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BRESOV

Breeding for Resilient, Efficient and Sustainable Organic Vegetable production

Deliverable No. D4.2

Protocol and guidelines to maximize organic seed production and to control the sanitary quality of produced seeds in tomato, brassicas and snap bean

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PREFACE

With the goal to increase organically managed farmland to 25 % by 2030 together with the upcoming requirement to use exclusively organic seeds for organic farming, more seeds produced under organic conditions are needed on the market. Seed production under organic conditions is even more challenging than in conventional production systems, mainly due to the restricted access to regular products for fertilization and plant protection. To increase the number of healthy seeds, we tested different agronomic factors which could influence the number of seeds produced in several organic farms in Europe and provide recommendations on relevant factors in this deliverable. Organic farming prohibits the use of conventional chemicals to control pests and diseases, so alternative solutions such as natural compounds as well as mechanical treatments were evaluated on seed against seed-borne pathogens. The optimal protocols and treatments to control main seedborne pathogens affecting tomato, broccoli and snap bean are presented in this deliverable. The results are relevant for breeders, seed multipliers and farmers active in organic (Crenn *et al*., 2021).

PART 1: Protocol and guidelines to maximize organic seed production for broccoli, tomato and snap bean

1. INTRODUCTION

The objective of the protocols and guidelines are to maximise organic seed production for tomato, *brassica* and snap bean by providing seed producers with protocols and recommendations for relevant agronomic factors, which can influence the amount and quality of their seed production.

Seed producers aim to produce a high quantity and quality of seeds. This includes to produce a high number of seeds with high germination rates. In farming for seed production, production cycles are often longer than for production for feed or food. This increases the risk of production losses due to abiotic and biotic stresses. Especially in organic production systems, where the use of conventional pesticides and fungicides is prohibited, the challenges to produce large amounts of high-quality seeds are even higher.

Based on the input of stakeholders, the following six factors were identified and tested in three different climatic regions throughout Europe:

- Plant density (Tomato, *brassica* and snap bean)
- Use of microorganisms in combination with amino acids and foliar application of nutrients (Tomato, *brassica* and snap bean)
- Fruit harvesting regime (Tomato)
- Grafting on rootstocks (Tomato)
- *Rhizobium* symbiosis (Snap bean)
- Transplantation time (*Brassica*)

We assessed plant growth, appearance of abiotic and biotic stresses and measured seed number, Thousand Seed Weight (TSW) and seed germination rates of different varieties to determine the effect of these different agronomic practices on seed production.

2. HOW TO ASSESS THE IMPACT OF AGRONOMICAL FACTORS ON SEED YIELD AND QUALITY?

2.1. General methodology for field experiments on seed production

The goal of the BRESOV seed production field trials was to assess the effect of different agronomic factors on seed production in *brassica* (mainly broccoli and cauliflower), tomato and snap bean. We aimed at increasing the number of seeds produced, while maintaining a good quality and high level of seed germination rate.

The BRESOV trials were conducted in open field trials (all three crops), tunnel (*Brassica*, tomato) and greenhouse (tomato and *brassica*) in three different climatic regions in Europe: in Brittany, France, in Aargau, Switzerland, and in Sicily, Italy.

In general, the seedling or seeds were planted or sown in a randomized split plot design (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This means that the fields were divided into three blocks representing three repetitions. Within each block, the field was divided in three main plots representing the nutrition protocols (e.g., control, treatment 1 with a specific dose of microorganisms and treatment 2 with another dose of microorganisms = **main effect**). Within each main plot, we tested different genotypes (4-8 depending on the crop and trial = **split effect**). However, the genotypes within each repetition were randomized, which means that they were randomly mixed to reduce potential neighbouring effects. For example, the tomato trial conducted by P6-FiBL in Switzerland followed a randomized split block design with three repetitions (blocks) per genotype and treatment. Each repetition consisted of 12 plants per genotype, of which each six plants were assigned to the first treatment (frequently harvested) and 6 to the second treatment (less frequency harvested).

With this experimental setup in different regions, we were able to better distinguish the variation in seed production introduced by the agronomic practices, the different repetitions (which may be introduced by micro-climatic or different soil conditions within the field), the different experimental locations/years, the genotypes and their interaction effects.

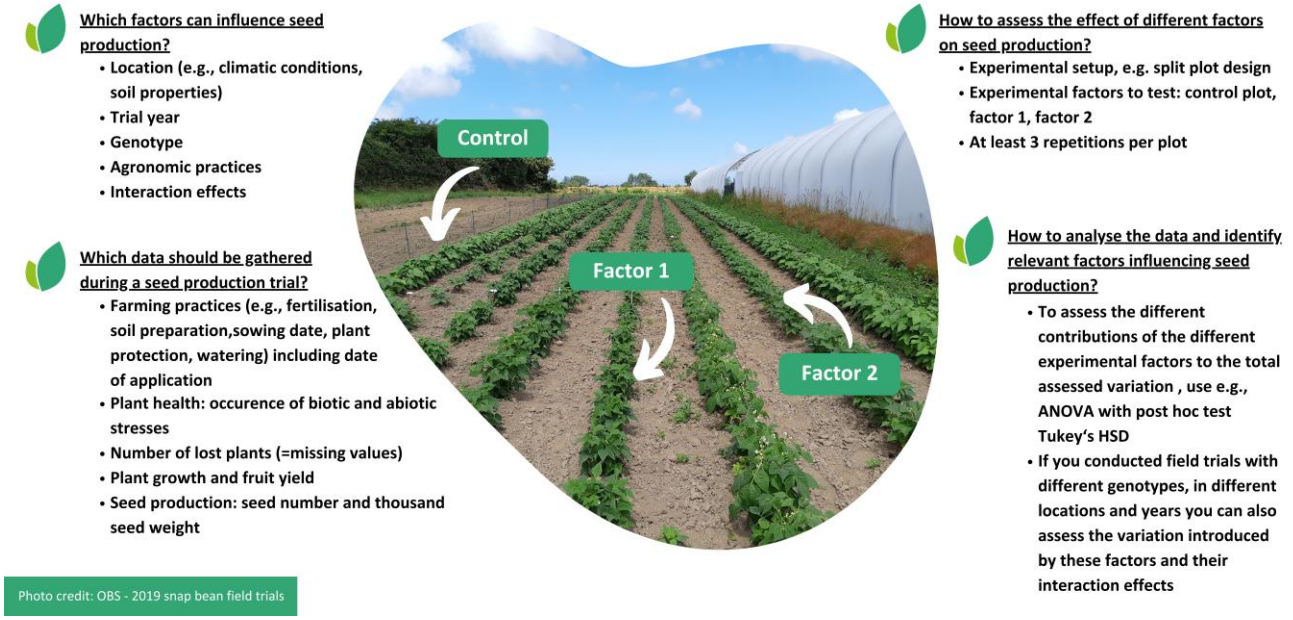


Fig. 1 General experimental setup and data assessment for seed production field trials.

2.2. Seed quantity assessment

To calculate the effect of different factors on seed production, it is important to assess the number of plants per plot (including e.g., the number of plants lost or not yielding seeds) and to collect dry pods (snap bean), siliques (broccoli) or ripe tomato fruits for seed extraction.

In the seed tomato trials, a subsample of about 1 kg of fruits was extracted by smashing the fruits and mixing with approximately 10 % water. Afterwards, the mixture was left for fermentation at 25 °C for three days, before the fruit flesh and other residues were washed off the seeds in a sieve. The seeds were dried on filter paper before counting (detailed method of tomato seed extraction and Schwitter *et al.* 2022).
For *brassica* and snap bean, the seeds were extracted from the dry pods and counted. Next to seed number, the Thousand Seed Weight (TSW) was measured. With the number of harvested plants, seed number and TSW, we extrapolated the medium number of seeds per plant (*Brassica* and snap bean) and the medium number of seeds of the first two bunches of tomato or over a specific harvest period.

2.3. Seed germination protocols for small seed lots on paper or on soil

High germination rates and normal development of germinated plants are criteria to assess the quality of seeds. The seed quality can be evaluated by seed germination tests. In the Council Directive 2002/55/EC of 13 June 2002 on the marketing of vegetable seed it is defined that cauliflower seeds being sold must exhibit at least 70 % germination rate, and at least 75% for broccoli, tomato and snap bean seeds.

There are two different ways of assessing seed germination at small scale, depending on availabilities of a growth chamber or greenhouse: on paper and on soil.

2.3.1. **Seed germination protocol on paper** (BRESOV practice abstract 5)

If it is possible to control the temperature and light in a growth chamber, seed testing on paper is ideal. 150 seeds were divided into three lots of fifty seeds each. Seeds were placed in three aluminum containers containing absorbent paper moistened with distilled water (Fig. 2). Seeds were covered by absorbent paper and the aluminum containers were placed in the dark at optimal temperature (~20 °C for *brassica* and snap bean, ~25 °C for tomato). Seedling assessment took place when the cotyledons appeared and one true leaf was developed. The number of normal and non-germinated plantlets were scored. Following standards of the International Seed Testing Association (ISTA), for tomato the final counting took place fourteen days after sowing, eight days for snap bean and ten days for *brassica*. Out of the three replicates, the average number of normal seedlings was calculated by adding the number of normal seedlings (NNSs) of each replicate and by dividing by three:

Average number of normal seedlings = $(NNS_{Rep1}+NNS_{Rep2}+NNS_{Rep3})/3$

The germination rate was calculated as follows:

Germination rate [%] = (Average number of normal seedlings/ Number of seeds sown) x 100

Fig. 2 Seed germination assessment on wet absorbent paper and broccoli germinated seedlings.

2.3.2. **Seed germination protocol on soil** (BRESOV practice abstract 13)

To test the seed germination rate of seed lots, a simple soil-based method can be performed as an alternative to the paper-based method (see practice abstract 5). Seeds were sown in 150 cell seedling starters with 50 seeds per replicate having three replicates per seed lot. To test different seed lots, the different replicates of each lot were placed in different seedling starters. The seedling starters were filled in with a soil suitable for small pots and with high drainage ability. One seed was placed in each pot and covered with soil. The seedling starters were watered by avoiding to float the seeds up. The temperature was kept at around 18 °C for *brassica*, 25 °C for tomatoes and snap beans and the soil moist. Seedling assessment and calculation of germination rates took place when the cotyledons appeared and one true leaf was developed as described in 2.3.1 (Fig. 3). Classification of germinated seedlings into the different category was done by following the protocol of the ISTA handbook of seedling evaluation specific to each crop type (ISTA, 2006).

