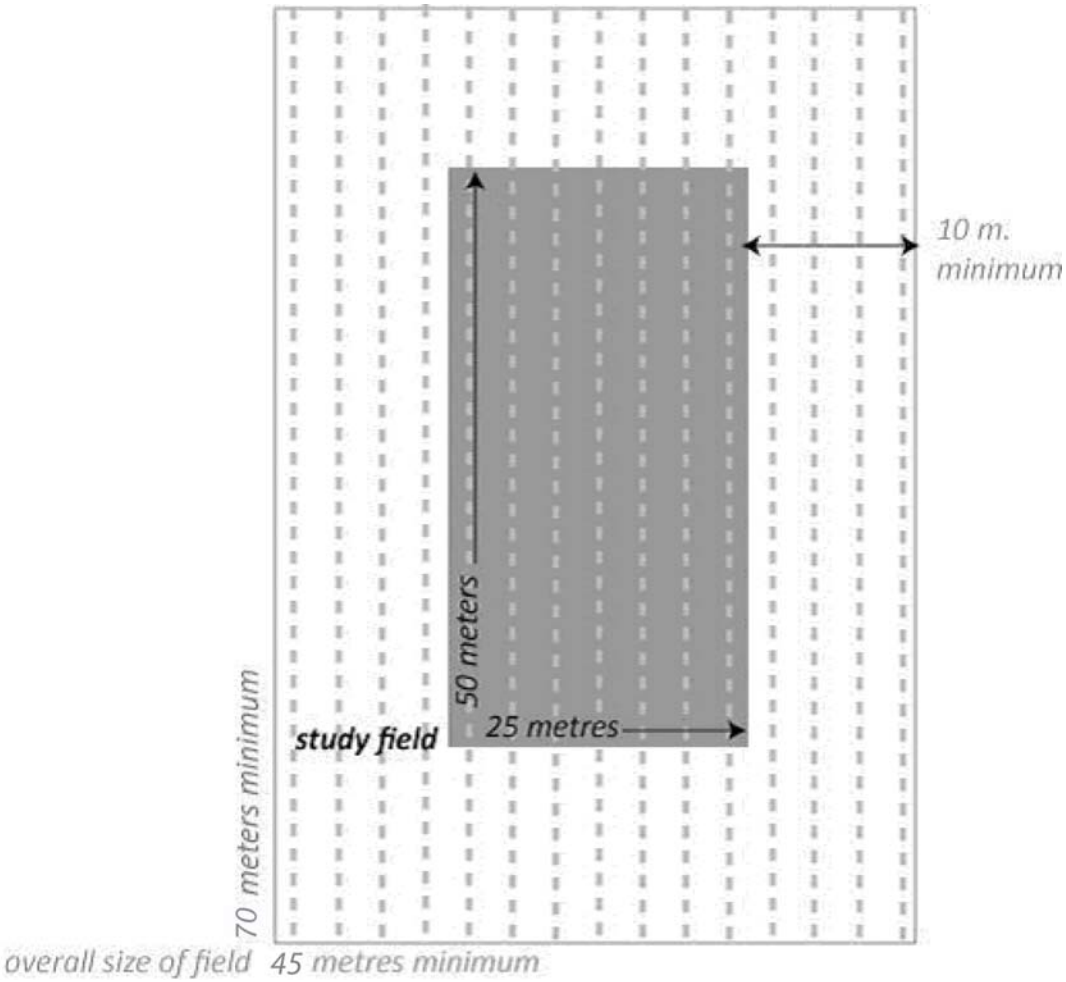

Figure 2. Location of the experimental site for data collection in a standard field .

For orchard crops, it is the tree panting pattern that will dictate the size of the experimental site as an area 50 m x 25 m may be far too small and not encompass but a single tree. By using the tree as the individual unit, rather than a distance of row or an area, it is possible to lay an experimental site that will permit the establishment of plots for data collection (Figures 6 and 9).

Finally, when the study 'field' consists of a set of patches of plants of the focal crop species – such as for cucurbits grown in home gardens – the experimental site will consist of a subset or all of these patches, the actual number of patches being adjusted so as to enable the collection of data over an adequate number of sampling units as indicated in the next section.

