TÖÖVÕTULEPING Nr. 379

ERA-NET CORE Organic II

“BICOPOLL - Targeted precision biocontrol and pollination enhancement in organic cropping systems”

Täppisbiotõrje ja tolmeldamine mahepõllumajanduses

01.12.2011 – 01.02. 2015

Lõpparuanne

Projekti koordinaator: Heikki Hokkanen
Projektis osales 13 partnerit 7 riigist, sh Eesti Maaülikool kui alateema „WP3- Teised mesilaseliigid kui taimekaitsevahendi siirutajad“ juhtasutus

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Töö eesmärk

selgitada kimalaste kasutamise võimalused biopreparaatide siirutajatena ja tolmeldajatena aedmaasikal avamaastiku tingimustes.

Tulemused

Kimalased biopreparaadi siirutajatena avamaastiku tingimustes

Katsed näitasid, et kimalaste (*Bombus terrestris*) abil maasikaõitele kantud biofungitsiid Prestop Mix vähendas oluliselt hahkhallitusse nakatunud marjade osakaalu – töödeldud lappidel oli haigestunud vilju 2,6 korda vähem kui kontroll-lappidel (Muljar 2010, Muljar et al 2012, 2014, 2015, Muljar Mänd 2011abc, De Mayer et al 2013, Menzler-Hokkanen et al 2013, Mänd Karise 2013, Karise Mänd 2013, Starast et al, 2013, Hokkanen et al 2014)

Tolmeldamise mõju saagile

Tänu biotõrjega kaasnevale tolmeldamisele suurenes mõlema meil enamkasvatatud maasikasortide saagikus: risttolmleval sordil 'Polka' suurenes kümne vilja mass 27 % võrra ning peamiselt isetolmleval sordil 'Sonata' 5 % võrra. Seega kaasnes entomovektor-tehnoloogia kasutamisega nii hahkhallituse tõrjeks sobiva preparaadi Prestop Mix siirutamine õitele kui ka samaaegne lisatolmledamine. Tolmeldamise efektiivsus sõltus konkretsete aedmaasika sortide omadustest (Karise et al 2012, 2014, 2015).

Kimalasperede arvukus istandikus

Optimaalse kimalasperede arvukuse hindamiseks suurendati aedmaasika istandikes perede arvukust: kimalasperede arv varieerus kolmest kuni kaheteistkümne pereni hektaril. Optimaalseks osutus 6 peret/ha. Kõik kimalased, kes istandikus õisi külastasid olid pärit vaid katsepesadest, kuna looduslikes kimalasperedes pole sel ajal töökimalased veel koorunud. Katsed näitasid, et vähemalt kuni 100 m kaugusele tarudest ei tähendatud haigestunud viljade osakaalu tõusu, seega tuleks tarud paigutada istandikku 200 meetriste vahedega tagamaks tõrjeks piisav biopreparaadi siirutamine õitele (Karise et al, 2014, Muljar et al 2011, 2012).

Aedmaasika õietolmu osatähtsus kimalaste korjes avamaastiku tingimustes

Kimalaste poolt tarru toodud õietolmukämbud sisaldasid keskmiselt 22% aedmaasikaõietolmu. Selgus, et 1/3 töölistest kogus õietolmu peamiselt või ainult aedmaasikalt. Kimalaste kogutud õietolmu liigilise koosseisu analüüs näitas, et aedmaasikas toidutaimena ei ole alati tolmeldajate esimene valik. Aedmaasika õietolmu osakaal sõltus tugevasti konkureerivatest taimedest. Enamkülastatud taimedeks olid valge iminõges *Lamium album* ja mitmed roosõielised, mis alustasid õitsemist aedmaasikaga samal ajal. Valge iminõges on tuntud hea nektari- ja õietolmutaimena, mis tolmeldajaid ligi meelitab. Lisaks asusid katsepõllu läheduses õitsevad puuvilja- ja marjaaiad, millega võib seletada roosõieliste taimeliikide õietolmu sagedast esinemist korjes. Kultuurtaimedest oli kimalastele atraktiivsemateks nektari- ja õietolmutaimedeks taliraps ja valge ristik.

Kimalaste kasutamisel biotõrjepreparaadi siirutajatena ja ka tolmeldajatena, peab tähelepanu pöörama ümbritseva maastiku erisusele, kuna see mõjutab kimalaste õietolmukorjet aedmaasikal ja sellest tulenevat biopreparaadi taimede õiteni kandmise efektiivsust. Uurimistöö tulemustest järeldub, et kimalaste efektiivsus tolmeldajatena on suurem aladel, kus konkureerivate taimede arvukus on madal (Muljar et al, 2012, Muljar Mänd 2011d, Dreyersdorff et al 2014, 2015, Mänd Karise 2013).

Järeldused

- Kimalased osutusid efektiivseteks biopreparaadi Prestop Mix siirutajateks mitte üksnes kasvuhoone tingimustes vaid ka avamaal aedmaasika istandikes, vähendades oluliselt aedmaasika viljade haigestumist hahkhallitusse.

- Biopreparaadi efektiivse õitele siirutamise tagamiseks tuleks pered paigutada aedmaasika istandikesse 200 meetriste vahedega.

- Entomovektor-tehnoloogiaga kaasnev lisatolmeldamine suurendas viljade massi ja seega ka saaki. Tehnoloogia tasuvus oli suurem risttolmlevatel sortidel.

- Kimalased osutusid olulisteks aedmaasika tolmeldajateks ja biopreparaatide siirutajateks hoolimata sellest, et istandikud paiknesid heterogeenses põllumajandusmaastikus, mis pakkus tolmeldajatele rohkelt alternatiivseid toidutaimi. Tarru toodud õietolmust moodustas aedmaasika õietolm keskmiselt 22%. Kolmandik korjekimalastest kogus ainult või peamiselt aedmaasika õietolmu.

- Kimalaste kogutud õietolmu liigilise koosseisu analüüs näitas, et aedmaasikas toidutaimena ei ole siiski alati tolmeldajate esimene valik. Aedmaasika õietolmu osakaal sõltus tugevasti konkureerivatest taimedest. Meie tingimustes osutusid sugukondade *Rosaceae* ja *Laminaceae* esindajad peamisteks aedmaasika konkurentideks. Samas külastasid kimalased ka talirapsi, valget ristikut ja lupiini.

- Enomovektortehnoloogia, kus kasutatakse kimalasperesid biopreparaatide siirutajatena, sobib rakendamiseks hahkhallituse tõrjes aedmaasikal meie kohalikes tingimustes. Antud biotõrje meetod on heaks alternatiiviks hahkhallituse keemilisele tõrjele nii Eesti integreeritud-, keskkonnasõbraliku- kui mahetootmisega tegelevate tootjate jaoks. Lisaks haiguse allasurumisele suureneb ka aedmaasika saagikus tänu putuktolmlemisele Tolmeldamise efektiivsus sõltub konkreetse aedmaasikasordi omadustest.