Fig. 3 Exemplary pictures for normal (left plantlet in each picture) and abnormal (right plantlet in each picture) cauliflower seedlings.

3. SUMMARY OF THE SEED PRODUCTION TRIALS

3.1. Snap bean

The trials on snap bean were conducted over four years in two different climatic regions, Brittany in France and Sicily in Italy, on eight different genotypes. Agronomic factors tested were plant density (both sites), plant nutrition (both sites), *Rhizobium* inoculation (Brittany) and plant nutrition with additional foliar application (Sicily). TSW was highly influenced by the cultivar chosen, contributing to 71 % to the total variation assessed in the trials, while the agronomic factor contributed to 12 % of the variation. The seed number per sqm was mainly influenced by the agronomic factor tested, which contributed to 20 % of the total variation.

3.1.1. Multi-site trials: plant density and plant nutrition

In Brittany and Sicily, three different plant densities and different doses of microorganism application in combination with nutrient were tested.

Seed production was significantly higher per sqm for plants with the highest chosen plant density of 23.8 plants/sqm (Brittany) and with the medium density in Sicily (Rizzo *et al*., 2023). Therefore, we recommend to transplant snap beans for seed production in Brittany in the following planting scheme: 5 plantlets per spot (bulk) with distance within rows of 0.30 m and distance between rows of 0.70 m, which allows mechanical weed control. Under the climatic and soil conditions of Sicily, plants grown with 14.3 plants/sqm yielded best results, also with regard to germination rate. With 14.3. plants/sqm, 5 plantlets were sown in bulks with a distance within rows of 0.5 m and between rows of 0.7 m.

Treatment with microorganisms and micronutrients did significantly increase snap bean seed production by application of D50%: Product applications were done at indicated BBCH stages for snap bean (BBCH stages snap bean, Feller *et al.*, 1995). We applied two different products of ITAKA Crop Solutions: 3KO® containing *Trichodermus* (*T. Arzianum, T. asperellum, T. atroviride*) and *mycorrhiza* (*Glomus mosseae, G. intraradices)* and ACE®, which supplied nutrients and amino acids to the plants (Tab. 1).

Tab. 1 Application scheme for application of microorganisms and amino acids in snap bean.

Snap Bean		D50%	
BBCH stage	Number of Applications	Product name: 3KO	Product name: ACE
9	1	2 kg/ha	32 kg/ha
15 to 19	1	1 kg/ha	16 kg/ha
25 to 29	1	1 kg/ha	16 kg/ha
51 to 59	1	0.5 kg/ha	8 kg/ha
71 to 75	1	0.5 kg/ha	8 kg/ha
	Total:	5 kg/ha	80 kg/ha

3.1.2. Single-site trial: *Rhizobium* inoculation

Seed production was significantly increased when seeds were inoculated with *Rhizobium* prior sowing in Brittany. Legumes benefit from a symbiosis with naturally occurring soil-borne bacteria, which fix nitrogen present in the air for plants to use. With seed inoculation, an increase in seed production by about 40 % was achieved. We tested RhizoFix® RF-60, Feldsaaten Freudenberger on snap beans. The product is ready to use and should be applied right before sowing. The product was evenly poured (or sprayed) onto the seed and then the treated seeds were well mixed before storage in a cool dark place (BRESOV Practice abstract 10 and BRESOV Practice Abstract 11). The recommended agronomic practices to increase seed yield in snap bean are presented in Table 2.

Tab. 2 Recommended agronomic practices for snap bean.

Recommended agronomic factor Snap bean	Single-site (S)/ multi-location (M) trial	Best results within BRESOV experiments
Plant density	M (France, Italy)	Brittany: 23.8 plants/sqm (5 plantlets per spot (bulk) with distance within rows of 0.30 m and distance between rows of 0.70 m) Sicily: 14.3 plants/sqm (5 plantlets are sown in bulks with a

		distance within rows of 0.5 m and between rows of 0.7 m) CAVE: Seed germination rates (nutrition protocol and Rhizobium inoculation can have beneficial effect)
Microorganism application in combination with amino acids	M (France, Italy)	Sicily: D50% (3KO, ACE, ITAKA srl)
Rhizobium symbiosis	S (France)	Seed treatment with RhizoFix® RF-60, Feldsaaten Freudenberger

3.2. Tomato

The trials on tomato were conducted over four years in three different climatic regions, France, Switzerland and Italy, on four to eight different genotypes. Determinate plants were grown in the field in Sicily, while indeterminate plants were grown in the greenhouse in all three different sites. Agronomic factors tested were plant density (France and Italy), plant nutrition (France and Italy), different harvesting regimes (Switzerland), grafting (France) and plant nutrition with foliar application (Italy). In the French and Italian trials, cultivars contributed to 37 % variation of TSW taken trials from the two regions together. The agronomic factor tested contributed over all to 5 % of the total variation. The seed number per sqm was mainly influenced by the cultivar, which contributed with 36 %. The agronomic factor contributed with 10 % and the interaction effect between cultivars and agronomic factor with 23 %. The detailed results of the Swiss trial are available in the annexed publication (Schwitter *et al.*, 2022), and similarly to the other trials, genotypes were a significant factor for total fruit yield and TSW. Frequent harvest resulted in 25 % more fruit and therefore total seed yield.

3.2.1. Multi-site trials: plant density and plant nutrition

In indeterminate tomatoes grown in greenhouse, higher plant densities showed to decrease the number of seeds harvested per plant, but taken the higher number of plants per sqm into account, increased total seed number per sqm. This is why we recommend to increase the plant density to 5 plants/sqm. We transplanted the plants with a distance between rows of 0.5 m and within rows of 0.2 m. Each plant was grown with a single stem.

Although the total contribution of the agronomic factor to the variation of TSW was low, the application of D100%+F increased TSW in comparison to D50% (Malgioglio *et al.,* 2021).

3.2.2. Single-site trials: grafting and harvesting regime

Grafting increased yield and seed production in our trial in France, regardless of variety and rootstock tested (Floury *et al.*, 2022). No difference was established between rootstocks. Grafting increased seed production by about 60 %. Increase in seed production by grafting was related to the increase in yield, i.e., number of fruit and fruit weight, rather than the number of seeds per kg of fruit which was reduced by grafting (BRESOV practice abstract 9).

Different fruit harvesting frequencies were applied to test their effect on seed quality and germination rate. We applied two different harvesting regimes: In freq 1, mature fruits were regularly harvested twice a week, counted and weighed to assess fruit production. After three weeks, seeds from mature fruits harvested on extraction day were extracted. In freq 2, fruits with a mixed ripe maturity level were harvested after three weeks on extraction day only, counted, weighed and seeds were extracted. Seed production differed between genotypes and extraction time-points. Different harvesting procedures, and with that different fruit maturity levels, did not affect TSW and seed germination. Additionally, cool storage of tomato fruits prior to seed extraction was tested. Cooling fruits for 2-3 weeks before extraction did not affect germination rate negatively. The findings allow seed producers to choose based on their needs to rather harvest the fruits frequently or in bulk without compromising seed quantity or quality (for detailed information see the publication on the effect of tomato harvesting frequency, Schwitter *et al.* 2022, BRESOV Practice abstract 14, and Herforth-Rahmé, Joelle & Patricia Schwitter (2023) as well as Schwitter P. & Herforth-Rahmé J. (2023) publications in farmers' and Swiss agricultural research listed in the reference)).

The recommended agronomic practices to increase seed yield in tomato are presented in Table 3.

Tab. 3 Recommended agronomic practices for tomato.

Recommended agronomic factor Tomato	Single-site (S)/ multi-location (M) trial	Best results within BRESOV experiments
Plant density	M (France, Italy)	Greenhouse, indeterminate production: 5 plants/sqm (distance between rows of 0.5 m and within rows of 0.2 m. Each plant was grown with a single stem).
Microorganism application in combination with amino acids/ and foliar nutrition	M (France, Italy)	Sicily: D100%+F (3KO, ACE, ST02213, Micro7213, ITAKA srl)
Different harvesting regimes	S (Switzerland)	Regular harvesting or bulk harvests every three weeks do not influence TSW or seed germination. Both can be used based on seed producer's needs.
Cooling fruit before seed extraction	S (Switzerland)	Cooling tomato fruits for 2-3 weeks before seed extraction did not affect germination rate negatively and can save on number of extractions by pooling several harvests together.
Grafting	S (France)	Grafting increases fruit production and with that seed production. Commercially available rootstock comparable to tested BRESOV rootstock.

3.3. *Brassica*

The trials on *brassica* were conducted over four years in two different climatic regions, Brittany in France and Sicily in Italy, on four to eight different genotypes. Agronomic factors tested were plant density (Brittany and Sicily), plant nutrition (Brittany and Sicily) and plant nutrition with foliar application (Sicily). The cultivar contributed to 45 % variation of TSW taken all experiments together. In addition, the cultivar x treatment 10 % cultivar x year 6 %, agronomic factor tested contributed over all to 6 % and the location to 6 % to of the total variation. In contrast to snap bean and tomato, the seed number per sqm was also mainly influenced by the cultivar x year interaction (42 %) and the cultivar chosen, which contributed by 39 %. The agronomic factor tested contributed with 4 % and cultivar x treatment with 7 %. A considerable contribution to the total variation was not represented by the other factors taken into consideration (Year, location and their interaction effects with cultivars and agronomic factor tested). Therefore, cultivar choice is a highly relevant factor for increasing seed production especially for *brassica*.

3.3.1. Multi-site trial: plant density and plant nutrition

In Sicily, the experimental trials conducted during the five years, concerned to individuate the best sowing date or transplanting date. Especially for the sprouting type and for the biannual landraces /cultivars of broccoli the sowing or the transplant from the end of June to the beginning of August, permit a good vegetative growth of the plant and then a good establishment of the inflorescence and then consequently a good seed production. In relation to the best plant crop density varying from 4 plants sqm for sprouting types and Mediterranean landraces and 6 plant sqm for the apical dominance types, the nutrition protocols by amino acids and microbial consortia improve the seed yield and germinability. In Brittany, the highest plant density with 6 plants per sqm yielded the best results. Therefore, we recommend a planting density of 0.5 m distance between rows and 0.3 m distance within rows. The application of microorganisms did not yield in a significant difference in seed production in Brittany as for the other crops. Similarly, to the results of other crops, the application of microorganisms showed significant differences in the trials conducted in Sicily (Detterbeck *et al.*, 2021).