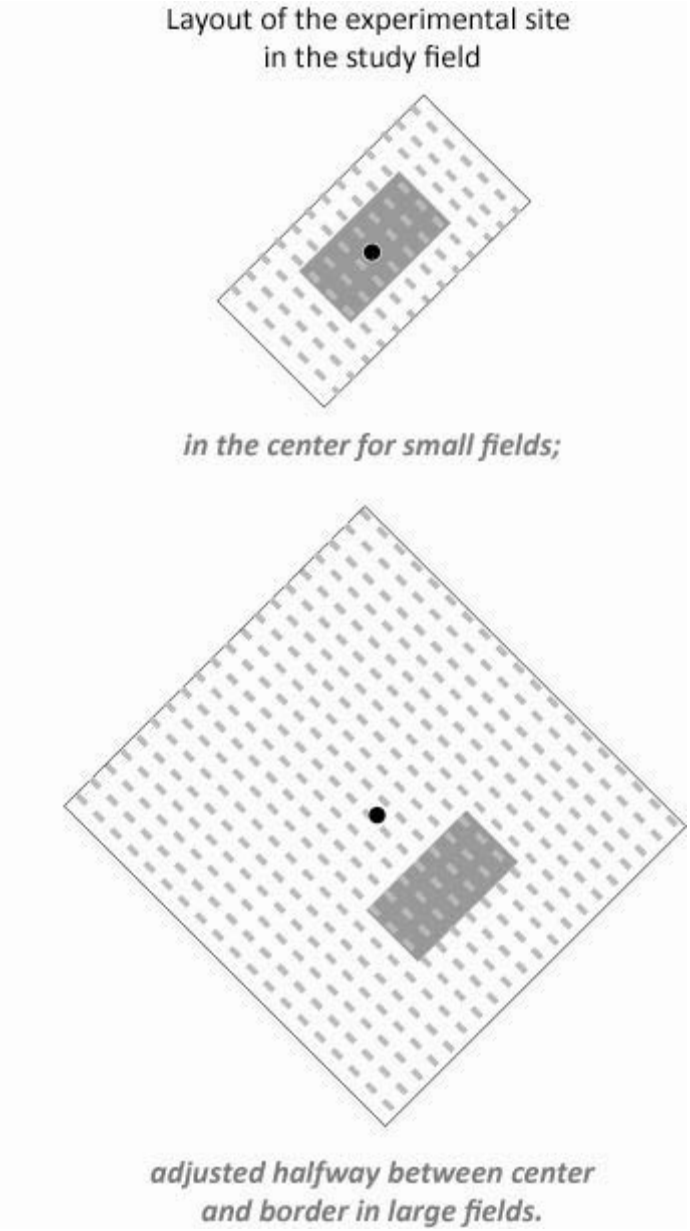

Figure 3. Layout of the experimental site in relation to the size of the study field.

As a reminder, it is very important that the management of all experimental units (field or plot or plant) be as similar as possible (except for the pollinator treatment). This means that they are planted with the same crop variety at more or less the same time, are managed in a uniform fashion and receive the same level of inputs (fertilizer, weeding, pest control, etc).

Section 6. Pollinator dependent variables and data collection

In section 4, we indicated for each treatment the kind of data that should be recorded to characterize each study field (namely the stocking rate of pollinator units, the distance to the closest patch of natural habitat, and/or the proportion of natural habitat in a 1 km radius

around the study field). These will provide the values of the independent variables that are used at each site.

For each study field, it will be essential to record all information deemed important to characterize this field as well as the cropping system used so as be able to justify that all or a subset of the study fields can validly be compared among themselves : field size, soil type and preparation, field immediate surrounding (hedge bordering the field or not), fertilizer application, planting date, genetic material (variety, source of seeds), planting density, planting pattern (for dioecious and self-incompatible species), list of main weed species in bloom & % soil cover of these weeds at the time of crop flowering, main management practices (irrigation, pesticide applications), and harvesting date (see data recording sheet in ANNEX 1).

Even when using well contrasted treatments either based on pollinator supplementation or landscape context, there is no guarantee that the response on the crop will match the intensity of the treatment exactly in either pollinator abundance or diversity. For this reason, data will have to be recorded on a regular basis to assess the impact of the pollinator treatment on the abundance (pollinator density) and the diversity (species richness or broader categories) of pollinators in the focal crop throughout its main blooming period. The response of the crop plants in terms of production output will then have to be measured to be able to measure the effects of the pollinator treatment.

Dependent variables	details
Pollinator density (pollinator/floral unit)	Pollinators (usually bees) /100 to 500 flowers or flowering units depending upon the focal crop (flower size) and the density of open flowers (scan sampling)
Pollinator diversity (species richness or broader categories)	Pollinator catch along fixed transect on the flowers of the focal crop (with insect net)
Agronomic yield	Production per unit area (expressed as kg of output and number of produce units – fruits and/or seeds – per m^2, acre or ha)
Quality of production	Any characteristics of the produce that may affect its price and marketability (e,g, average weight or size for fruit such as apple (*Malus x domestica* Borkh) or seeds such cashew nut (*Anacardium ocidentale* L.); grade for strawberry; oil content and oil quality for seed from oilseed crops; germination rate for planting seeds)
Economic yield	Expressed in local currency; production per unit area multiplied by the sale price paid to the producer per unit production

6.A Data collection for measuring pollinator density

These measurements should be recorded in the experimental sites only under good weather conditions for foraging bees: Temperature ≥ 15°C, low wind, no rain, and dry vegetation (Westphal et al. 2008). Recordings whould be made from the onset of the main blooming period, that is when ≥ 10% of the plants have started to bloom with flowers at anthesis (that is with open corolla).

Pollinator density will be measured by scan sampling a fixed number of open floral units in each of the 4 plots located in each experimental site (Figures 4, 5 and 6- see symbols for scan sampling) and the data will be recorded in appropriate data sheets on at least 4 dates during the main flowering period (ANNEXES 2, 3, 4 and 5). For orchards, a plot will consist of at least 2 trees (ANNEX 3), and when a pollenizer variety is present, a plot will consist of at least 2 trees of each type (ANNEX 4). When there is no plot, the required number of flowers will be surveyed over the whole experimental site, that is on the selected patches of plants (ANNEX 5).

The recordings will be done by scan sampling as there is no duration attached to the observations but rather an insect will be recorded or not depending on whether it is present at the time a given flower is first seen. Scan sampling was selected because it provides the most reliable way to assess pollinator density on flowers (Levin et al. 1968). This sampling will be done by walking slowly along a set path, in between rows when rows are present, and recording the numbers of pollinators seen when looking at individual floral units one by one in sequence. The term 'floral unit' is used here to mean an individual flower whenever practical. Else, whenever individual flowers are too small or too tight together to be observed one at a time, the floral unit will be an inflorescence like a flower head for crops with a tight inflorescence such as sunflowers (*Helianthus annuus* L.) or buckwheat (*Fagopyrum esculentum* Moensch) or even a loose panicle such as cashew nut trees or mango trees. The number of floral units to scan in each plot will be set at the start of the experiment, but should be adjusted based on the density of floral units so that it does not take more than 15 minutes to scan a plot and should also be adjusted to take into account the size and relative attractiveness of the floral units to avoid having too many null values. For example, for large nectariferous flowers such as those of cotton *Gossypium hirsutum* L., passionfruit (*Passiflora* spp.) or pumpkin, the scan of 100 flowers per plot usually provides reliable estimates (ANNEX 5). It is also the case for the large inflorescence such as those of mango trees or sunflowers, and for crops with often have few open flowers per plant on a given day even at peak bloom such as French beans, *Phaseolus vulgaris* L., or strawberry (ANNEX 3). For crops with smaller and more abundant flowers such as apple and cantaloupes (*Cucumis melo* L.) as well as smaller inflorescence such as those of buckwheat, 200 to 250 floral units per plot are usually needed (ANNEXES 2 and 4). Finally for crops with small flowers such as those of most Brassicaceae like canola (*Brassica napus* L.) or mustard (*Brassica campestris* L.), the number of floral units scanned per plot should be increased to 400 or 500 to avoid too many zero values. Pollinator density will be recorded in reference to a fixed number of floral units at anthesis rather than a fixed area or length of row so as to take into account the level of flowering and also be able to draw management recommendations subsequently by linking pollinator density on a per flower basis with production results.