<u>Avaldatud publikatsioonid</u>

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<u>BICOPOLL projekti tulemuste käsitlemine EMÜ õppes</u>

BICOPOLL projekti tulemusi käsitletakse EMÜ õppekursustes:

PK1036/PK1214Taimekahjustajad ja nende tõrje
PK0519 Keskkonnasäästlik taimekaitse
PK0202 Bioloogiline mitmekesisus
PK0250 Bioloogiline mitmekesisus ökosüsteemides
PK1504 Ökoloogilise põllumajanduse põhiprintsiibid

Projekti baasil kaitstud üliõpilastööd

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28. veebruaril 2015

Marika Mänd	Aret Vooremäe
Projektijuht	EMÜPõllumajandus- ja keskkonnainstituudi direktor
(allkirjastatud digitaalselt)	(allkirjastatud digitaalselt)

LISA

ERA-NET CORE Organic II

"BICOPOLL - Targeted precision biocontrol and pollination enhancement in organic cropping systems"

WP 3. Other bees as vectors

Final report

Final project summary

The BICOPOLL project has progressed according to the original plan, and in many instances, much more has been achieved than what was originally anticipated. On the research and demonstration side BICOPOLL has progressed very well. The consortium has identified several aspects of the technique, which could be (and some have been already) improved, and the consortium is looking forward to the coming field season to study these in reality. During 2012 field demonstration trials on strawberry cultivations were carried out in four countries: Finland, Estonia, Italy and the UK, while demo trial sites have been established in several other countries for trials during 2013 (e.g., Norway, Åland, Turkey, Belgium, Germany, Slovenia). Demonstration trials in Sweden and Denmark are being prepared, but may not be available yet in summer 2013.

A conservative estimate for Finland is that the uptake of the entomovector technique by growers is rapid, and in 2012 close to 500 ha of strawberry cultivation was using the technique (over 10% of growers and of the growing area). The excellent control results from the Finnish case have been presented at several international and national conferences. Further field results from BICOPOLL partner trials in summer 2012 showed that in Italy – despite extremely difficult weather conditions – significant reduction over untreated control (mean 39% mould) by biocontrol alone (13%) was achieved, and similarly by the combined treatment (11%), while chemical control (26%) did not differ significantly from the untreated control. In the UK trial entomovectoring by bumble bees resulted in control of the grey mould, which was as good as by the chemical control. In Estonia, field studies at very low pathogen pressure nevertheless showed significantly less grey mould, and higher marketable berry yields in plots entomovectored either by honey bees, or by bumble bees (separate field studies). So far, all field tests using entomovectoring and *Gliocladium catenulatum* (Prestop Mix) have shown excellent control results, and we intend to broaden these trials to include still other BICOPOLL partner countries in 2013 and 2014.

Several project partners have participated in applications for further funding on the topic (e.g., from the last calls in FP7). Active dissemination of project results in scientific, professional, and general media has been pursued. A significant development is the plan to publish a scientific book on the BICOPOLL topic as an outcome for the project; the publishing contract has already been signed (with Springer, to be included in their series "Progress in Biological Control"). A series of practical "how-to-do" handbooks/pamphlets for use by farmers in each of the participating countries (in their national languages) is also being planned.

WP 3. Other bees as vectors

- The data provides strong evidence that bumblebees can effectively vector a MCA to reduce significantly *B. cinerea* incidence not only in greenhouse strawberries but also in open field conditions where the landscape is heterogeneous with many competing crops and wild flowers.
- In greenhouses equal amounts of CFUs were recovered on the flowers up to 21 m. There were no significant decrease in disseminated CFUs between first, second and third flowers visited.
- In open fields the data provides proof that bumble bees are able to disseminate the Prestop Mix evenly to strawberry flowers at least within 100 m radius from the hive. The triple hives positioned over the fields with the distance of 200 m from each other is enough to guarantee the even distribution of bumble bees.

- In open field conditions the corbicular pollen gathered from homing bumblebees contained on average 22% of strawberry pollen and 1/3 of the foragers visited mostly or only strawberry during one foraging trip. This allows suggesting that bumble bees are effective strawberry pollinators and MCA disseminators.

- Beside strawberry the competing pollen in corbiculas originated from plant families *Rosaceae* and *Laminaceae,* in addition to those in different years the oilseed rape, white clover or lupines might affect strawberry pollination. The strawberry fields where the samples were gathered were surrounded by orchards and gardens which provided plentiful alternative food resources.
- The experiments show that *Osmia cornuta* can be used to collect powdery preparation at very high extent – 10^6 CFUs for each body part. This means that potentially each osmia-bee can transport several millions of potential inoculum cells to the visited flowers, even when the charge is reduced to the half of the initial one.
- During the primary dissemination there is high variability of CFUs on flowers due to the behavior of the bee. Still there is no significant variation between the first six flowers visited.
- The secondary dissemination by other insects visiting treated flowers improves the even distribution of BCAs.
- The data confirms that bee vectored BCA results in the sufficient numbers of CFUs on stamens which are the crucial part in flower to prevent the infection. Hence the spraying technique can be considered as wasteful.

WP 3	Other bees as vectors

Responsible partner: P3, EULS, WP manager Marika Mänd

Original description of work:

Objectives of WP3 are

- To determine the efficiency of bumble bees in vectoring BCA to strawberries, and in enhancing pollination, in the greenhouse and in open field cultivations
- To evaluate the specific requirements in the use of commercial bumble bees for targeted biocontrol and pollination on strawberries
- To assess the potential of using solitary bees (*Osmia* spp.) for targeted biocontrol and enhanced pollination in open field strawberry and pome fruit orchard situations

Task 3.1 Bumble bees as vectors in greenhouse conditions (UGHE)

3.1.1. Assessment of *Bombus terrestris* foraging

Study on the reliability of *B. terrestris* as vector will focus on their foraging behaviour under greenhouse conditions. Measured parameters include flight time, handling time, and number of flower visits per bee. All bumble bee workers will be labelled with opalith plates at the start of the experiment (before opening of the hive).

3.1.2. BCA dissemination capacity of *Bombus terrestris*

a) *Effect of distance to target flowers*: *B. terrestris* hives containing a queen, 20 workers and her brood, and the dispenser developed by Mommaerts et al. (2010) are used. Strawberry plants at various distances, with individually labelled flower buds are used for the test. Flowers and the bee are both individually collected after the first bumblebee visit. Plants placed at 5m-10m-15m-20m from the hive are assayed. 20 flowers/site are collected and analysed; the experiment is replicated twice.

b) *Effect of multiple bumblebee visits*: A similar experiment as above, but bumblebees are allowed to visit the flowers during the entire life-span of a flower. Thereafter all flowers are collected and the amount of BCA per flower is determined.

3.1.3. Efficacy in grey mould control

The efficacy of *B. terrestris* as vector to control *Botrytis cinerea* in the greenhouse is evaluated as under 3.1.2, using *Gliocladium catenulatum*. At each distance 20 plants with 100 labelled freshly opened flowers are inoculated with 2000 *B. cinerea* spores. Bee disseminated biocontrol is introduced at varying times, and the efficacy of control scored at two time points: at picking, and 2 days after picking (Mommaerts et al., 2011b).

Task 3.2 Bumble bees as vectors in open field conditions (EULS)

3.2.1. Management of bumble bee foraging

Commercially-produced bumble bee colonies (*B. terrestris*) are employed. Bee densities and colony locations in the strawberry field are manipulated to estimate the optimal number of colonies. Foraging distances are determined by recapturing marked individuals along transects, and by following individual bees. Foraging parameters (flight time, handling time, number of flowers visited) of individual bees are quantified in the open field. Foraging preferences are determined by sampling the pollen loads of returning foragers and the pollen stored in nests. Relative flower densities, reward availabilities and competition by other pollinators are analysed.

3.2.2. Dispersal distance and control effect of BCA

The number of BCA (*Trichoderma* and *Gliogladioum*) inocula on flowers is determined at varying distances from the hives. The proportion of healthy and infected fruits is calculated. Based on the experimental data and literature, a meta-analysis will be performed to evaluate the specific requirements for using commercial bumble bees for targeted biocontrol and pollination on strawberry.