3.3.2. Single-site trial: plant nutrition and foliar application and transplantation time

Application of microorganisms and micronutrients in combination with foliar nutrient application increased plant vigour seed production in trials in Sicily (Treccarichi *et al.*, 2022). Plants were grown at a density of 4 plants/sqm with a basic organic fertilization of 140 kg/ha N, 123 kg/ha P, 105 kg/ha K. Product applications were done at indicated BBCH stages for *brassica* (BBCH stages *brassica*, Feller et al., 1995). Four different products from ITAKA Crop Solutions were applied: 3KO® containing *Trichodermus* (*T. Arzianum, T. asperellum, T. atroviride*) and *mycorrhiza* (*Glomus mosseae, G. intraradices*), ACE®, which supplies nutrients and amino acids to the plants, ST02213® (foliar amino acids and plant extracts) and Micro7213® (microelements) (Tab. 4).

Tab. 4 Application scheme for application of microorganisms, amino acids and foliar nutrients in *brassica*.

Brassica			**Product application and doses (ITAKA Crop Solutions)**		
BBCH stage	N° Applications	3KO	ACE	ST02213	Micro7213
12 to 19 (transplantation)	1	3 kg/ha	48 kg/ha	/	/
39	1	3 kg/ha	48 kg/ha	5 kg/ha	3 kg/ha
49	1	2 kg/ha	32 kg/ha	5 kg/ha	4 kg/ha
59	1	1 kg/ha	16 kg/ha	5 kg/ha	4 kg/ha
69	1	1 kg/ha	16 kg/ha	5 kg/ha	4 kg/ha
	TOTAL:	10 kg/ha	160 kg/ha	20 kg/ha	15 kg/ha

The trial conducted in Brittany with different transplantation time-points (3 in total, each two weeks apart in autumn) did not significantly affect seed production.
The recommended agronomic practices to increase seed yield in *brassica* are presented in Table 5.

Tab. 5 Recommended agronomic practices for *brassica.*

Recommended agronomic factor *brassica*	**Single-site (S)/ multi-location (M) trial**	**Best results within BRESOV experiments**
Plant density	S (France)	6 plants/sqm (0.5 m distance between rows and 0.3 m distance within rows).
Microorganism application in combination with amino acids and foliar nutrition	S (France, Italy)	Sicily: D100%+F (3KO, ACE, ST02213, Micro7213, ITAKA srl)

4. GERMINATION RESULTS

Taken all snap bean trials together, the most prevalent factor contributing to 30 % of total assessed variation in germination rate was the interaction between genotype and agronomic factor. The agronomic practice tested contributed with 19 %. Overall, the germination rates in snap bean trials were sufficiently high to meet the germination rates for marketing. However, in the trial with the highest plant density led to lower seed germination rates in Sicily. We therefore recommend the medium density for seed production

in Sicily. In addition, the treatment with microorganisms and foliar application could have a beneficial effect on germination rates based on our data.

In tomato, due to the assessed data, the variation introduced by the location and/or the agronomic factor (40 % in total) could not be distinguished. However, we hypothesize that the high temperatures present in the Sicilian trials could have had a negative effect on seed germination rate, why it is recommended to prevent high temperatures in tomato seed production.

Regarding contribution to total assessed variation in germination rate in *brassica*, the cultivar x location contributed with 24 % and the agronomic factor chosen with 22 %. Overall, seed germination rate was sufficiently high in all *brassica* trials conducted and met 75 % of germination rate, which is needed for marketing of seeds in the EU.

5. RECOMMENDED AGRONOMICAL FACTORS TO INCREASE SEED PRODUCTION IN ORGANIC

To increase seed production in *brassica*, tomato and snap bean we identified several influencing factors. In our trials, the TSW was mainly determined by the choice of cultivar in all the three crops. In tomato and *brassica*, variation in seed number per sqm was also mainly influenced by genotype and interaction effects between the genotype and agronomic practice (tomato) and year (*brassica*). In snap bean, the variation was evenly distributed between different factors analysed. Therefore, we recommend to choose cultivars suited to the specific climatic conditions for ideal seed production.

Overall, the agronomic practices contributed to around 4-22 % of the total variation, depending on crop and characters identified. In all three crops, different planting densities had a significant effect on seed production. Here, we can recommend the medium to highest densities that were tested for the three crops. Although the seed production per plant was reduced for some crops at the highest densities, the seed production per sqm was nevertheless significantly higher compared to the lower densities.
In tomato, harvesting regime as well as fruit cooling had no influence on seed production and quality except for the fact that regularly harvested plants produce more fruits. We therefore recommend to follow the strategy that suits best to the producer needs: small seed production of different varieties can benefit from regular harvest and storage of fruits for a pooled extraction to save time and resources, while larger producers can harvest every three weeks and extract the seeds after removing obviously bad fruits.

Some agronomic factors were tested at a single location only, but positively influenced seed production. This included inoculation with *Rhizobium* in snap bean and grafting in tomato. In general, we recommend to increase the productivity and vigour of the plants. Healthier, more vigorous plants produce more fruits and with that seed production is increased.

Application of microorganisms, amino acids and foliar application showed positive effects in Sicily in contrast to the trials conducted in France. We hypothesize that this could either be due to the different climatic conditions, due to different soil conditions (marginal soil in Sicily in comparison to long cultivated organic, well-nourished soils in Brittany) or due to a combination of these effects. Another hypothesis is that the strains used in ITAKA products are adapted to the Sicilian region and are therefore more effective there.

6. DEVIATIONS

Due to extreme weather conditions (e.g., heat) in Sicily, partially bad soil conditions and pathogen damages, some trials did not yield good number and healthy seeds. Although most trials were planned in a replicated form between different locations (e.g., density trials), some results are only presented for single locations. The data of the stressed plants was not included in the analysis of the data presented in this deliverable.

PART 2: Protocol and guidelines to control the sanitary quality of organically produced seeds in tomato, broccoli and snap bean

1. INTRODUCTION

The general objective of the work described here was to determine the tools and products to control the sanitary quality of organic seed lots. Organic farming prohibits the use of conventional chemicals to control pests and diseases, so alternative natural compounds as well as mechanical treatments were evaluated on seed for protection against seed-borne pathogens. The review of the detection tools for main seed-borne pathogens of tomato, broccoli and beans (3-5 pathogens per crop) and of the available seed treatments that are applicable in organic seed production (D4.1) was used as a basis to further investigate methods and products to control seed pathogens under organic conditions such as Biocontrol Agents (BCAs) and Natural Compounds (NCs).
Experiments were shared among partners as presented in Table 6. The results are presented in this deliverable D4.2. Protocols with recommendations will be made accessible to the stakeholders, in collaboration with WP6.

Tab. 6 Plant species, seed borne pathogens, activity and involved BRESOV partners.

Species	Seed-borne pathogen*	Pathogen detection tool **	Seed treatment product evaluation
Tomato	*Clavibacter michiganensis subsp. michiganensis*	UNICT (v)	UNICT
	Pseudomonas syringae pv. tomato	UNICT (v)	UNICT
	Xanthomonas euvesicatoria pv. perforans	UNICT (v)	UNICT
	ToMV	VEG (v)	-
	Fusarium oxysporum f.sp. radicis	ITAKA (v)	ITAKA

	lycopersici		
Broccoli	*Xanthomonas campestris pv. campestris*	VEG (d)	VEG
	Alternaria spp	VEG (d)	ITAKA
	Phoma lingam (Leptosphaeria maculans)	ITAKA (v)	ITAKA
Snap bean	*Colletotrichum lindemuthianum*	FiBL (v)	FiBL
	Pseudomonas savastanoi pv. phaseolicola	FiBL (v)	FiBL
	Fusarium solani f.sp phaseoli	ITAKA (v)	ITAKA
*same as in D4.1		**validation (v) development (d)	

2. METHODS FOR IDENTIFICATION AND/OR DETECTION AND QUANTIFICATION OF SEED BORNE PATHOGENS

In deliverable D4.1 a review of the detection tools available for the main seed-borne pathogens of the three crops was reported. Partners decided to validate existing methods or develop methods within the project. The choice of the method was based on available official protocols and scientific literature. The methods were either molecular-based methods (PCR-based -real time PCR- conventional PCR-RT PCR) or methods based on *in vitro* culture with molecular identification. Each partner used the most suitable detection protocols for their respective pathosystem of study. General information and methods are reported in tables 7 and 8.

Tab. 7 Pathogen detection methods used for tomato, broccoli and snap bean seed.

Species	Seedborne pathogen	Identification/ detection method	Reference
Tomato	*Clavibacter michiganensis subsp. michiganensis*	Real-time PCR	https://worldseed.org/wp-content/uploads/2017/07/Tomato_Cmm_July2017.pdf
	Pseudomonas syringae pv. tomato	Real-time PCR	Peňázová *et al.*, 2020
	Xanthomonas spp.	Real-time PCR	https://worldseed.org/wp-content/uploads/2022/01/2017_Protocol_Tomato_Xanthomonas_spp_v5.pdf
	ToMV	Real-time PCR	Boben *et al.*, 2007
	Fusarium	PCR	Hirano and Arie, 2006

	oxysporum f. sp. radicis lycopersici		
Broccoli	*Xanthomonas campestris pv. campestris*	Real-time PCR	ISF
	Alternaria spp.	Real-time PCR	Iacomi-Vasilescu *et al.*, 2002; Guillemette & Simoneau 2004
	Phoma lingam (Leptosphaeria maculans)	PCR	Liu *et al.*, 2006; Fernando *et al.*, 2016
Snap bean	*Colletotrichum lindemuthianum*	Real-time PCR, ISTA germination/grow-out	ISTA
	Pseudomonas savastanoi pv. phaseolicola	Real-time PCR, ISTA method (plating, suspension, number of pathogen propagules (cfu))	ISTA
	Fusarium solani f. sp. phaseoli	PCR	Arif *et al.*, 2012

Tab. 8 Oligonucleotides and probes used for pathogen diagnosis and detection.