In practical terms, this monitoring will be done by an observer with two hand counters, one in each hand, who scans the flowers that are well exposed as well as those that may be somewhat hidden. For orchard trees, depending on their height, the use of binoculars might

be useful so as to be able to identify the broad categories of foragers in all parts of the trees. It is essential that there be no bias resulting from the observer in recording pollinator density in control versus treated fields or when moving along a potential gradient of pollinator density. To this end, the same observer should do the recording in all the study fields of a given focal crop in a given location, or on all the plots along a gradient when a gradient design is used. When this is not possible and several observers are doing the recording, they should alternate between the fields with the different treatments so as to even out any difference due to the observer. One hand counter will be used to record the number of observed floral units while the other counter will be used to record the number of pollinators seen in these floral units. If possible, this basic method can be refined by using several hand counters to record separately different pollinator groups when these are of particular interest for the focal crop (e.g. ANNEX 5 : the data sheet for recording pollinator density in Nepal on squash flowers has different columns to record separately Western honey bees, Eastern honey bees, bumble bees, and other wild bees, while syrphid flies and other pollinators have been pooled under a single 'Other' column because they are known to be of lower pollination effectiveness). These measurements should be done at a fixed hour over two to four times per day depending upon the length of the period of anthesis of the focal crop flowers and the period when pollinators are active: For squash (*Cucurbita pepo* L.) and pumpkin (*Cucurbita maxima* Duch.) flowers that wilt by noon and sometimes earlier, it is usually not possible to go beyond two recording periods per day (ANNEX 5), while for flowers that stay open and are visited over the whole day and which are easily scanned, such as cantaloupe and mango flowers, recordings can be done over four periods during a day – (ANNEXES 2 and 3). Apple flowers usually do not open very early and so their scanning can be done only twice during the day (ANNEX 4). In all cases, the standard time closest to the solar time should be used so as to have comparable results among countries.

Each study field will be monitored only once on a recording day, but the time of recording of pollinator density will change among the different fields on each date of recording so that every study field has its pollinator density recorded at least once over all time periods during its blooming season. For this reason, the interval between two consecutive recordings will vary depending upon the flowering phenology of the crop. For determinate crops with a short flowering cycle that lasts only 10 to 15 days such as apple trees, for example, bee counts should be done every 3 to 4 days, while for indeterminate crops such as cotton or mustard, bee counts can be done on a weekly basis so as to cover the whole flowering season. This counting frequency should also be adjusted based on the weather since bee counts can only be made whenever the conditions are adequate for bee foraging (maximum daily temperature ≥ 15^{o}C, low wind and no rain, and crop plants dry).

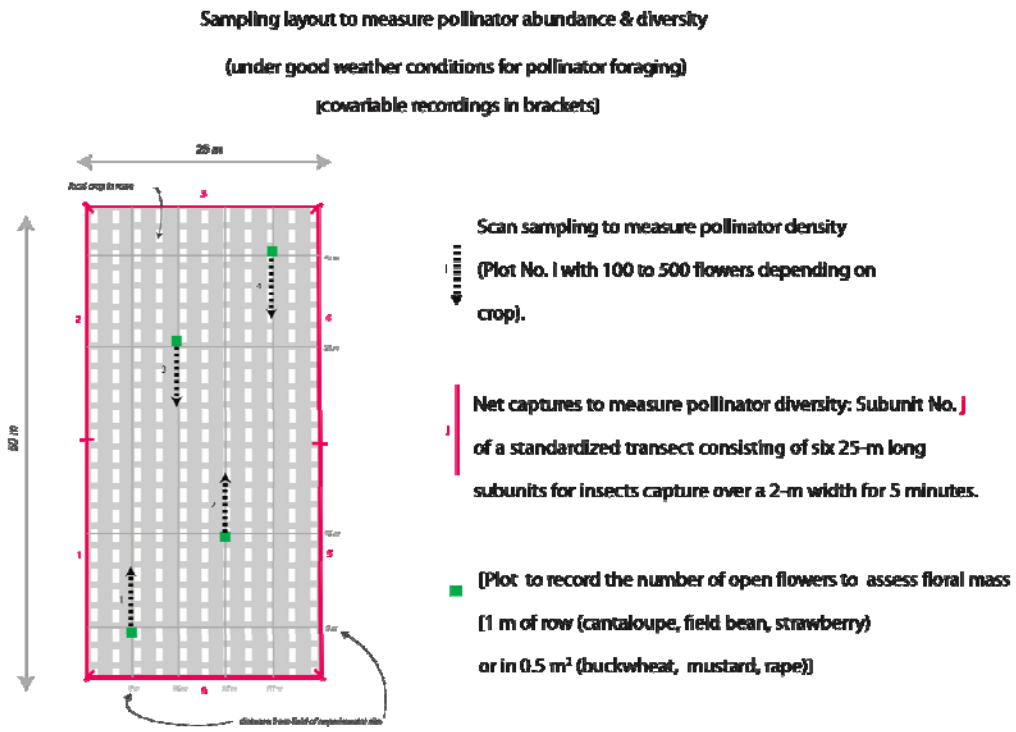

Figure 4. Layout to measure pollinator abundance and diversity, as well as flower density if appropriate as a covariable, in the experimental site of a study field