Task 3.3 Solitary bees as vectors (ITACAA, UHEL)

3.3.1 Rearing and management of the solitary bees *Osmia cornuta* and *Osmia rufa*

Cocoons of *O. cornuta* and *O. rufa* will be overwintered under controlled conditions, and introduced slightly before initiation of flowering to a strawberry field and pear orchard, and manipulated to ensure the emergence of females and males at the onset of blooming. In May-June nesting tubes will be retrieved from the field. In September, cocoons of the new generation will be extracted and the reproductive success evaluated.

3.3.2. Efficacy of *Osmia* in delivering BCA from dispenser to target flowers (primary dissemination)

After the establishment of an *Osmia* population and the insertion of the dispenser, flowers at increasing distances from the nesting shelter (10, 50, 100, 200 m) and in the four directions (North, South, East, West) will be sampled and plated to evaluate the amount of BCA on the floral organs. Non-visited flowers from net-protected buds will act as negative controls. Sampling will be repeated three times during blooming.

3.3.3 Efficacy of *Osmia* in disseminating BCA from flower to flower (secondary dissemination)

The efficacy of pollinators in transferring the BCA from spray-inoculated flowers to the stigmas of newly opened ones will be determined at different time intervals after inoculation till the end of blooming. Flower samples will be collected and analyzed for the BCA; untreated and treated flowers will act as controls.

Report on results obtained and changes to the original plan/WP aims:

A- results obtained:

1. Bumblebees as vectors in greenhouse conditions

At UGHE the study was conducted in strawberry production greenhouses. Queen-right bumblebee hives equipped with special dispensers containing biofungicide Prestop-Mix were introduced to the study area. The *Botrytis* infection was measured in bumblebee vectored Prestop-Mix treated and untreated strawberries. In addition, the bumblebee effectiveness in depositing CFUs on flowers was studied. The most important results:

1.1. Assessment of *Bombus terrestris* foraging

The flying activity of bumble bees from different hives was homogeneous ($\alpha = 0.013$). The mean number of bumble bees moving in or out the hive varied between 11.0 and 25.3 bees per 30 minutes.

The foraging activity of *B. terrestris* in the greenhouse was the same during the 4 time intervals, which confirms the efficacy of *B. terrestris* as a pollinator and vector in the greenhouse. No significant differences ($p > 0.05$) in foraging intensity between nests with and without an empty built-in dispenser was detected, which indicates that the dispenser does not interfere with the foraging behaviour of the vector and does not induces abnormal behaviour (e.g. grooming). In order to be useful in the entomovector system the dispenser and MCA powder have to guarantee no impairment with the natural foraging behaviour when the dispenser is connected to the hive. A more simple design of a two-way dispenser seems to be most appropriate for use in the entomovector system so that no loss of foraging activity is observed. In addition it is of crucial importance that the powder formulation is safe for the vector (prevention of grooming behaviour etc.). The presence of the powder in the dispenser didn't had a significant effect on the in and out frequency of flying either ($p>0.05$) compared with the 5 nests with an unloaded dispenser, and this was the case for all time points (Figure1).

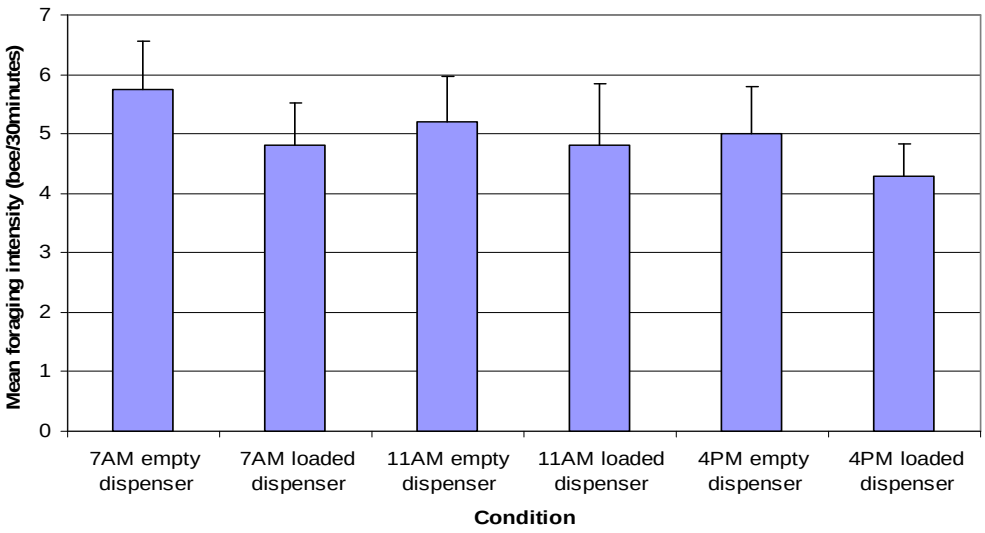


Figure 1. The mean foraging intensities (bees/30min) between a loaded and empty built-in dispenser over 3 different time intervals i.e. 7 AM, 11 AM and 4 PM. Data were analysed via an independent sample t-test ($p = 0.05$) and were not significant between 7 AM empty/loaded dispenser ($p = 0.387$), 11 AM empty/loaded dispenser ($p = 0.76$) and 4 PM empty/loaded dispenser ($p = 0.469$).

1.2. BCA dissemination capacity of *Bombus terrestris*

During first 60 s of the flight the bumblebees lost 81% of Prestop Mix they had achieved. The CFU/flower was found to be 23.4 ± 0.4, 14.7 ± 6.3 and 16.5 ± 2.0 for dissemination to the first, second and third visited flower respectively. This confirms that MCA is delivered in 3 consecutively visited flowers, wherein the numbers were highest in the first flower and then decreasing for the second and third flower visited. Though, this reduction was marginal ($p = 0.069$) and a relatively high amount of deposited spores was still achieved. Since flowers will be visited multiple times by different worker bees and over several days (Goulson 2010), it can be postulated that the amount of CFU/flowers will accumulate.

For the subsequent greenhouse experiment equal amounts of CFUs were recovered on the flowers in the 3 zones (zones 0–8 m, 8–18 m and 18–21 m) with the Mommaerts dispenser causing a mean deposition of 97.5 ± 41.3, 77.3 ± 15.4 and 105 ± 38 CFU/flower respectively. Though it should be noted that there were also zero values scored in each zone. The homogeneously spreading of the MCA powder formulation into the flowers with an amount of around 100 CFU/flower indicates that strawberry flowers can be protected over an area of 21m around the hive.

With the newly commercially available 'Flying doctors' dispenser also a homogeneous dissemination of the product was achieved (Figure 2), i.e. no significant differences ($p > 0.05$) between the 4 distances (0.5, 1, 2, 4m) was noticeable and this was the case after 1 day foraging and after 4 days foraging (8, 6, 6, 5 CFU/flower and 124, 123, 84, 148 CFU/flower respectively). After 4 days, the amount of MCA accumulated in the flowers because of consecutively visits. The results demonstrated that, with the use of the newly developed dispenser, bumblebee workers carried higher amounts of MCA which could be sufficient to suppress a medium to high infection pressure.