Target	Primer and probes (P)	Sequence (5'-3')	Target region
Clavibacter michigansis subsp. michiganensis	MVS21 F	CTAGTTGCTGAATCCACCCAG	not known
	MVS21 R	TACCGCTTGACTCTCGTTTC	
	MVS21 P	FAM CTGCCACCCGATGTTGTTCC TAMRA	
Xanthomonas spp.	Primer XDF	TCGACGGCACCTTCGACTACG	XopD
	Primer XDR	CTGGAGCTTGCTCCGCTTGG	
	Probe XDFAM	FAM-CCTCATCAGGGATCGTCTTGCCCCA AG C BHQ1	
Pseudomonas syringae pv. tomato	PST-hrpL_e_fwd	TTTCAACATGCCAGCAAACC	hrpL
	PST-hrpL_e_rev	GATGCCCCTCTACCTGATGA'	
	PST-hrpL_TP	FAM -	

		GCTGAACCTGATCCGCAATCAC - BHQ1	
Colletotrichum lindemuthianum	ClF432	GGA GCC TCC TTT GCG TAG TAAC	ITS region
	ClR533	ACC TGA TCC GAG GTC AAC CTT GTT	
Pseudomonas savastanoi pv. phaseolicola	SSRP_F	GACGTCCCGCGAATAGCAATAATC	Phaseolotoxin gene
	SSRP_R	CAACGCCGGCGCAATGTCG	
Fusarium solani f. sp. solani	Tef-Fs4sf	ATCGGCCACGTCGAC TC	translation *elong ation factor* 1-alpha
	TEF-Fs4r	GGCGTCTGTTGATTGTTAGC	
TOMV	ToMV-F	TTGCCGTGGTGGTGTGAGT	
	ToMV-R	GACCCCAGTGTGGCTTCGT	
Fusarium oxysporum f.sp. radicis lycopersici	Sprl–F	GAT GGT GGA ACG GTA TGA CC	
	Sprl–R	CCA TCA CAC AAG AAC ACA GGA	
Xanthomonas campestris pv. campestris	XCC-F	GTGCATAGGCCACGATGTTG	Detection *of X. campestris pv. campestris* only
	XCC-R	CGGATGCAGAGCGTCTTACA	
	XCC-Pr	/56-FAM/ CAA GCG ATG TAC TGC GGC CGT G /3IABkFQ/	
	DLH153-F	GTAATTGATACCGCACTGCAA	Detection of *X. campestris pv. campestris* and *X. campestris pv. raphani*
	DLH154-R	CACCGCTCCAGCCATATT	
	P7	/5SUN/ATGCCGGCGAGTTTCCACG/ 3IABkFQ/	
Alternaria brassicicola	Abra1	AAGGCGAGTCTCCAGCAAACTG	
	Abra2	ACTCACCTCAGCAGCATCTGCTGT	
Alternaria brassicae	115 sens	AACCCTATAGACCCACGTCGACTA	
	115 rev	GATGGTACGCAAGGCTTGGT	
Leptoshaeria maculans	LmacF	CTTGCCCACCAATTGG-ATCCCCTA	ribosomal RNA region
	LmacR	GCAAAATGTGCTGCGCTCCAGG	

3. RESULTS

3.1. Pathogen detection protocols

3.1.1. Tomato pathogens

3.1.1.1. *Clavibacter michiganensis subsp. michiganensis*

A real-time PCR molecular assay was performed to quantify the DNA of *C. michiganensis subsp. michiganensis* (Cmm). Bacterial DNA was extracted from overnight (ON) culture in Nutrient Broth 10^8 CFU ml^{-1}. For seedlings or seed DNA extraction samples were homogenized in extraction bags. All DNA extractions were performed using a C-TAB protocol. The detection of *Cmm* by qPCR was based on primers and probe described on the International Seed Federation (ISF) protocol. In order to quantify the DNA in the samples standard curves were performed by serial dilutions in water and plant DNA. The reaction was carried out using the QuantiNova Probe PCR Kit (QIAGEN) Master Mix, in a volume of 15 µl. Primers were added at a concentration of 0.5 µM while the probe had a concentration of 0.25 µM. The reaction protocol included a hot start of 2 minutes at 95 °C, 40 cycles from 95 °C for 15 seconds and from 60 °C for 30 seconds.

The results showed good amplification efficiency and correlation index, and a discrete sensitivity for the pathogen, approximately from 10 ng to 1 pg of DNA corresponding approximately from 10^8 to 10^3 CFU/ml. Standard curves also showed that plant DNA did not interfere with the DNA amplification of the pathogen (Fig. 4).

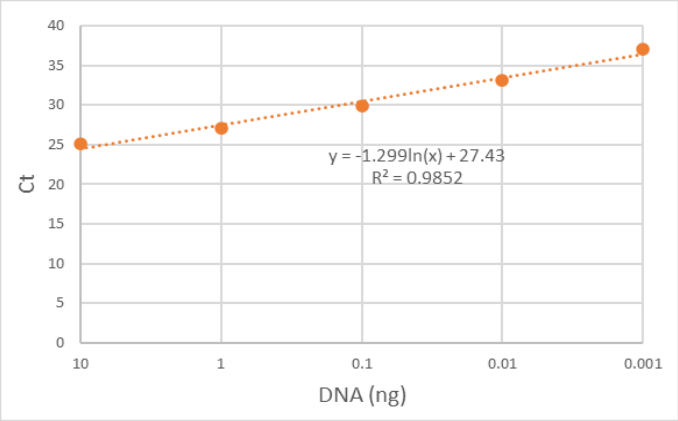


Fig. 4 Amplification efficiency and correlation index for *Clavibacter michiganensis subsp. michiganensis* detection.

3.1.1.2. *Pseudomonas syringae pv. tomato*

A real time PCR molecular assay was performed to quantify the DNA of *P. syringae pv*. tomato (Pst). Bacterial DNA was extracted from ON culture in Nutrient Broth 10^8 CFU ml^{-1}. For seedlings or seed DNA extraction samples were homogenized in extraction bags. All DNA extractions were performed using a C-TAB protocol. The detection of Pst by qPCR was based on a published protocol (Tab.7 and **Fehler! Verweisquelle konnte nicht gefunden werden.**). The protocol was originally based on a multiplex assay for the detection of tomato bacterial pathogens. Since the protocols of the other two pathogens were already validated at international level, we only worked on Pst protocol. In order to quantify the DNA in the

samples standard curves were performed by serial dilutions in water and plant DNA. The reaction was carried out using the QuantiNova Probe PCR Kit (QIAGEN) Master Mix, in a volume of 15 µl. Primers were added at a concentration of 0.6 µM while the probe had a concentration of 0.3 µM. The reaction protocol included a hot start of 2 minutes at 95 °C, 40 cycles at 95 °C for 15 seconds and at 65 °C for 30 seconds.

The results were not satisfactory as the fluorescence of the control was always very low whatever the primer/probe concentration and the amplification protocol. However, the sensitivity for the pathogen was approximately from 10 ng to 10 pg of DNA corresponding from 10^8 to 10^4 CFU/ml, standard curves showed also that plant DNA did not interfere with the DNA amplification of the pathogen (Fig. 5).

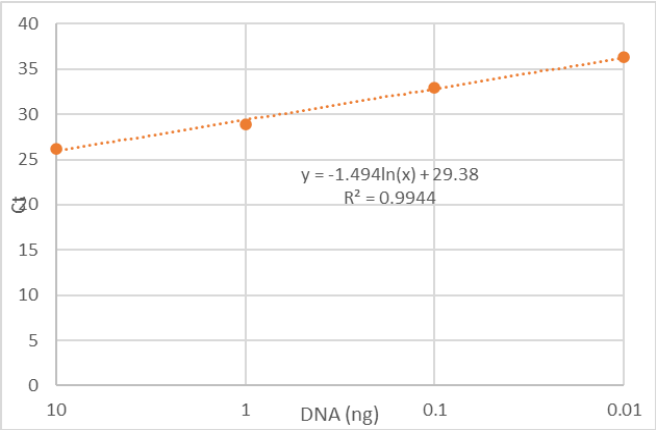


Fig. 5 Amplification efficiency and correlation index for *Pseudomonas syringae pv.* tomato detection.

3.1.1.3. *Xanthomonas euvesicatoria pv. perforans*

A real time PCR molecular assay was performed to quantify the DNA of *Xanthomonas euvesicatoria pv. perforans* (Xep). Bacterial DNA was extracted from ON culture in Nutrient Broth 10^8 CFU ml^{-1}. For seedlings or seed DNA extraction samples were homogenized in extraction bags. All DNA extractions were performed using a C-TAB protocol. The detection of Xep by qPCR was based on primers and probe designed for XopD gene (**Fehler! Verweisquelle konnte nicht gefunden werden.**) as described on the International Seed Federation (ISF) protocol for the detection of *Xanthomonas* spp. pathogen on tomato. In order to quantify the DNA in the samples standard curves were performed by serial dilutions in water and plant DNA. The reaction was carried out using the QuantiNova Probe PCR Kit (QIAGEN) Master Mix, in a volume of 15 µl. Primers were added at a concentration of 0.4 µM while the probe had a concentration of 0.4 µM. The reaction protocol included a hot start of 2 minutes at 95 °C, 40 cycles from 95 °C for 15 seconds and from 60 °C for 15 seconds.

The results showed good amplification efficiency and correlation index, and a discrete sensitivity for the pathogen, approximately from 10 ng to 1 pg of DNA corresponding from 10^8 to 10^3 CFU/ml. Standard curves also showed that plant DNA did not interfere with the DNA amplification of the pathogen (Fig. 6).

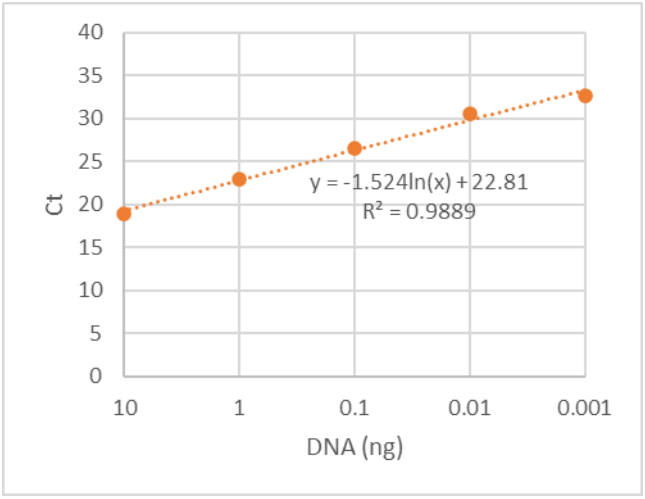


Fig. 6 Amplification efficiency and correlation index for *Xanthomonas euvesicatoria pv. perforans* detection.