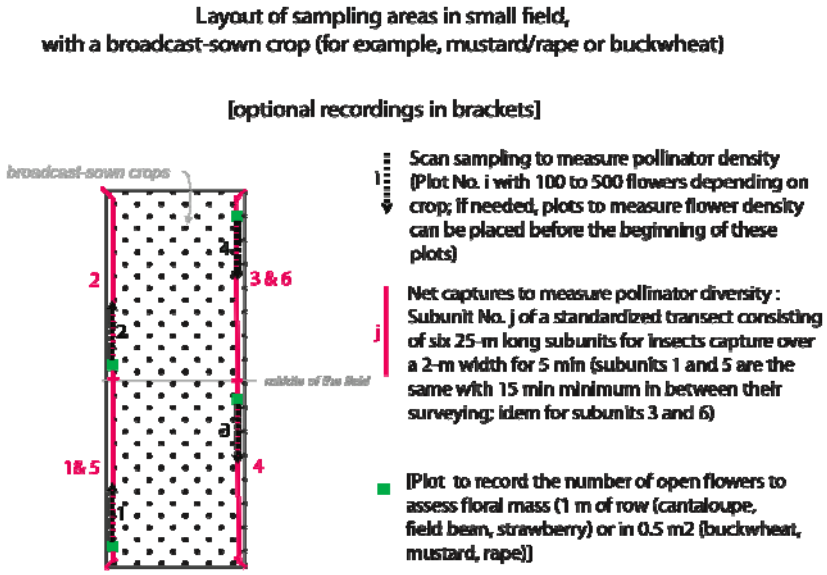

Figure 5. Layout of the sampling units to measure pollinator density and diversity in a study field too small to set a separate experimental site

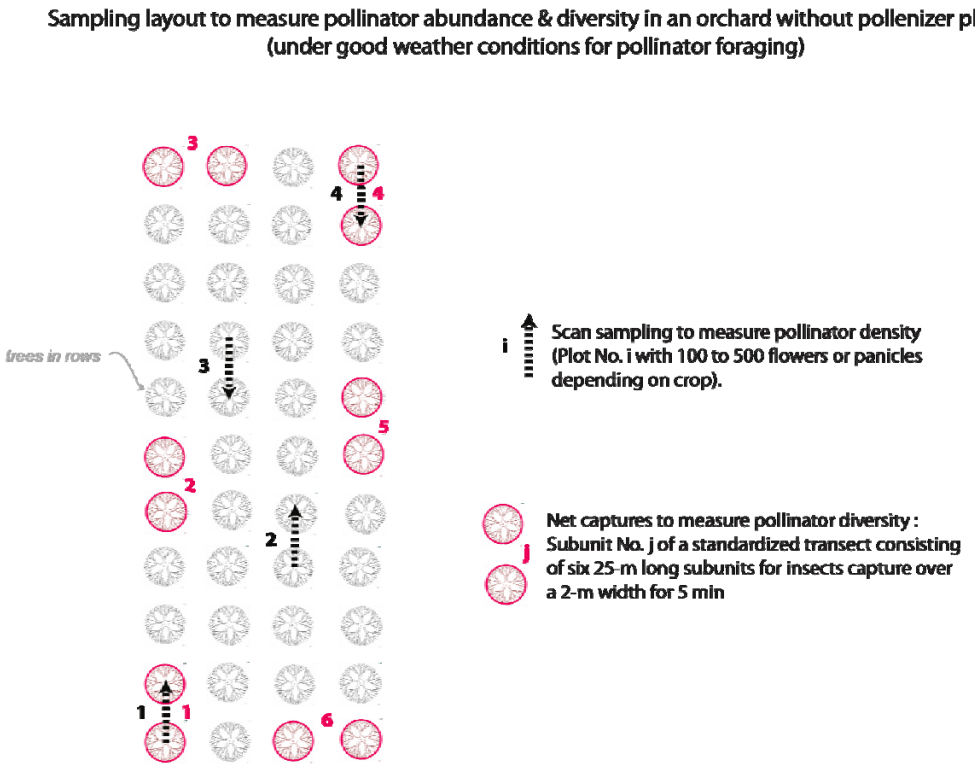

Figure 6. Layout to measure pollinator abundance & diversity in an orchard without pollenizer plants

6.B Data collection for measuring pollinator diversity

These recordings will be made right after the recording of pollinator density inasmuch as possible and they should also be conducted in the experimental sites only under good weather condition for foraging: that is, temperatures ≥ 15°C, low wind, no rain, and dry vegetation. Because honey bees can be very abundant and their presence and abundance will be recorded with the pollinator density, *Apis* bees can be caught during the net captures to assess pollinator diversity to make sure that are, indeed, *Apis* bees, but they will not be recorded in the appropriate sheets. Some examples of these data sheets are presented in ANNEXES 6 & 7 for an herbaceous row crop and ANNEX 8 for an orchard crop.

To assess pollinator diversity in herbaceous crops, insects visitors that are suspected to be effective pollinators (most commonly bees – Apiformes – and syrphid flies that are also called drone flies (Syrphidae)) will be caught with insect nets along six 25 m long and 2 m wide transects over 5 minutes each, for a total of 30 minutes per study field (Figures 4 and 5 - see symbols for net captures). In orchard crops, insects visitors that are suspected to be effective pollinators, will be caught with insect nets in six plots of a pair of adjacent trees (Figure 6). Again 5 minutes of surveying will be spent on each plot, for a total of 30 minutes per study field, and the surveying will be done by walking slowly around each tree. Depending on the

height of the tree, the use of a telescopic net or a small ladder in the field might be useful so as to be able to sample the foragers in all parts of the trees.