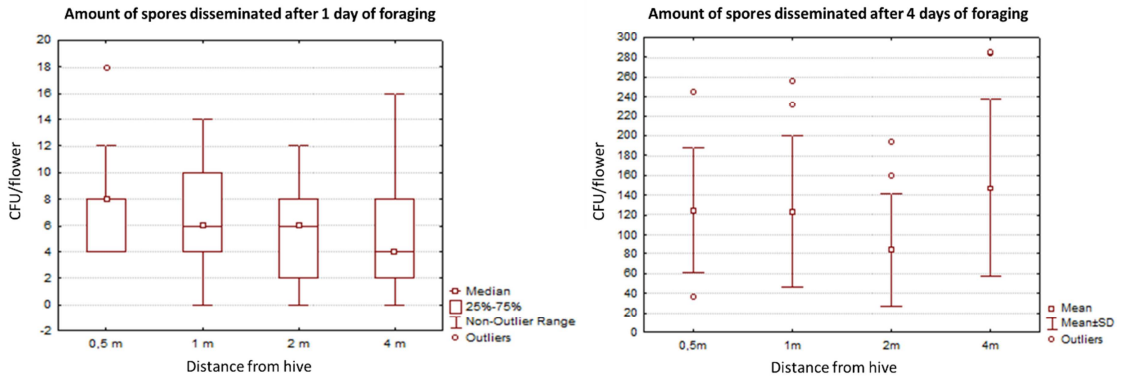


Figure 2. CFU/flower after respectively 1 day and 4 days of foraging at a distance of 0.5m, 1m, 2m and 4m from the hive ($p > 0.05$). No significant differences between the distances were found. The number of spores was significantly higher ($p > 0.001$) after 4 days of foraging.

1.3. Efficacy in grey mould control

Vectoring of Prestop-Mix by bumblebees resulted in a higher crop production, as 71% of the flowers developed into healthy red strawberries at picking (pre-harvest yield) as compared with 54% in the controls. In addition, these strawberries were better protected, as 79% of the picked berries remained free of *B. cinerea* after a 2 day incubation (post-harvest yield), while this percentage was only 43% in the control.

The number of flowers resulting in healthy fruits was highest for the treatment with Prestop-Mix and the one with Prestop-Mix + Maizena-Plus (Table 1). The mean pre-harvest yield was 72 ± 17% and 71 ± 9% respectively in contrast to 54 ± 21% and 51 ± 9% in T1 an T2 respectively. This means that the protection in the first 2 treatments were significantly lower. The same trend was observed for the post-harvest yield: T3 and T4 reached a mean post-harvest percentage of 67 ± 13% and 79 ± 17% respectively, while it was 43 ± 13% and 50 ± 10% for T1 and T2 respectively.

Considering total yield (pre-harvest x post-harvest yield), it was more than 2 times higher than the total yield in the controls. It can be concluded that strawberry fruits were better protected caused by satisfactory levels of MCA transport by the bumblebee workers into the flowers. Furthermore it should be noted that for T4 with the use of a carrier material only half the amount of Prestop-Mix was needed to obtain the same yields as T3.

Table 1. The mean percentages of pre-harvest (directly after picking) and post-harvest (incubated 48 h) yield of 4 different treatments with use of entomovector system in a strawberry greenhouse. Maizena-Plus was used as inert carrier.

Treatment	Yield % (Pre-harvest)	Yield % (Post-harvest)
T1 (Control)	54 ± 21	43 ± 13
T2 (Bees + Maizena-Plus)	51 ± 9	50 ± 10
T3 (Bees + Prestop-Mix)	72 ± 17	67 ± 13
T4 (Bees + Prestop-Mix + Maizena-Plus)	71 ± 9	79 ± 17

2. Bumblebees as vectors in open field conditions

At EULS, the study was conducted in two strawberry farms. Bumblebee hives with Prestop-Mix containing dispensers were placed near strawberry fields. Two treatments were established: bee-delivered Prestop-Mix treatment and untreated control. Healthy and *Botrytis*-infected berries were counted from treated vs untreated plots as well as at different distances from the

hives. The field data such as flower densities, nectar and pollen amount in strawberry flowers, flower visitors on strawberries etc. were collected. Pollen pellets from returning forager bumblebees were gathered and identified. The most important results:

2.1. Management of bumble bee foraging

a) Bee densities and colony locations

No of colonies on the field varied from 3 to 12 colonies per ha. Still, the number of bumble bees per 100 m transects was low. We saw no significant differences in the numbers of bumble bees counted per 100 m transects between the fields (p= 0.07).The average number of bumble bees counted per 10 m transect sections varied from 0.045 to 0.12. Bumble bees dispersed evenly over the field (2012: p=0.2; 2013: p=0.64; 2014: p=0.17). We are sure that these bumble bees observed on flowers belong to the hives we have placed in the fields since at that time of year no workers from wild populations of bumble bees from *B. terrestris* group are present yet.

Conclusion here: the triple hives positioned over the fields with the distance of 200 m from each other is enough to guarantee the even distribution of bumble bees.

b) The foraging parameters

The average flower handling time of *B. terrestris* was 4.8 ± 0.3 seconds. The time bumble bees need to find the next flower (3.9 ± 0.3 s) is significantly shorter than needed for handling the flowers (p = 0.006) (Figure 3). Bumble bees change often rows when foraging on fields. This is the reason why it is impossible to follow individual bees in open fields to track the length of foraging trip and how many flowers visited by one trip.

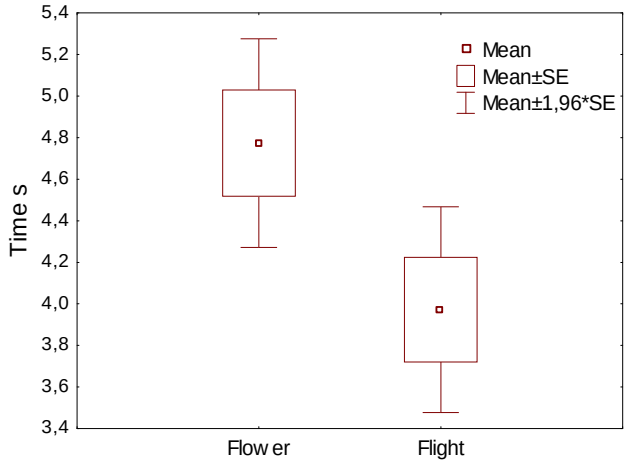


Figure 3. the mean time bumble bees spent in flowers or flying between flowers.

c) Bumble bee foraging preferences in open field

The corbicular pollen gathered by the bumble bees contained on the average 5.9 - 25.7% of strawberry pollen and 1/3 of the foragers visited mostly or only strawberry during a foraging trip. The strawberry pollen forage may vary significantly between the years (Kruskal-Wallis: X^2=14.8,df=2, p=0.0006) (Figure 4).

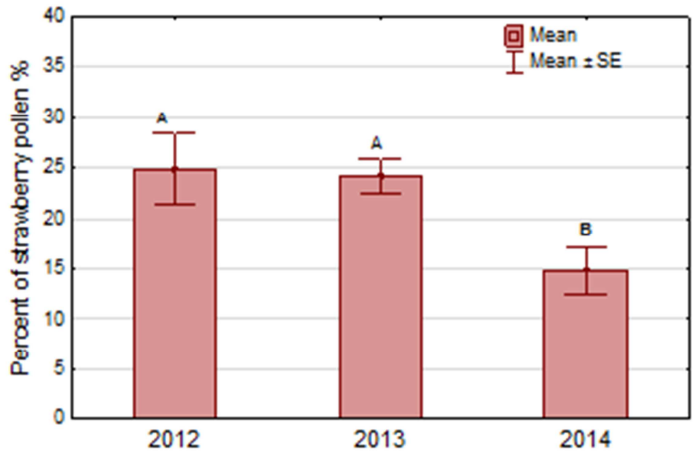


Figure 4. The average percentage of strawberry pollen in corbiculas of homing bumble bees in different years. The different letters upon the columns indicate statistically significant differences.