3.1.1.4. Tomato mosaic virus (ToMV)

For artificial contamination of seeds, seeds were incubated during 1 hour and 30 minutes in a concentrated inoculum of ToMV (Cp=9 for the inoculum), and dried on filter paper. A mixture of 1 (or more) contaminated seed was mixed with 1000 seeds to prepare the 1:1000 to 10:1000 mixtures.
Seeds were washed in 10 mL of buffer composed of 0.9 % NaCl and 0.02 % Tween20 during 2h 30 with agitation. Supernatants were lyophilized and stored at -80 °C until extraction.

The first step of the protocol consisted in extracting RNA (Nucleospin RNA plus, Macherey Nagel), according to the manufacturer instructions. In a second step, cDNA was synthesized by reverse transcription with a SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer instructions after RNA denaturation.
RNA denaturation: Mix 1 was prepared in a total volume of 20 µL with 10 µL RNA sample, 1 µL of Oligo(dT)15 (500 µL/mL) and 1 µL dNTP (10 mM of each). Mix 1 was incubated at 65 °C during 5 minutes, and kept on ice until next step.
Reverse transcription: Mix 2 was prepared in a total volume of 20 µL with 12 µL of Mix 1, 4 µL of 5X First-Strand Buffer, 0,1 M DTT and Ultra-pure autoclaved water. Mix 2 was incubated at 42 °C during 2 minutes, and kept on ice until next step. Then, 1 µL of Superscript II RT was added to Mix 2 and incubated at 42 °C during 50 minutes and 70 °C during 15 minutes. In a third step real-time PCRs was performed in 8 µL reaction volume with 5 µL Master Mix Takyon (Eurogentec), 0.1 µL of each primer at 10 µM (ToMV-F and ToMV-R, as described in **Fehler! Verweisquelle konnte nicht gefunden werden.**), 2 µL of cDNA and 2.8 µL of MilliQ water.
Real-time PCR conditions were as follows: Initial denaturation at 94 °C for 3 min, 45 cycle of amplification (94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 1 min). Melting curves indicates a Tm of 82,3 °C.

With this protocol, detection in seed lots is possible in mixture of 1:1000 infected:healthy seeds.

3.1.1.5. *Fusarium oxysporum f.sp. radicis lycopersici*

Total genomic DNA was extracted from 100 mg of fresh mycelium from a 7-days *Fusarium* culture in PDA using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada) according to manufacturer protocol. The DNA was eluted in 50 µl of buffer of the Kit, quantified by using NanoDrop 1000 spectrophotometer (Thermo Scientific) and finally stored at –20 °C until PCR amplification. A primer pair based on pgx4 gene was used to obtain an amplicon of 941 pb from pathogen (Hirano and Arie, 2016). PCR reaction contained 1X GoTaq® G2 Colorless Master Mix (Promega), 0.4 mM of each primer and 1 µL of template in a total volume of 25 µL. Reactions were performed in a thermal cycler (Multigene Optimax, Labnet International, Inc.) with the following protocols: DNA denaturation for 5 min at 94 °C, amplification (30 cycles) at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 10 min.

3.1.2. Broccoli pathogens

3.1.2.1. *Xanthomonas campestris pv. campestris*

The first step of the protocol consisted in extracting DNA (Nucleospin Food, Macherey Nagel), according to the manufacturer instructions.
In a second step, real-time PCRs was performed in 8 µL reaction volume with 5 µL Master Mix Takyon (Eurogentec), 0.1 µL of each primer at 10 µM (XCC-F, XCC-R and XCC-Pr; DLH153-F, DLH154-R, P7; respectively for detection of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* or *Xanthomonas campestris* pv. *campestris*, as described in Table 8), 2 µL of DNA and 2.8 µL of MilliQ water. Real-time PCR conditions were as follows: Initial denaturation at 94 °C for 3 min, 45 cycles of amplification (94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 1 min). For interpretation of the data, please refer to **Fehler! Verweisquelle konnte nicht gefunden werden.**.

Tab. 9 Interpretation of Real-time PCR signal detection.

XCC F/R/Pr	DLH153/154/P7	Interpretation
+	+	Detection of ***Xanthomonas campestris pv. campestris***
-	+	Detection of ***Xanthomonas campestris pv. raphani***

Limit of quantification has been tested with two different strains of *Xanthomonas campestris* pv. *campestris*, and vary from Cp=32 to Cp=23, corresponding to 1.10^2 to 1.10^3 cells/mL.

With this protocol, detection in seed lots is possible in a mixture of 1:1000 infected:healthy seeds.

3.1.2.2. *Alternaria spp.*

For artificial incubation of seeds, seeds were incubated during 1h and 30 minutes in a concentrated inoculum of *Alternaria brassicicola* or *Alternaria brassicae* (2.106 spores/mL), and dried on filter paper. A mixture of 1 (or more) contaminated seed was mixed with 1000 seeds to prepare the 1:1000 to 10:1000 mixtures. Seeds were washed in 10 mL of buffer composed of 0.9 % NaCl and 0.02 % Tween20 during 2h30 with agitation. Supernatants were lyophilized and stored at -80°C until extraction.
A protocol for detection of *Alternaria brassicicola* or *A. brassicae* using Real-Time PCR is proposed:
The first step of the protocol consists in extracting DNA (Nucleospin Food, Macherey Nagel), according to the manufacturer instructions. In a second step, real-time PCR is performed in 8 µL reaction volume with 5 µL Master Mix Takyon (Eurogentec), 0.1 µL of each primer at 10 µM (Abra1 and Abra2; 115 sens and 115 rev; depending the species targeted, as described in Table 8), 2 µL of DNA and 2.8 µL of MilliQ water. Real-time PCR conditions are as follows: Initial denaturation at 94 °C for 3 min, 45 cycle of amplification (94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 1 min). For *A. brassicicola*, melting curves indicated a Tm of 84.8 °C. Limit of quantification is Cp=29, corresponding to 2,102 spores/mL. For *A. brassicae*, melting curves indicated a Tm of 84,9 °C. Limit of quantification was Cp=32, corresponding to 1,3.101 spores/mL.

With this protocol, detection in seed lots is possible in mixture of 1:1000 infected:healthy seeds.

3.1.2.3. *Phoma lingam (Leptosphaeria maculans)*

Total genomic DNA was extracted from 100 mg of fresh mycelium from a 10- days *Fusarium* culture in PDA or from broccoli seeds using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada) according to manufacturer protocol. The DNA was eluted in 50 µl of buffer of the Kit, quantified by using NanoDrop 1000 spectrophotometer (Thermo Scientific) and finally stored at –20°C until PCR amplification. A primer pair based on ribosomal RNA region was used to obtain an amplicon of 331 pb (Liu *et al.*, 2006; Fernando *et al.*, 2016). PCR reaction contained 1X GoTaq® G2 Colorless Master Mix (Promega), 25 pmol of LmacF 5' and 50 pmol of LmacR, and 1 µL of template in a total volume of 25 µL. Reactions were performed in a thermal cycler (Multigene Optimax, Labnet International, Inc.) with the following protocols: DNA denaturation for 2 min at 95 °C, amplification (30 cycles) at 95 °C for 15 sec, 6 8°C for 30 sec, and 72 °C for 60 sec, and final extension at 72 °C for 10 min. The 331 pb PCR product was analysed by agarose gel electrophoresis in 1.0 % (wt/vol) agarose at 90 V for 50 min. An amplicon of 331 pb was amplified from DNA of *Leptoshaeria maculans* CBS260.94. Detection limit of the assay tested on 10-fold serial dilution of DNA of the fungus on total seed DNA (from 50 ng to 50 fg) was 50 pg μL^{-1} (Fig. 7).

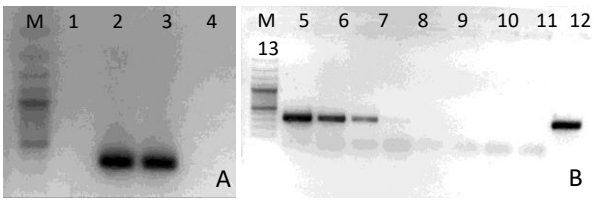


Fig. 7 PCR product of 331 bp was obtained from *Leptoshaeria maculans* CBS260.94. (A) Sensitivity assay: the detection limit was 50pg μl^{-1} (B) M, Thermo Scientific Gene Ruler 100 pb Plus DNA ladder, 1 empty; 2,3 DNA of *L. maculans*; 4, negative control; 5, 50ng μl^{-1}; 6, 5ng μl^{-1}; 7, 500pg μl^{-1}; 8, 50 pg μl^{-1}; 5pg μl^{-1}; 9, 500fg μl^{-1}; 10, 50 fg μl^{-1}; 10, 5 fg μl^{-1}; 11, negative control; 12 positive control *L. maculans.*

3.1.3. Snap bean pathogens

3.1.3.1. *Colletotrichum lindemuthianum*

A molecular detection method described by Gadaga *et al.* (2018) was tested using the pure strain, symptomatic leaves, healthy leaves, and naturally infected seeds. It was extremely difficult to detect the pathogen on the seeds. Therefore, DNA extraction from the seeds had to be further developed. Finally, the pathogen detection on the seeds was accomplished through a Quick-DNA Plant/Seed DNA MiniPrep kit (Zymo Research, Irvine CA, USA) with reduced sample amount and increased lysis level.
The ISTA grow-out method for the detection of *C. lindemuthianum* (7-006: Detection of *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* (bean) seed) in bean seeds was used to determine the infection rate of seeds. More information on the application and validation steps of these methods is found in Gamper Cardinali, Carlo (2022), Gamper Cardinali, Carlo and Herforth-Rahmé, Joelle (2023) publications and conference poster, listed in the references section.

3.1.3.2. *Pseudomonas savastanoi pv. phaseolicola*

The molecular detection on seeds was done with DNA isolated through a Quick-DNA Plant/Seed DNA MiniPrep kit (Zymo Research, Irvine CA, USA) in a reduced sample amount (i.e., small powder sample from well homogenized mealed seed(s)), and the set of primers described by Seok Cho *et al.* (2010).