The insects will be killed with killing jars using either potassium cyanide and/or ethyl acetate (the former kills the insects very quickly but is dangerous to use while the second takes more time, but has the advantage of making the bees pull their tongue prior to death and tongue length and characteristics are important characters to identify bees; it is also possible to use a cyanide killing jar with a few drops of ethyl acetate placed on tissue paper inside the jar so as to have the advantages of both methods). After capture, each specimen will be mounted in the evening following collection or, if available, placed in a fridge for 24-28 hours to get rid of the cadaveric stiffness and subsequently mounted. Mounting will be done on pins following usual entomological procedures and each specimen will receive a tag with the collection date, exact location of collection, focal crop name and name of collector as follows :

22 February 2010 Kosi Katarmal Uttarakhand, INDIA on flowers of Brassica campestris Ranbeer S. RAWAL

If immediate mounting is not possible, specimens will be pooled by study field and date of capture and placed in a small jar along with the tag information listed above written in soft pencil on a small piece of paper. All such jars will then be stored either in a freezer at -20°C or in 70% ethanol until they can be mounted adequately. Storage in freezer should be preferred if at all possible as specimens stored in 70% ethanol need a special procedure to dry them and mount them in a way that they can be identified properly (for further help on this, see the videos on http://www.youtube.com/swdroege).

Once mounted, specimens will then be identified to the species level if possible or else at least to the same taxonomic level as used to record the density of insect pollinators (ANNEXES 2, 3 and 4). Because taxonomic expertise on bees is not readily available in most places, it may be necessary to send the specimens to various experts. The precise data on the diversity of non-*Apis* pollinators will therefore usually not be readily available after specimens are caught and initial analyses may have to be done on the categories listed in the data sheet rather than on species diversity. It is noteworthy that a further step is now available as a key to the bee families of the world is available on the internet (http://www.yorku.ca/bugsrus/BFoW/Images/Introduction/Introduction.html). This resource should be used as much as possible to better asses bee diversity in the 'wild bee' category.

6.C Data collection for co-variables

Covariables are variables that are usually not related to the independent variables, but which may contribute to explain the values of the dependent variables and also help in the interpretation and analyses of the results. By collecting information on these dependent variables, the investigator may gain a more precise picture of the level and key characteristics of the pollination service and this is why they are also listed below. Their recording will depend upon the time available for the experiment, in particular the flower density may be quite time consuming to assess, but, together with forager density data, it will

provide some independent assessment of the characteristics of each field in terms of overall plant vigor and yield potential as well as overall crop attractiveness.

possible covariables	details
Flower density : the number of floral units at anthesis (with corolla open) per unit area of study field on a given date (ANNEXES 9-10)	Provides an assessment of the quantity of flowers to be pollinated and also, together with the size of the field and the pollinator density, a mean to assess the total floral mass present, the total amount of resources (nectar and pollen) available to pollinators on the study field, and the total size of the pollinator population foraging in the field
Age of trees (or diameter of trunk at given height)	Assessment of the production potential
Weather conditions (included in the data sheet to record forager density – see ANNEXES 2, 3 and 4)	Impact on foraging activity of pollinators

If deemed important, the recording of flower density needs to be done at the same time as the recording of the pollinator density and diversity so that the three variables can be related to assess the overall population of pollinating insects in the study field. This measurement is usually best done after the other two and when the flowers at anthesis can easily be distinguished from buds as well as wilted flowers. Flowering units are defined here as previously in section 6.A and a flowering unit is considered at anthesis whenever at least one of its flower is at anthesis. From that day on, a flowering unit is considered to be at anthesis until all of its constitutive flowers are wilted and therefore no longer at anthesis. Wilting is often noticeable by the closing of the corolla (as in cucurbits and liguliflorae Asteraceae such as chicory *Cichorium intybus* L. and lettuce *Lactuca sativa* L.) or the dropping of the petals (as in almond, apple, kiwifruit, rape, and strawberry) though in some species the stigma can remain receptive after the corolla has dropped (e.g. strawberry, personnal observation). Wilted flowers should not be included in the count. It is noteworthy that in some species, most noticeably Asteraceae such as sunflowers, the wilting of the disc florets is not straightforward to see and one usually considers that the anthesis of a head is over when all the ray florets have their stigma exposed (Asteraceae are protandrous).

For herbaceous crops planted in rows and where rows are well defined throughout the season, the number of floral units at anthesis is recorded on plots that cover a set length of row. This length varies with the planting density and the floribundity of the crop, but it is best set so that at peak bloom the numbers of floral units per plot can be recorded within 15 min at most by a trained observer. This usually amounts to 1 m of row for crops such as strawberries and cantaloupes, while plots of 3 to 5 m of row can usually readily be examined in crops like cotton and sunflower that have a low floribundity or large inflorescences. When the rows are no longer identifiable at the flowering stage, it is best to record the number of floral units at anthesis in the fixed area of a square or circular frame. Just as for the length of row, the size of this area will depend upon the plant density and the crop: for squash, a frame of 1 or even 2 m^2 is usually necessary to avoird having too many null values. For crops with

many smaller flowers such as buckwheat, mustard and rape, a frame of 0.5 m^2 is usually large enough.

Orchard trees are a real challenge to assess the floral mass and there is no easy way to solve it. But for these crops, it is not always necessary to have absolute numbers of floral units per unit area, and often a relative assessment of the flowering stage is what is really important. The recording plots are usually made of a single or two trees (a production tree and a pollenizer tree for self-incompatible species). If branches are easily accessible, the flowering may be followed over one main branch or two on each tree in the plot. If this is not possible, then a photograph taken at a fixed spot can be taken on the occasion of each recording of pollinator density to assess the flowering in rough relative terms.