The amount of strawberry pollen gathered depends on the competing plant species flowering at the same time in the foraging distance of bumble bees. Bumble bees usually forage on several food plants simultaneously. About 1/3 of bumble bees foraged on more than one plant species. The most often visited plant species was *Lamium album* L. (Figure 5) which is a very common nectar-rich weed in Estonian agricultural landscapes and in vacant land. The flowering period of this species starts before and stops after the flowering of strawberry. The flowers of species belonging to the family Rosaceae are also attractive to bumble bees and these may compete with strawberry for pollination. The white clover *Trifolium repens* L. also may compete with strawberry, but this species flowers about a week later than strawberry does. White clover is very often grown between the rows of strawberry and the suggestion is to mow it periodically to control the competing effect of it. Oilseed rape is considered as very attractive forage plant for different bee species. Still when it was present in Rõhu 2014, it proved to be just slightly more attractive than strawberry, maybe because of the longer distance (400 m) to the closest oilseed rape field. Also *Lupinus polyphyllus* may attract bumble bees away from the strawberry. In Estonia this plant is growing in small patches here and there, but the flowering period is more variable and only in some years it overlaps the strawberry flowering.

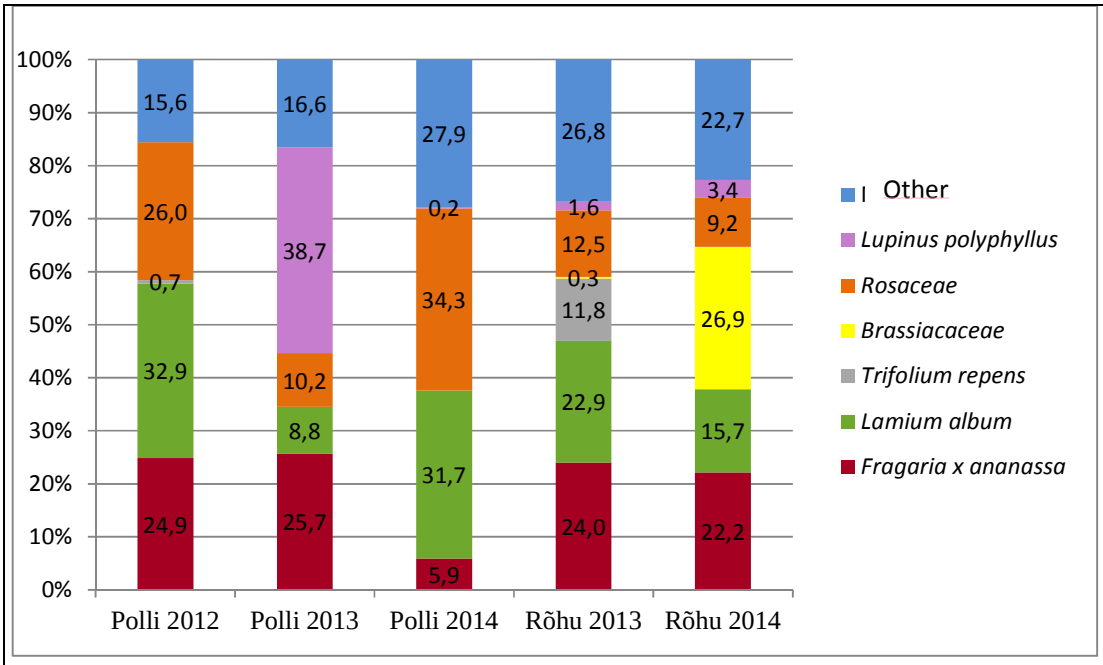


Figure 5. The proportions of different pollens gathered by bumble bees in three years and two experimental fields. Into the group "Other" are classified plant species which percentage did not exceed 5%

d) Competition by other pollinators, reward availabilities

Bumble bees formed 8% of all strawberry flower visitors counted on transects. Honey bees 29%, solitary bees 14%, syrphid flies 6%. 43 % of all flower visitors belong to groups that are not gathered as pollinators.

2.2 Control effect of BCA and dispersal distance

a) Control effect of BCA

The Prestop-Mix vectored by bumble bees decreased the Botrytis infection significantly in two years out of three (2012 and 2013) (Figure 6). In 2012 the percent of moulded berries was 3 times and 2013 2.2 times lower than in plots isolated from bees hence were without Prestop-Mix treatment. Still in 2014 the weather conditions favoured the *Botrytis* growth and there was no difference between treated or untreated plots.

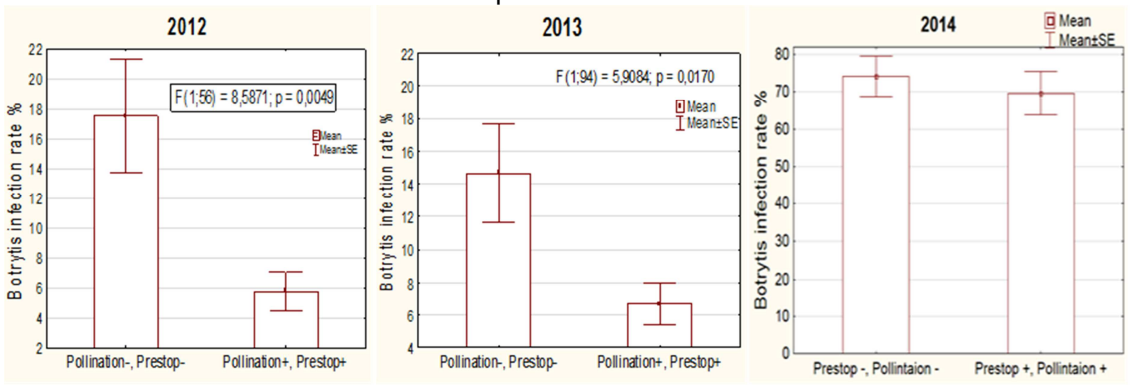


Figure 6. The mean percentage of Botrytis infected berries in three different years on plots treated or not treated with bumble bee vectored Prestop-Mix. Note the scale difference for the year 2014. Means and SE of means are presented on the figures.

The total yield per 12 plants was significantly higher in plots treated with Prestop-Mix (Figure Q). Still, in 2014 the Botrytis pressure extremely high and therefore no effect was observed also on yield.

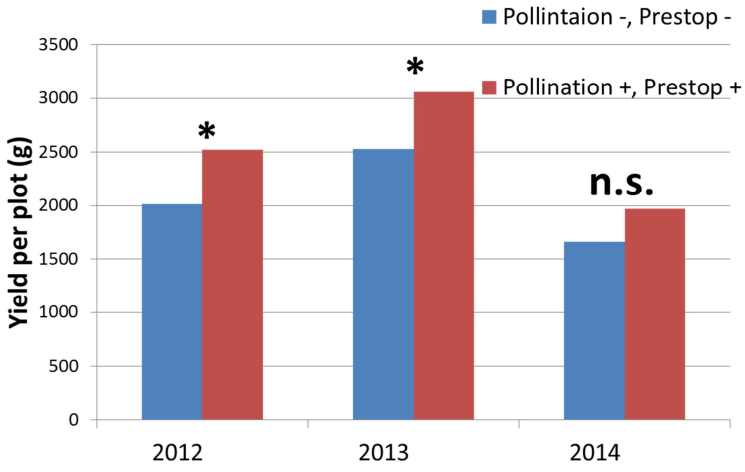


Figure Q. The average yield (g) per plot (12 plants) in different years.

b) The dispersal distance:

There were no differences in Botrytis infection rate in 0, 25, 50, 75 and 100 m from hives (Figure 7). Similar results were obtained in each year: 2012: P= 0.9, 2013: P=0.7 2014: p=0.1.