3.1.3.3. *Fusarium solani f. sp. phaseoli*

Total genomic DNA was extracted from 100 mg of fresh mycelium of a 10- days *Fusarium* culture in PDA or from snap beans seeds using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada) according to manufacturer protocol. The DNA was eluted in 50 µl of buffer of the Kit, quantified by using NanoDrop 1000 spectrophotometer (Thermo Scientific) and finally stored at –20 °C until PCR amplification. A set of specific PCR primer pairs for *Fusarium solani* was used to amplify a 658 pb of translation elongation factor 1-alpha TEF-1α (Arif *et al.*, 2012).

PCR reaction contained 1X GoTaq® G2 Colorless Master Mix (Promega), 400 µM of each primer, and 1 µL of template in a total volume of 25 µL. Reactions were performed in a thermal cycler (Multigene Optimax, Labnet International, Inc.) with the following protocols: DNA denaturation for 5 min at 94 °C, amplification (40 cycles) at 94 °C for 60 sec, 58 °C for 60 sec, and 72°C for 120 sec, and final extension at 72 °C for 10 min. The PCR product was analyzed by agarose gel electrophoresis in 1.0 % (wt/vol) agarose at 90 V for 50 min.
An amplicon of 658 pb was amplified from DNA of *F. solani f. sp. phaseol*i CBS 265.50. Detection limit of serial dilution of fungal DNA were carried out in seed DNA to obtain a final concentration ranging from 50 ng to 5 pg. The protocol was able to detect the pathogen up to 500 pg μL^{-1}.

3.2. Seed product treatment evaluation

ITAKA provided six seed treatment products based on growth promoting microorganisms and natural compounds; two other natural treatments available in organic farming were also tested (Tab. 10). UNICT proposed a trialling plan for all three crops with timelines. The trials were set up under controlled

conditions. Each partner was free to use more genotypes according to the availability (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

Tab. 10 Microbial based and natural products used for seed coating.

BRESOV CODE	Treatment	Composition	Dilution
1	KONCIA XP191EV	*Bacillus subtilis, B. megaterium, Pseudomonas lurida, Glomus spp.*	1:10
2	KONCIA KMS1943	*B. subtilis, B. megaterium, B. amyloliquefaciens, P. fluorescens, P. putida, Streptomyces griseus, P. lydicus, Trichoderma arzianum, T. asperellum, T. atroviride, Glomus spp.*	1:10
3	KONCIA KSK1967	*Streptomyces griseus, P. fluorescens, P. chlororaphis, Glomus spp*	1:10
4	KONCIA KFC1980*	*B. subtilis, B. megaterium, Azotobacter vinelandii, Glomus spp*	1:10
5	CH193EV	ChitosaN based	1:100
6	CR192EV	Mustard oil-glucosinolates and propolis	1:100
NA	Warm water	Physical treatment, soaking seeds in warm water at 50°C for 10 min.	NA
NA	Acetic acid	Organic acid; Soaking seeds in acetic acid at 5% for 30 minutes	5:100

Tab. 11 Commercial cultivars and genotypes of tomato, broccoli and snap bean used in the trials.

Species	CV/genotype	Source	Partner
Tomato	cv San Marzano Nano	Bioseme s.c.a.r.l.	UNICT, ITAKA
	cv Rio Fuego	Bioseme s.c.a.r.l.	UNICT, ITAKA
	BT10210	UNICT	UNICT, ITAKA
	BT10050	UNICT	UNICT, ITAKA
Broccoli	cv Natalino	Bioseme s.c.a.r.l.	ITAKA
	cv Rasmus	BRESOV ITAKA	ITAKA
	Gentleman	ITAKA	ITAKA
	Sparaceddu	UNICT	ITAKA
	CNBRO09	UNICT	ITAKA

	Pollux	CAILLARD	Vegenov
	Samson	SAKATA SEED	Vegenov
	Steel	SEMINIS	Vegenov
	Larsson	RIJK ZWAAN	Vegenov
	BH86-1	UNICT	Vegenov
	BR354	UNICT	Vegenov
	BR320	UNICT	Vegenov
	BR325 Ramoso calabrese	OBS	Vegenov
Snap bean	cv Ferrari Mangiatutto	Bioseme s.c.a.r.l.	ITAKA
	cv Marconi - Grano bianco	Bioseme s.c.a.r.l.	ITAKA
	SBP302	UNIVPM	ITAKA
	SBP303	UNIVPM	ITAKA
	SBP307	UNIVPM	ITAKA
	Purple Teepee	Bingenheimer Saatgut AG	FiBL
	La Victoire	Sativa Rheinau AG	FiBL
	Maxi	Bingenheimer Saatgut AG	FiBL
	Helda	Bingenheimer Saatgut AG	FiBL
	Borlotto Mercato OL	Sativa Rheinau AG	FiBL
	Canadian Wonder	Sativa Rheinau AG	FiBL

3.2.1. Tomato

3.2.1.1. Bacterial pathogens

Experimental method and design. Trials on tomato were conducted over three cycles by using organic seeds of different cultivars/genotypes as reported in **Fehler! Verweisquelle konnte nicht gefunden werden.**. The data come from independent trials.

Bacterial inoculum was produced from the three seed bacterial tomato pathogens according to Table 6: *Xanthomonas euvesicatoria* pv. *perforans* strain NCPPB 4321 (Xep), *Pseudomonas syringae pv*. tomato strain PVCT28.3.1 (Psto), *Clavibacter michiganensis* subsp. *michiganensis* NCPB 2973 (*Cmm*). For the preparation of the bacterial inoculum single colonies from 48 h cultures either of Xep, Psto or *Cmm* on nutrient glucose agar NAG were transferred to Luria Broth (LB) medium. After approximately 24 h incubation at 28 °C under shaking at 180 rpm bacterial suspensions were centrifuged at 6000 g for 20 minutes, the supernatant discarded and the pellet consisting of bacterial cells suspended in sterile distilled water to obtain a suspension with OD_{600} 0.1 (approximately 10^8 CFU/ml).

Grow out test. The seeds were soaked in the bacterial suspension for 10 minutes under shaking and vacuum and then dried over-night on sterile filter paper. Control seeds were soaked in an equal volume of sterile distilled water for 10 minutes. The day after the seeds were treated with microbial and natural compounds according to the general protocol. The set-up trials and molecular tests were realized on 'San Marzano Nano' in grow-out tests. Artificially inoculated and treated seeds were placed in Petri dishes on wet paper filter disks and incubated in climatic chamber until cotyledons emerged (45 seeds per treatment; 15 seeds per replicate). The germinated seeds were recorded up to two weeks (a seed was considered germinated when the cotyledons were completely opened).
Symptoms were detected only in Xep inoculated seeds. Black spots induced by Xep on cotyledons were observed under a stereomicroscope 10 days after sowing, and disease severity was evaluated by using an arbitrary disease scale of 5 scores based on the number and the extension of the spots: 0 = no symptoms; 1 = 3-5 pin point black spots; 2 = from 5 to 8 pin point spots; 3 = large black and/or confluent spots; 4 = extensive withering of the cotyledons.

Bacterial titer in sprouts. To evaluate the effect of the seed treatments on inoculated seeds also in absence of disease symptoms the real time PCR protocols described above were used. The experiments were performed as 'sweat-box assay' where the seeds were placed on a commercial peat mix covered by a thin layer of vermiculite, a total of 90 seeds per treatment (three replicates of 30 seeds) were used. Plant DNA from seedlings (1 g per replicate) obtained from seeds treated and water control C+ was extracted by the C-TAB protocol and utilized as template for the qPCR assay according to the described protocols for the three bacteria. Results were compared with 10-fold serial dilutions of the bacterial target in plant DNA.

Biopriming assay. Seeds treated with the experimental products were sown in plant trays 4x6 pots (7 x 8 x 5 cm) with a commercial peat mix. Plants (at least three leaves fully expanded) were inoculated with a bacterial suspension (10^8 CFU/ml) of either Xep or Pst sprayed on the leaves until run off. After 10 dpi (days post inoculation), 10 leaves per plant were collected randomly; leaves were scanned and observed to count the lesions typically of the bacterial spots from Xep or Pst (Fig. 8). Disease severity was evaluated as the number of lesions per cm^2 of leaf area calculated with Image J software. The percentage of diseased area was calculated starting in the second trial. Since *Cmm* is a xylematic pathogen, the plants were inoculated with a bacterial suspension (10^6 CFU/ml) of *Cmm* by injection of 10 µl of inoculum at the axil of the third true-leaf. The disease severity was recorded weekly based on a 5 points arbitrary scale (Bella *et al*., 2012)

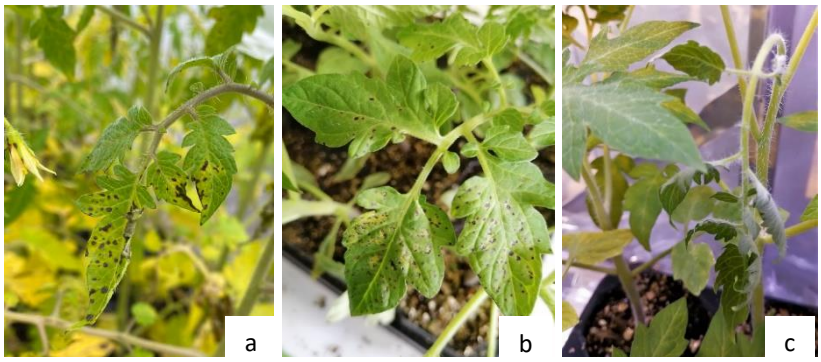


Fig. 8 Disease reproduction by spray inoculation of Xep (a), Pst (b), and stem inoculation of *Cmm* (c).

3.2.1.2. *Xanthomonas spp.*

The experimental trials were realised with a strain of *X. euvesicatoria* pv. *perforans* one of the *Xanthomonas* species causing tomato bacterial spot. Only with this pathogen it was possible to reproduce symptoms on tomato upon seed inoculation. 'San Marzano Nano' cultivar was used in the trials. Typical symptoms of the disease were evaluated on emerging cotyledons approximately ten days after sowing. All biological treatments (Microbial Compounds) and both NCs reduced the number of spots on cotyledons as confirmed by the significant reduction of the disease severity compared to seedlings obtained from non-treated seeds (Fig. 9). The results indicate that both MCs and NCs may have an effect on seed infection. DNAs from sprouts obtained from seeds treated with 2K (microbial consortium) and 5K (chitosan based) was extracted, then tested by real time PCR to compare the inoculum with the positive control. The results show a reduction of inoculum in 2K-treated plants (Rizzo *et al.,* 2021a).