The layout of the plots or area for quadrat location to measure the flower density is presented in Figures 4 and 5. Also an example of data sheets to record the flower density of an herbaceous crop and the flowering phenology of an orchard crop are provided in ANNEXES 7 and 8, respectively.

Section 7. Production dependent variables and sampling units

Many variables have been used to assess the impact of pollination level on crop output. These include variables related to pistil characteristics (e.g., number of conspecific pollen grains per stigma, number of pollen tubes per style, and the proportion of fertilized ovules), to the initiation of fruits (e.g., fruit set and seed set), to agronomic yield expressed in weight or number of produce per unit area, and economic yield expressed as gross or net return per unit area in local currency.

7.A. Agronomic yield

Yield variables are usually not available until a long lag time after flowering and many factors not related to the pollination level during flowering can interfere with the production output and thereby confound the effects of the pollinator treatment. Also yield data are not always easy to record. In particular, plants with indeterminate flowering may require repeated harvesting of the marketable produce over the whole production season (e.g., vegetables such as green bean, eggplant, pepper, tomatoes and zucchini, and also some fruits such as mango and strawberry). Also, for perennial crops, the harvest should be measured over two seasons to avoid the confounding effect of alternate bearing on different trees and orchards. Yet, because our protocol is aimed at gathering data meaningful for farmers, despite their shortcomings yield variables will be the only ones we will consider here.

As indicated previously (section 2), crop plants can compensate for pollen limitation with longer flowering periods and greater flower production. Also fruit set and seed set can be resource-limited, and thereby the results obtained by increasing pollination levels on a subset of flowers on a plant may result in larger fruit and more seeds from those flowers, but not greater overall production on a plant basis (Knight et al. 2005). As a consequence of these two important mechanisms, **it is essential when considering agricultural output that the whole plant be used as smallest sampling unit rather than individual flowers or a sample of flowers regardless how large**. Therefore our proposed yield measurements are based on the whole plant as smallest sampling unit, that is the yield will be calculated on the basis of a sample of individual plants, a set of plots or the whole field. For each, we will examine below the pros and cons. Those applying the protocol can select the best sampling

units for their focal crop and study fields to measure the agronomic yield and the quality of the output, the only requirement being that the same sampling units be used in all study fields inasmuch as possible

7.A.1 Individual plants

Pros and cons:

Natural yield unit from a farmer's standpoint (especially for trees);
Biological unit, reflecting an integrated response to the treatment;
Applicable in mixed cropping systems;
Provides intrafield variability (usable with gradient within field)

Needs plant density at harvest to calculate yield;
Does not control for resource allocation between years unless recorded over several years;
Not possible for some crops when plants are highly intermingled at harvest (buckwheat, rape);
Variability among plants often very large;
Mechanical harvest usually not possible except for some tree crops;

7.A.2 Plot (unit length of row or unit area of study field)

Pros and cons:

Useful when when individual plants are too intermingled (buckwheat, rape)
By recording plot size, result are directly expressed in yield units meaningful for farmers ;
Amenable to mechanical harvest;
Provides intrafield variability (usable with gradient within field)

May require more work than individual plants for harvesting;
Not applicable in mixed cropping systems

7.A.3 Whole field

Pros and cons:

Can often be obtained directly from farmer;
Direct measurement of commercial yield over the whol study field;
Meaningful for farmers and the public

Farmers may be reluctant to provide data;
No measurement of intrafield variability (not usable for gradient within field);
Between field variability can easily confound the link to pollination level (water availability; fertilizer; pest control)

From a practical standpoint, whenever possible it is best to obtain the yield data from individual plants or from plots. The layout for such sampling units is presented in Figure 7, 8 and 9 and some examples of data sheets to record such data are provided in ANNEXES 9 to 10. When individual plants are harvested as in mixed planting systems, it is best to harvest adjacent plants that are located in the same general area as the proposed plots (Figure 5). In general, it is best to harvest a minimum of 2 plants per plot (e.g., trees for orchard crops) up to 10 plants or more per plots for herbaceous determinate crops. Produce should be harvested when fully mature and right before commercial harvest.

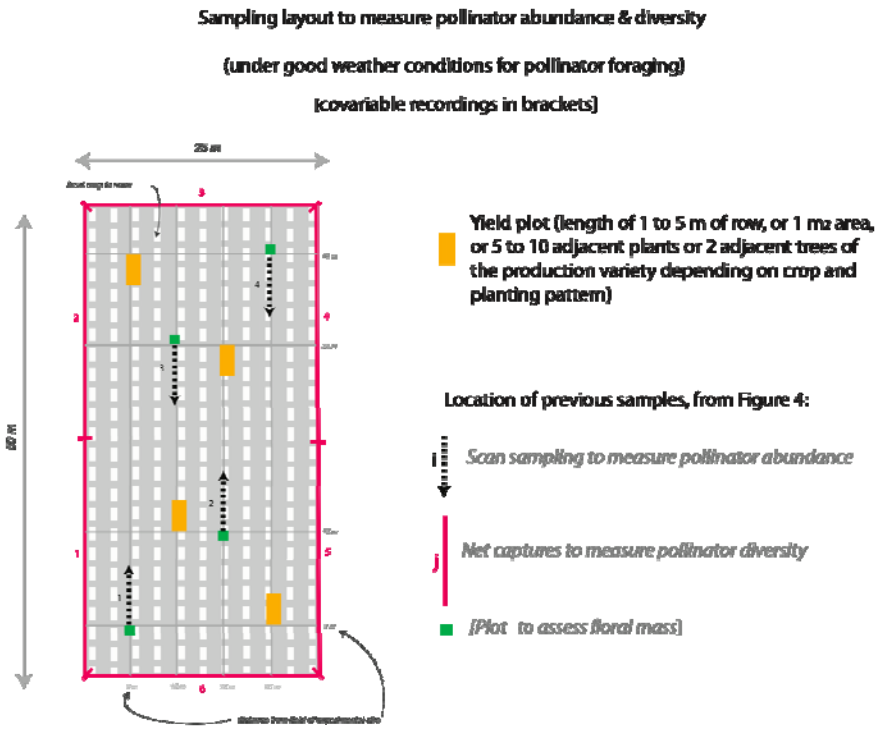

Figure 7. Layout of the sampling units to measure the yield based on individual plants or plots in the experimental site of a study field