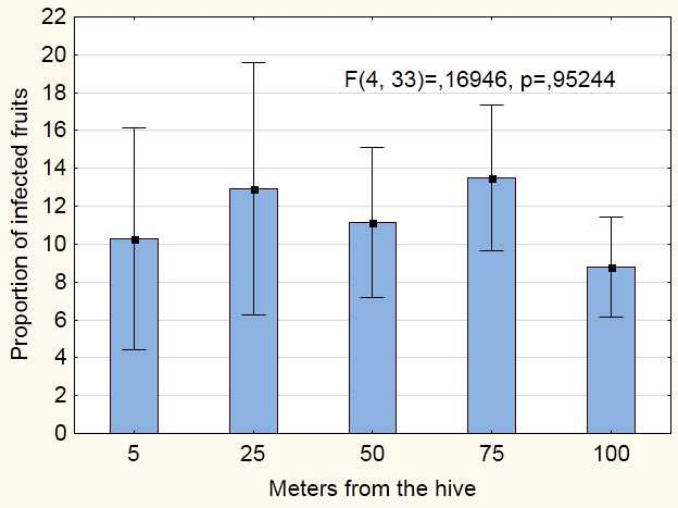


Figure 7. The proportion of infected berries

2. Solitary bees as vectors

3.1 Rearing and management of the solitary bees *Osmia cornuta* and *Osmia rufa*

At ITCAA, the rearing and management techniques were worked out. Cocoons of *O. cornuta* were overwintered under controlled conditions. Male and female cocoons were sexed on the base of their weight and dimensions, and two groups made of 50 males and 50 females were constituted. When the meteorological conditions turned favourable, males and females were brought gradually to 20°C under laboratory conditions for two days. It is important to synchronize the flowering of target crop and female emerging from cocoons. When the first males emerged from cocoons, the cocoons, protected in small polystyrene boxes with respiratory holes were brought to the pear orchard slightly before blooming initiation. At that moment, the nearby peach (*Prunus cerasus*) orchard was also starting flowering. In May-June

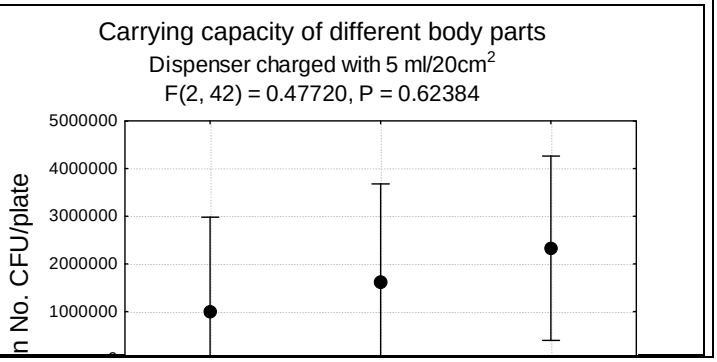
nesting tubes were retrieved from the field.

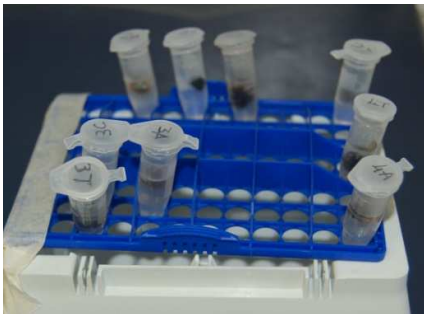
2.2. Efficacy of *Osmia* in delivering BCA from dispenser to target flowers (primary dissemination)

a) The BCA carrying capacity of *Osmia*

The experiments with *Osmia cornuta* conducted in 2012-13 showed that they can be used to collect powdery preparation at the exit of a shelter hosting the nesting materials, on which a dispenser is mounted.
The dispenser was loaded with 5 ml and 2.5 ml of Amylo-X® (Intrachem-Italia), a powdery biopreparation based on *Bacillus amyloliquefaciens* strain D747, containing 5x1010 CFU/g, an efficient antagonist of *Erwinia amylovora*, the causal agent of the pear fire blight. The powder was distributed at the base of the ramp through which the bees had to pass to get out, forming a layer of nearly 1 mm height. The layer width the bees had to walk through was around 1.5 cm for the first trial, and little narrower for the second trial.

Eight *Osmia cornuta* females exiting through the dispenser and crawling on the powdery preparation were captured, sacrified with ether, and their body was divided into three parts: head, thorax and abdomen. The body parts were treated according to a protocol for bringing into solution the bacterial cell attached to the hairs. Body parts were separately washed into an Eppendorf containing 1 ml solution of $MgSO_4$, and centrifuged for three minutes. A diluting series was performed till the 10^{-6} dilution: the dilutions 10^{-3} and 10^{-6} were used to inseminate Petri dishes containing a culture medium (Nutrient Agar) suitable for the development of *Bacillus amyloliquefaciens.* Plates were incubated at 36°C for 24 hours, then the number of developed colonies was counted. The results of both trials (dispenser charged with 5 or 2.5 ml of Amylox) showed that exiting bees loaded up the biocontrol agent at a very high extent – 10^6 CFU for each body part (head, thorax, abdomen). The statistical analysis (ANOVA) showed that the abdomen loaded up a significantly higher amount of CFU with respect to the head and the thorax (Figure 8).





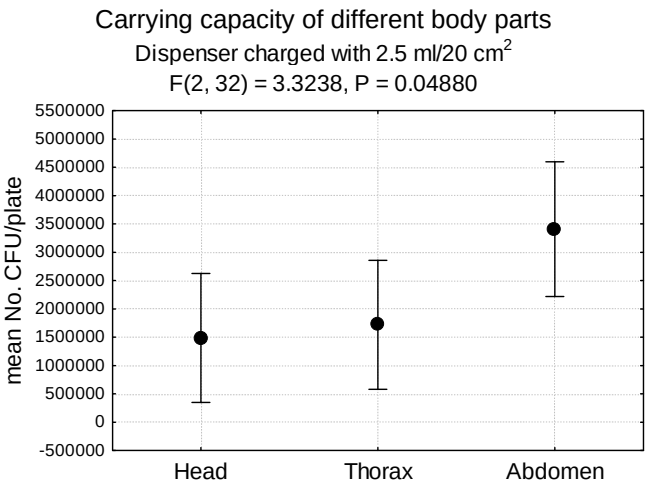


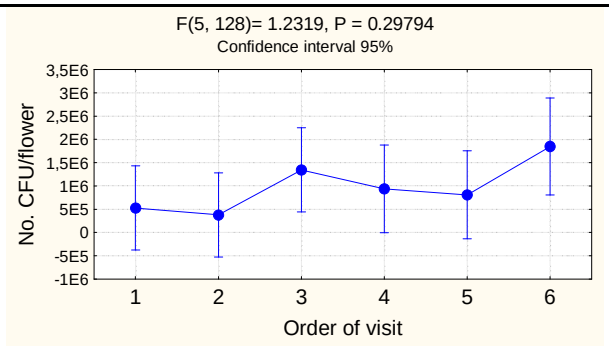
Figure 8. Carrying capacity of different body parts. Dispenser charged with 5 ml/20cm^2 and 2.5ml/20 cm^2 of Amylo-X® (Intrachem-Italia), a powdery biopreparation based on *Bacillus amyloliquefaciens* strain D747, containing 5x1010 CFU/g