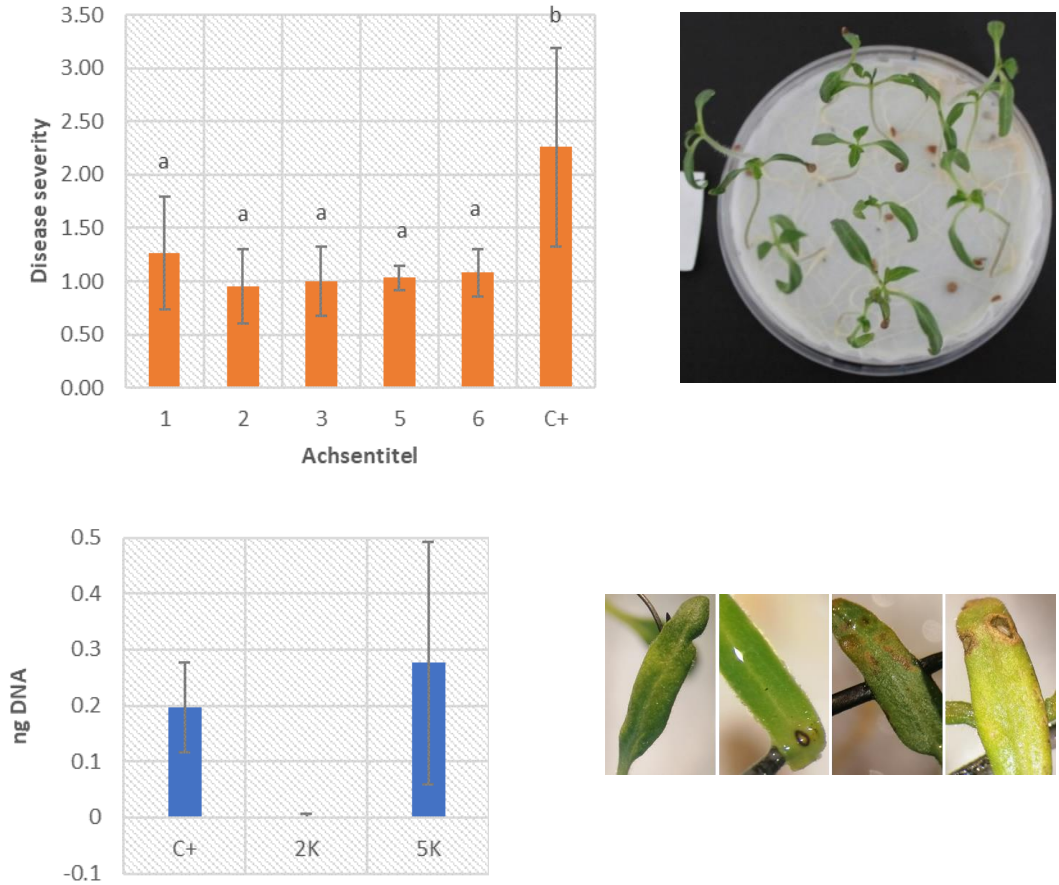


Fig. 9 Grow out test assay - Bacterial spot causal agent *Xanthomonas euvesicatoria* pv. perforans (Xep). Organic tomato seeds 'San Marzano Nano' were artificially infected and then treated with the microbial consortia and natural compounds. The bar charts in (a) show the disease severity evaluated by using an arbitrary disease scale of 5 scores (I cycle). 1, XP191EV; 2, KMS1943; 3, KSK1967; 4, KFC1980; 5, CH193EV; 6, CR192EV in (b) quantification of Xep by real-time PCR. Values followed by the same letters are not significantly different according to Student-Newmann-Keuls test at P=0.05.

Microbial consortia and natural compounds used to treat the seeds reduced bacterial spot-on tomato leaves although to a different extent in the genotypes and according to the trialling cycle (Rizzo *et al.*, 2021b; Emmanuello *et al.*, 2022). Only in a few cases the results were statistically significant. The

evaluation of the effect of biopriming showed that seed treatment CH193V, significantly reduced the number of spots on tomato plants cv 'San Marzano Nano' (I cycle) and 'Rio Fuego' (II cycle) (Fig. 10). Seed treatments KMS1943 and KSK1967 significantly reduced disease severity on cv 'San Marzano nano' (I cycle).

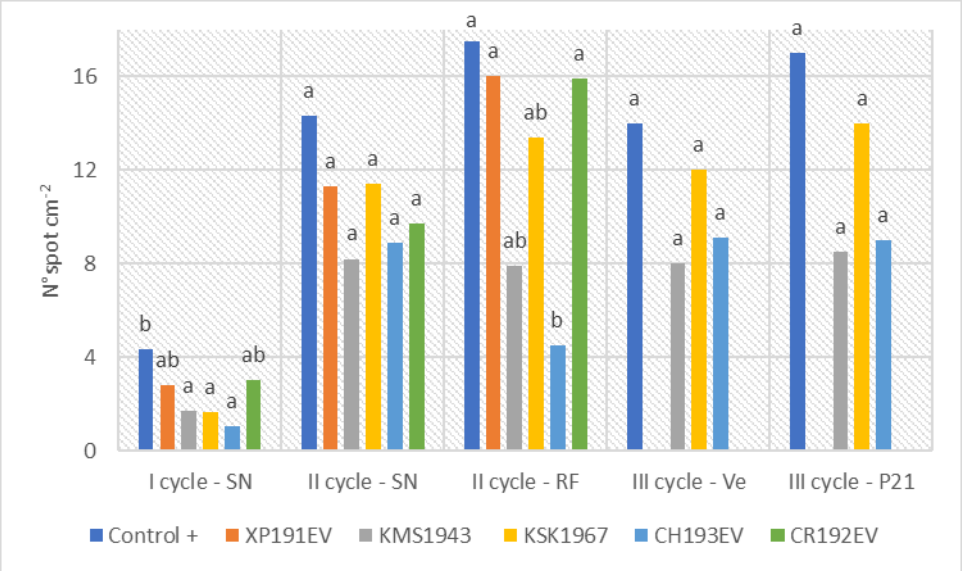


Fig. 10 Biopriming assay - Bacterial spot. *Xanthomonas euvesicatoria* pv. perforans (Xep). Seeds were treated with the microbial consortia and natural compounds and Xep was inoculated by spraying the bacterial suspension on the leaf. The bar charts show the number of spots per cm^2 ten days post inoculation in three independent experiments (I. II and III cycle). SN, San Marzano Nano; RF, Rio Fuego; Ve, del Vesuvio (BT10050); P21, Piennolo 21 (BT10210). Values followed by the same letters are not significantly different according to Student-Newmann-Keuls test at P=0.05.

3.2.1.3. *Clavibacter michiganensis subsp. michiganensis*

It was not possible to reproduce disease symptoms of bacterial canker caused by *Clavibacter michiganensis subsp. Michiganensis* (*Cmm*) from inoculating seeds even raising the bacterial inoculum up to 10^{10}cfu/ml. On 'San Marzano Nano' we attempted to evaluate *Cmm* titer in the grow-out test after the different treatments by qPCR. According to absolute quantification of the bacterial DNA some of the treatments resulted in a lower concentration in the seedling although results were not statistically significant due to the high variability of *Cmm* DNA in the control (Fig. 11).

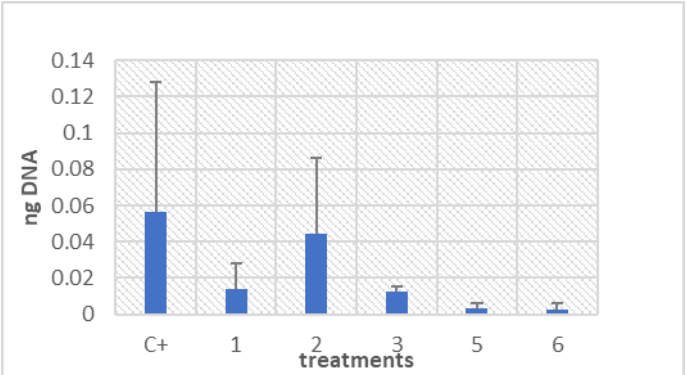


Fig. 11 Grow out test assay - Bacterial canker causal agent *Clavibacter michiganensis subsp. michiganensis* (*Cmm*). The bar charts show the *Cmm* DNA concentration in tomato seedlings assessed by qPCR.

A biopriming effect of seed treatment was therefore investigated: CH193EV significantly reduced bacterial canker severity on tomato plants cv San Marzano Nano (I and II cycle) and Rio Fuego II cycle (Fig. 12). Although non-statistically significant a reduction of the disease severity was observed also in the cultivars used in the III cycle. A reduction of disease severity was observed on cv Rio Fuego (II cycle) subjected to seed treatments with KSM1943 and KSK1967.

3.2.1.4. *Pseudomonas syringae pv. tomato*

It was not possible to reproduce disease symptoms of bacterial speck caused by *Pseudomonas syringae pv.* tomato *(*Pst) from inoculating seeds even raising the bacterial inoculum up to 10^{10} CFU/ml, neither was possible to quantify DNA in sprouts (see detection section). Microbial consortia XP191EV and KMS1943 and the natural compound CR192EV resulted in a bacterial speck reduction although only in a few cases the results were statistically significant (Rizzo *et al.*, 2021b; Emmanuello *et al.*, 2022) (Fig. 13).