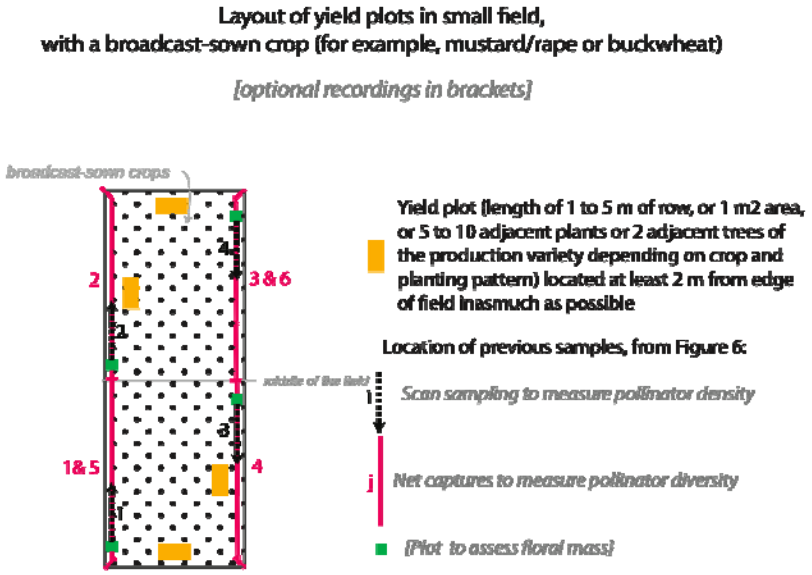

Figure 8. Layout of the sampling units to measure yield in a study field too small to set a separate experimental site

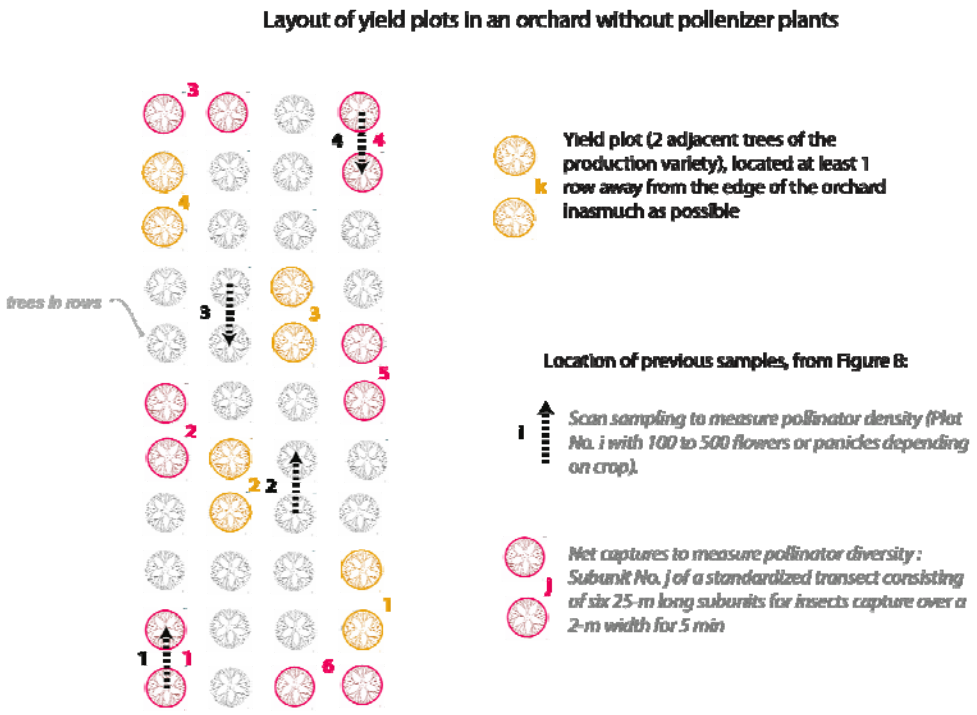

Figure 9. Layout of the sampling units to measure yield in in an orchard without pollenizer plants

Once a produce is harvested, it may be possible to measure quality characteristics of all or a sample of the production units if time, budget and available technology permits. No special protocol will be provided here for these measurements as they will clearly vary from one crop to another, be a function of the analytical tools available locally for these analyses, and they may also be context specific, that is dependent upon the requirements of a specific market. For example in Kenya, the pods of export-grade runner beans (*Phaseolus coccineus* L.) must be straight shaped and measure between 24 and 27 cm in length and anything smaller or beyond this range is considered a reject. Poor pollination leads to missing seeds resulting in sicle-shaped beans that are no longer acceptable for the export market.

Examples of quality figures than can be recorded are :

For fruit crops : average size (e.g. diameter, circonference, weight), number of filled seeds (e.g. apple; cucurbits), quality of the flesh consumed (sweetness, flavor);
For nut crops: average size (e.g. diameter, circonference, weight);
For oilseed crops : seed size, oil content, quality parameters of the oil;
For seed crops for planting : germination rate, quality parameters for seed industry

7.B Economic yield

If the price paid to the producer per production unit is known, it may also be possible to assess the yield of each harvesting unit (plant, plot or field) in economic terms, that is expressed in local currency or an international standard.