This means that potentially each osmia-bee can transport several millions of potential inoculum cells to the visited flowers, even when the charge is reduced to the half of the initial one.

b) Primary dissemination

Trials were run under semifield conditions in order to assess the efficacy of *Osmia cornuta* in carrying to the flowers the inoculum of the antagonist loaded up by passing through the dispenser. A net plastic tunnel (10 x 5 x 3 m was built, and early flowering plant were introduced in pots (*Brassica napus*, *Viburnus album*, *Prunus spinosa*, *Ranunculus* sp.). A nesting shelter with nesting materials was prepared, and 15 *Osmia cornuta* males and females were released. After females started nesting activity a simplified model of dispenser was mounted in the nesting shelter. Pear plants in pots were introduced in the tunnel to get female used to forage on pear at a certain distance. The day before the trial, all the pots containing feeding plants were taken out of the tunnel, and only two pear plants were left. In the evening, all the nesting females were closed in their nest by putting pressed cotton to close the entrance hole. On the day of the trial, one pear plant whose flowers had been numbered was introduced in the tunnel as the sole feeding plant. The dispenser was charged with 5 ml of the biocontrol agent, and one female per time was let free to get out by passing through the dispenser. The behaviour of eight females was observed, and the sequence of the visited flowers on the plant was recorded.

The flowers were then cut and treated according to the same protocol described above for the bees, in order to assess the number of CFU of the biocontrol agent transported and deposited by the bees on the flowers, according to the sequence of the visit (Figure 9).



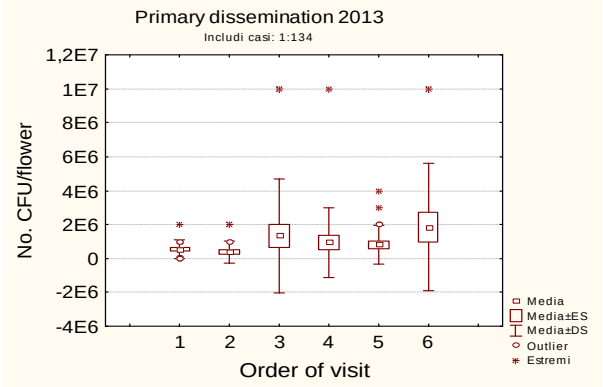


Figure 9. Efficiency of the secondary dissemination of BCA's.

The counting of the number of colonies developed in the plates showed that the amount of bacterial cells in the first 6 flowers were on average in the order of magnitude of 10^6. Data showed a very high variability due to the different behaviour of the bees. Behavioural differences concerned the way of exiting the dispenser (some of them walked on the top of the dispenser, avoiding the biopreparation at the bottom), and the kind of approach to the flowers, that in some cases was extremely rapid, probably because of the disturb created by the presence of the researchers.

3.3 Efficacy of *Osmia* in disseminating BCA

a) Secondary dissemination under semifield conditions

In 2013 trials were run under semifield conditions to assess the efficacy of *Osmia cornuta* to load up the inoculum from contaminated flowers and to transfer it to new flowers.

The experimental set up was the same of the trial described above for the primary dissemination. When the females were considered used to forage on pear plant in pots under the semifield conditions, all of them were closed inside their nests, in the evening. On the next morning, two new pear plants in pots were introduced in the tunnel and placed at 2 m from the nesting shelter. One plant was untreated and its flowers were numbered on the petals. Before being introduced in the tunnel, the other plant was sprayed with Amylox®, by using a manual device simulating the same pressure and volume used to spray under field conditions, at the same concentration indicated in the label. The untreated and treated plants were placed at 2 m from the nesting shelter and at a distance of 1m one from the other. One female per time was let free to forage on the pear plants, while two observers recorded its behaviour. The flowers visited on the untreated plant from a female coming from one flower visited on the treated plant were recorded and as soon as possible collected. The behaviour of 10 females was recorded. The untreated plants was substituted when too few flowers were remained or when it was not

possible to follow the sequence of the visits, and thus it was difficult to decide which flowers had been already visited.

Samples of flowers were collected also from the untreated plant before the visit of the bees to assess the natural presence of *B. amyloliquefaciens* or of other *Bacillus subitilis* strain whose shape and colour could be not distinguishable from those of the antagonist transferred by the bees.

All the flowers were treated according to the protocol described above in order to obtain dilutions of the washing solution to inseminate Petri dishes containing a medium suitable for *B. amyloliquefaciens* development. The secondarily inoculated flowers showed an amount of CFU of 10^4 magnitude order.

The countings of the colonies developed after 20-24 hours of incubation showed a statistically significant difference between the amount of CFU counted in the untreated plants before the visits of the bees and the flowers that received a visit of the bees that had previously visited the sprayed plant.

In fact the amount of antagonist-like bacterial cells on the untreated flowers was in the order of some tens of CFU (Figure 10).

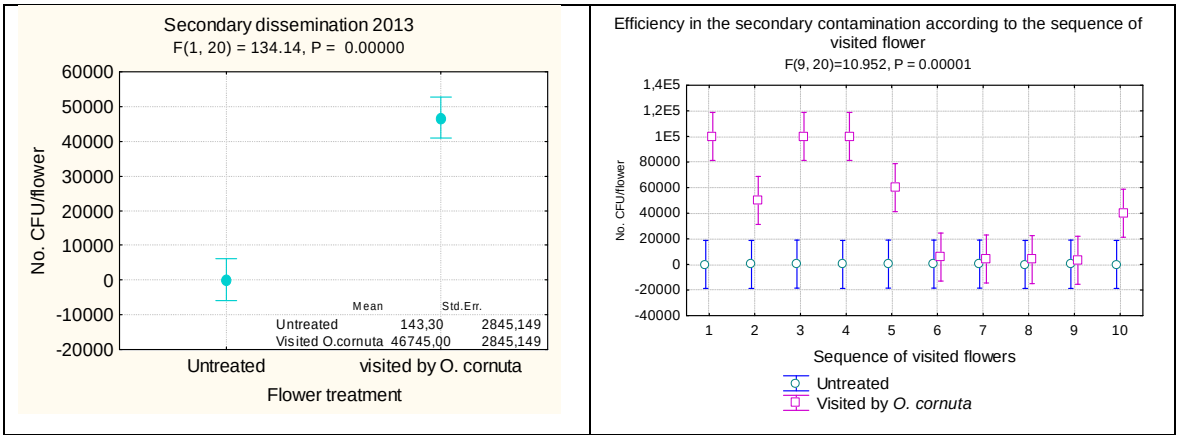


Figure 10. Efficiency of the secondary dissemination of BCA's.

b) Secondary dissemination under field conditions

In 2014 a field trial was run to assess the role of *Osmia cornuta* in the secondary dissemination of biocontrol agents in a pear orchard. At the beginning of march a population of *Osmia cornuta* was established in an orchard with two main varieties, 'Abate Fetél' and 'Max Red Bartlett'. The nesting shelter was placed at 15 m from a portion of the orchard with plants 12 years old, composed by 10 rows nearly100 m long, with 90 plants placed at a distance of 1.20 m one from each other. The *O. cornuta* release occurred 10 days before pear started flowering. When pear flowering reached the 50 % of opened blossoms, along the nearest 'Abate Fetél' row 30 branches on thirty plants with flowers were covered with plastic bags. Soon after, a spray treatment with Amylox®-(Intrachem Italia) was performed by using a portable pressurized pump. The dilution of the product was done according to the instructions of the label, and the volume of sprayed dilution was comparable to that one obtainable with the use of the normally used machinery for spray treatments. Three hours after the spray, the plastic bags were remove in order to let *O. cornuta* bees (and eventually other wild pollinators) visit all the flowers.