Pros and cons:

Meaningful variable for farmers & consumers;
Meaningful for government & policy makers;
May assist farmers to record proper documentation;
May also include non-market values, e.g. nutritional value

Farmers may be unwilling to share the price at which they sold their crop;
Very context specific;
Can be very volatile from one season to the next;
Lack of accepted methodology (interdisciplinary);
Link to pollination deficit may be tenuous & difficult to establish;
Usually beyond the control of individual farmers

If at all possible, the producer price should be obtained for the production of each study field so as to provide some input data for the economic analyses of the impact of adopting pollinator-friendly practices.

Section 8. Statistical analyses and general conclusions

Control sites without introduced pollinators will have a factor value set at 0 while fields with introduced pollinators using a fixed stocking rate will have a factor value set at 1 and the values of the dependent variables for the two groups will be easily contrasted using usual ANOVA procedures. When pairs or blocks of fields will be used, similar methods can be applied using adapted ANOVA procedures.

This will probably not be so with the distance to the closest patch of natural habitat or the proportion of natural habitat in a 2 km radius around each study field as those values are continuous and will probably vary from field to field along a gradient so that regression analyses may be more appropriate to analyze the results of the landscape treatments.

For large fields with a gradient of distances from the pollinator front, ANOVA with contrasts or regression methods should be used depending on the number of distances set from the pollinator front.

In all cases, it will also be of interest to look at the correlation between forager density and diversity on one hand and the yield variables on the other, as in Hoehn et al. (2008). This will be especially important in drawing appropriate management conclusions from the studies conducted using the proposed protocol.

This protocol aimed to address pollination as a production factor at the farm scale level. As such, one should also remember that, as a production factor, pollination needs to be fully integrated into the overall farm management system to optimize production in a holistic and sustainable way.

References

Beil M, Horn H, Schwabe A. 2008. Analysis of pollen loads in a wild bee community (Hymenoptera: Apidae), a method for elucidating habitat use and foraging distances. Apidologie 39 :456-467.

Buchmann SL, Shipman CW. 1991. Foraging distances flown by honey bee colonies: analyses using Mathematica software. Am. Bee J. 131 :771.

Chacoff NP, Aizen MA. 2006. Edge effects on flower-visiting insects in grapefruit plantations bording premontane subtropical forest. J. Appl. Ecol. 43 :18-27.

Delaplane KS., Mayer DF. 2000. Crop pollination by bees. CABI Publishing, New York, USA. 332 p.

Free, J.B. 1993. Insect pollination of crops. 2nd. ed. Academic Press, London. 684 pp.

Goulson D. 2003. Effects of introduced bees on native ecosystems. Ann. Rev. Ecol. Evol. Syst. 34 :1-26.

Hingston AB, McQuillan PB. 1999. Displacement of Tasmanian native megachilid bees by the recently introduced bumblebee *Bombus terrestris* (Linnaeus, 1758) (Hymenoptera : Apidae) Aust. J. Zool. 47 :59-65.

Hoehn P, Tscharntke T, Tylianakis JM, Steffan-Dewenter I. 2008. Functional group diversity of bee pollinators increases crop yield. Proc. R. Soc. B. 275 :2283-2291.

Kato M, Kawakita A. 2004. Plant-pollinator interactions in New Caledonia influenced by introduced honey bees. Am. J. Bot. 91 :1814-1827.

Klein AM, Steffan-Dewenter I, Tscharntke T. 2003. Fruit set of highland coffee increases with the diversity of pollinating bees. Proc. Roy. Soc. London (B) 270 :955-961.

Klein AM, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proc. Roy. Soc. B. 274 :303-313.

Knight TM, Steets JA, Vamosi JC, Mazer SJ, Burd M, Campbell DR, Dudash MR, Johnston MO, Mitchell RJ, Ashman TL. 2005. Pollen limitation of plant reproduction: pattern and processes. Annu. Rev. Ecol. Evol. Syst. 36 :467-497.

Levin, M.D., R.O. Kuehl, & R.V. Carr. 1968. Comparison of three sampling methods for estimating honeybee visitation to flowers of cucumbers. J. Econ. Entomol. 61:1487-1489.

McGregor, S.E. 1976. Insect pollination of cultivated crop plants. Agric. Handb. 496. U.S. Dept. Agric. U.S. Govt. Print. Off., Washington, D.C. 411 pp.

Pasquet RS, Peltier A, Hufford MB, Oudin E, Saulnier J, Paul L, Knudsen JT, Herren HS, Gepts P. 2008. Long-distance pollen flow assessment through evaluation of pollinator foraging range suggests transgene escape distances. Proc. Nat. Acad. Sci. USA 105 :13456-13461.

Rader R, Howlett BG, Cunningham SA, Westcott DA, Newstrom-Lloyd LE, Walker MK, Teulon DAJ, Edwards W. 2009. Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. J. Appl. Ecol. 46 :1080-1087.

Roubik DW. 2002. The value of bees to the coffee harvest. Nature 417 :708.

Sanzol J, Herrero M. 2001. The "effective pollination period" in fruit trees. Sci. Hort. 90 :1-17.

Steffan-Dewenter I, Tscharntke T. 2000. Resource overlap and possible competition between honey bees and wild bees in central Europe. Oecologia 122 :288-296.

Vaissière, B.E., J.O. Moffett, and G.M. Loper. 1984. Honey bees as pollinators for hybrid cotton seed production on the Texas High Plains. Agron. J. 76 :1005-1010.

Wilcock CC, Neiland MRM. 2002. Pollination failure in plants: why it happens and when it matters. Trends Plant Sci. 7 :271-277.