Three days after the spray, the flowers that in the meanwhile opened were collected, and separated per plant into sterile Petri, and brought to the laboratory. Here the flowers undergo a slightly different protocol for the isolation of the bacterial cells of the antagonist with respect to those described in other paragraph. The three flowers of the same plants were analysed together. Petals and sepals were detached from the rest of the flower by using sterilized

forceps. Sepals were eliminated while petals were placed in Eppendorf with 1 ml of $MgSO_4$ solution. The stamens, the pistils and the receptacle were placed in a different Eppendorf with 1 ml of $MgSO_4$ solution. Both samples were centrifuged for three minutes, and the washing solutions were directly used to inseminate Petri dishes containing Nutrient Agar. Then a dilution series was performed by taking 50 microliters of the petals+sepals washing solution and 50 microliters of the stamens+pistils+receptacles washing solution and placed them together in an Eppendorf containing 900 microliters of sterile water. The dilution series continued till the 10^{-6} dilution. The dilutions 10^{-3} and 10^{-6} were plated as described in other paragraph, and incubated at 36 °C. Picture of the plates were taken after 17, 20 and 24 hours, and the colonies developed in the medium were counted on the pictures.

Dilutions of the product were also performed to have at disposal the images of the biocontrol agent, and to estimate its concentration in the product. The dilution series was continued until the 10^{-12}, and the dilutions -6 and -12 were plated on Nutrient Agar in Petri dishes.

The efficacy of pollinators in secondary dissemination was assessed by comparing the number of CFU per flower in 30 flowers collected on thirty branches of different plants for each of the following conditions:

- Untreated flowers, collected just before the spray
- Sprayed flowers
- Secondarily inoculated flowers, protected by the plastic bags during the spray

The comparison (Mann-Whitney U test) between the numbers of CFU in the untreated flowers was statistically lower in comparison to the flowers exposed to the visit of pollinators, as reported in fig. 11.

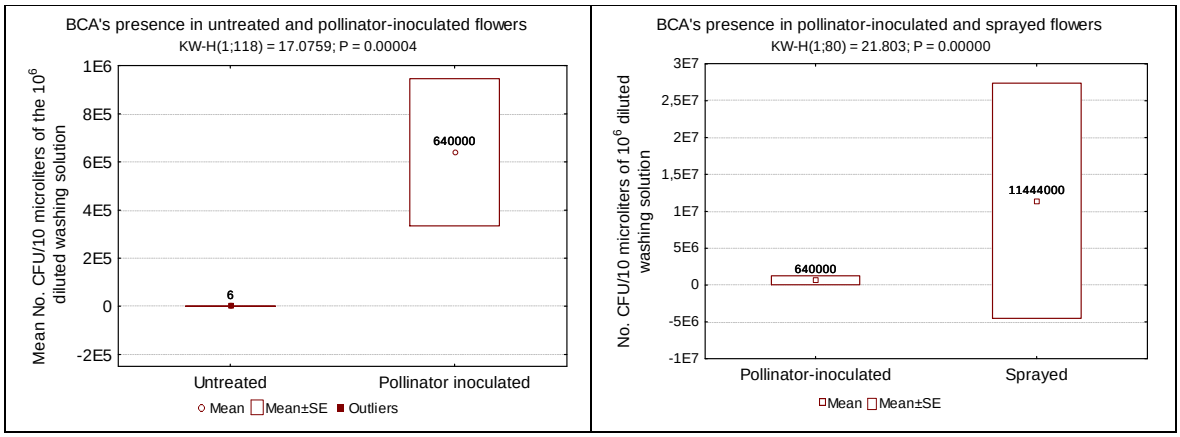


Figure 11. BCA's presence in untreated, pollinator-inoculated and sprayed flowers.

As expected, the sprayed flowers presented a significantly higher presence of the biocontrol agent with respect to the pollinator inoculated ones, being the order of magnitude of the CFU in the former was 10^6-10^7 while it reached the 10^5 order of magnitude in the latter

Interestingly, the statistical analysis (two-way ANOVA considering the treatment and the flower part as main factors, followed by the Tukey test for mean separation) showed that the presence of the biocontrol agent in the petals and stamens of the sprayed- and pollinator-inoculated flowers had a different distribution of the bacterial cells. In fact, in the sprayed flowers the presence of the biocontrol agent was the same in the washing solution obtained by the two matrixes, while in the pollinator-inoculated ones the there was a significantly higher number of CFU in the stamen and pistil's washing solution in comparison to the one obtained from the

Table 2. Tukey HSD test; comparison between the number of CFU in the petals and stamens
Approximate Probabilities for Post Hoc Tests Error: Between MS = 1743E2, df = 156.00

	Treatment	Flower part	{1} – 315.04	{2} – 801.29	{3} – 659.60	{4} – 777.0
1	Secondary dissemination	Petals		0.000045	0.002007	0.000017
2	Secondary dissemination	Stamens	0.000045		0.455966	0.994398
3	Spray	Petals	0.002007	0.455966		0.495424
4	Spray	Stamens	0.000017	0.994398	0.495424	

petals. The comparison between the number of CFU in the petals of sprayed and pollinator-inoculated flower did not put in evidence any difference between the presences of the bacterial agent on the stamens of the two matrixes (Table 2). This means that, despite of the higher concentration of the antagonist in the spray dilution used to treat the plant, the amount of active cell deposited on the crucial parts of the flower is not different from that one transported by *O. cornuta* + other pollinators, which loaded up the inoculum from previously sprayed flowers (Figure 12).

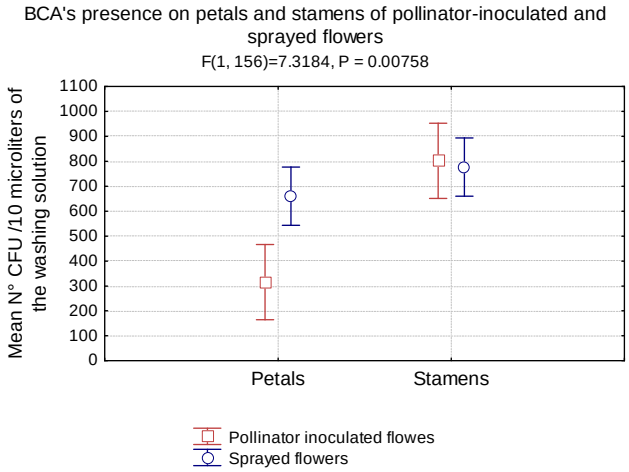


Figure 12. BCA's presence on petals and stamens of pollinator-inoculated and sprayed flowers.

These findings confirm that the delivery of the biocontrol agent is much better targeted to the reproductive parts of the flower, which are the most important ones for the prevention of the infection by *Erwinia amylovora*.

B- comments on deviations from the original plan:
No deviations necessary.