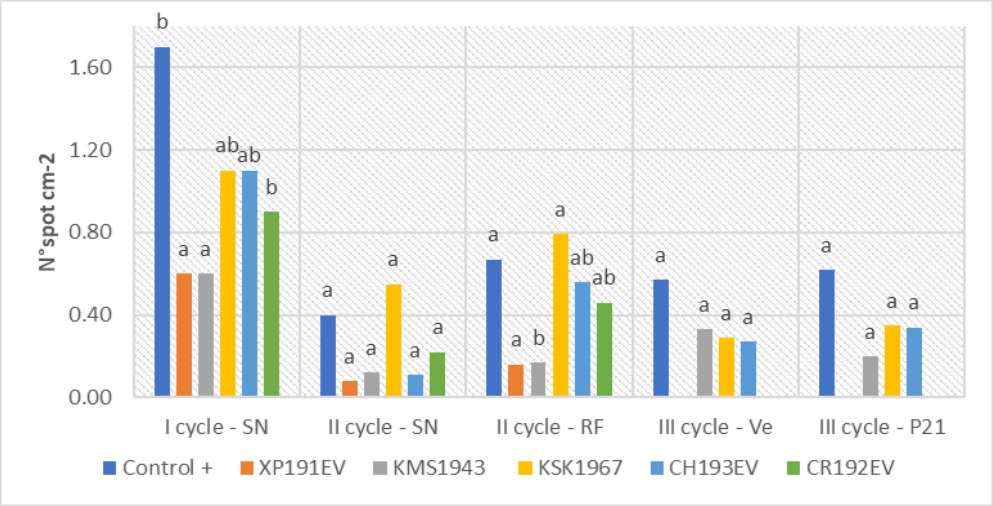


Fig. 12 Biopriming assay - Bacterial canker. *Clavibacter michiganensis subsp. michiganensis* (*Cmm*). Seeds were treated with the microbial consortia and natural compounds. *Cmm was* inoculated by injecting a bacterial suspension in the stem. The bar charts show the disease severity one month after inoculation in three independent experiments. SN, San Marzano nano; RF, Rio Fuego; Ve, del Vesuvio (BT10050); P21, Piennolo 21 (BT10210). Values followed by the same letters are not significantly different according to Student-Newmann-Keuls test at P=0.05

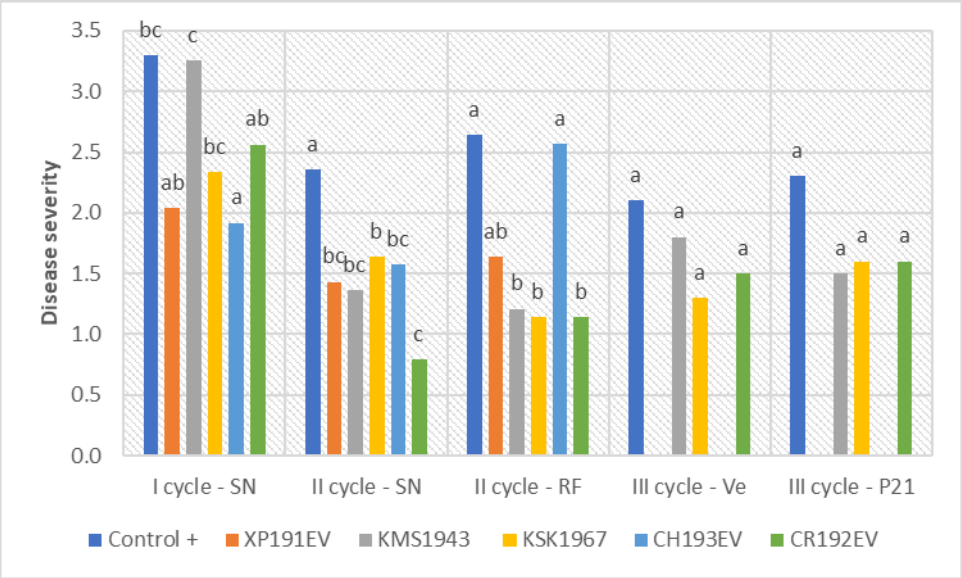


Fig. 13 Biopriming assay - Bacterial speck. *Pseudomonas syringae* pv. *tomato* (Pst). Seeds were treated with the microbial consortia and Pst was inoculated by spraying the bacterial suspension on the leaf. The bar charts show the number of spots per cm ten days post inoculation in three independent experiments (I. II and III cycle). SN, San Marzano nano; RF, Rio Fuego; Ve, del Vesuvio (BT10050); P21, Piennolo 21(BT10210). Values followed by the same letters are not significantly different according to Student-Newmann-Keuls test at P=0.05.

3.2.1.5. Fungal pathogen: *Fusarium oxysporum f. sp. radicis – lycopersici*

In vivo test. Trials on tomato were conducted over three years by using organic seeds of different cultivar/genotypes as reported Table 11. Therefore, the data come from independent trials carried out under different years on different cultivars or genotypes.
Seeds were artificially inoculated by immersion in a conidial suspension (10^6 conidia ml^{-1}) for 10 minutes under stirring. Seeds were artificially inoculated by immersion in a conidial suspension (10^6conidia ml^{-1}) for 10 minutes under stirring and left to dry overnight. Following treatment by the microbial consortia and natural compounds, the seeds were placed in plastic boxes or multicell polystyrene seedling trays containing a sterile commercial potting substrate. Plastic boxes were placed in a climatic chamber at 20°C and 16/8 photoperiod. Disease incidence and disease severity were recorded about 40 days after treatment. A disease index was calculated based on arbitrary 0-4 rating scale.

Results. Percentage of germination was similar in all trials and no differences were recorded between treatments and positive and negative controls. In the first year, on cv San Marzano Nano, artificially inoculated seeds treated with the microbial consortium KSK1967 and the two natural compounds CH193EV and CR192EV developed seedlings with a significantly lower disease severity (Fig. 14). Although single-trial results were not always comparable between the years for the same cultivar, the efficacy of these treatments were confirmed in three out five independent trials.

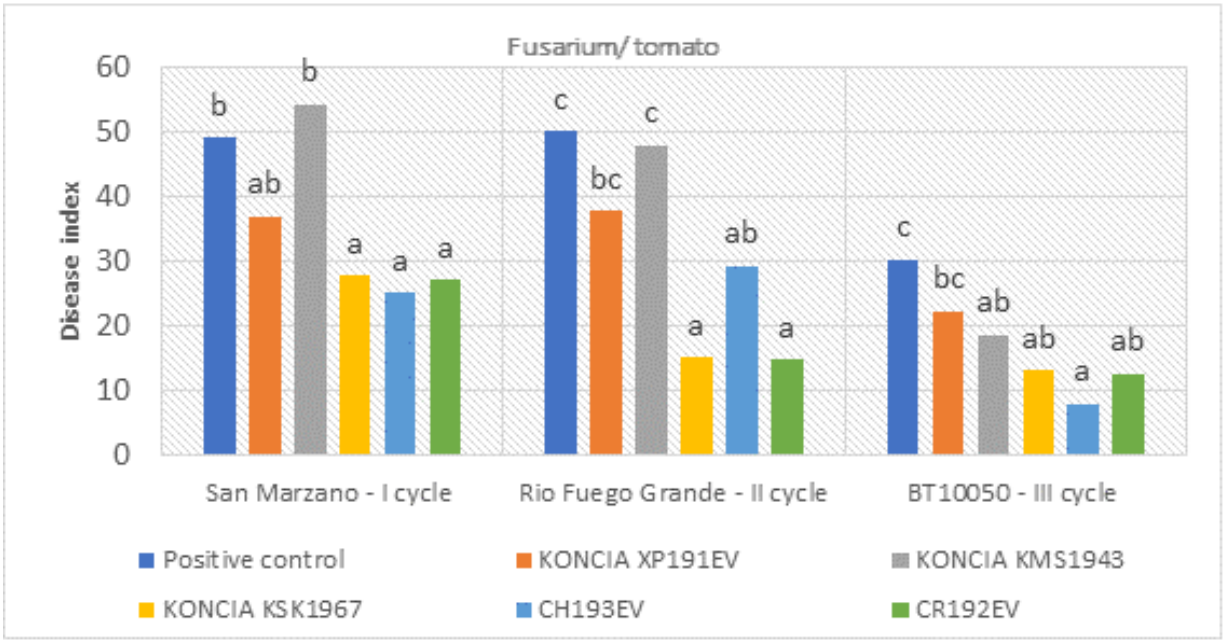


Fig. 14 Seed treatments KSK1967, CH193EV and CR192EV significantly reduced disease severity by *F. oxysporum f. sp. racicis-lycopersici* on tomato plants cv San Marzano (1st year-trial, I cycle), cv Rio Fuego (2nd year, II cycle) and BT10050 (3rd year-trial; III cycle).

Grow out test**.** In the second- and third-year trials**,** for the combination Forl/tomato, an *in vitro* test was also carried out. After treatments, artificially inoculated seeds were placed in petri dishes on wet paper filter disks and incubated in climatic chamber until cotyledons emerged (Fig. 15). In order to assess the effect of the treatments, 14 days after treatment, the isolation of the pathogen was attempted from each seedling, both from radicle and hypocotyl.

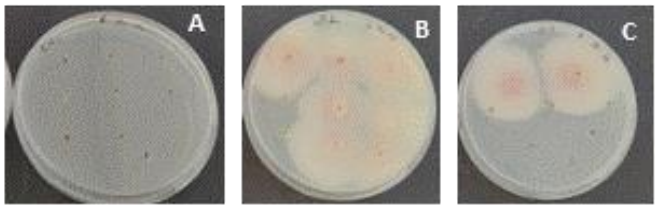


Fig. 15 Grow- out test on tomato/ *Fusarium*. Evaluation of the treatments on tomato seedlings was carried out by isolation of the pathogen on PDA with streptomycin sulphate both from both radicle and hypocotyl. Negative control, uninoculated, untreated (A); Positive control, inoculated with the pathogen (B) Treated with CH193EV (C).

Results. Values varied within cultivars, genotypes, and the years, but a reduction of positive isolations was obtained on seedlings that originated from seeds treated with Chitosan. The results were significantly different from positive control in the second-year trials of both cv S. Marzano Nano and cv Rio Fuego Grande, but not in the third-year trial on BT10210 e BT10050 (Fig. 14).

3.2.2. Broccoli

3.2.2.1. *Xanthomonas campestris pv. campestris*

Experimental method and design. The developed protocol provided guidelines to assess 5 seed treatment products towards *Xanthomonas campestris pv. campestris* on several broccoli genotypes (six commercial varieties and three lines provided by UNICT). *Xanthomonas campestris pv. campestris* inoculum (the Xc-12.001 strain of VEGENOV and/or the Xc-20-001 strain of UNICT) was obtained after multiplication on Petri dishes. Before inoculation, bacteria were re-suspended and calibrated at 1 x 10^9 CFU/ml (OD_{600} = 1) in a 0,9 % NaCl solution. Broccoli seeds were first scarified using sandpaper, then treated with each product following the method given above. Treated seeds were then inoculated with *Xanthomonas* by immersion in the suspension of bacteria during 24 hours under vacuum. Seeds were then sown in 7x7 cm pots (2 seeds per pot) and placed in the optimal conditions allowing the development of symptoms (16/20 °C and 12h photoperiod). To ensure *Xanthomonas* infection, a second inoculation was performed in the growing medium 7 days after sowing. For each trial, 10 replicates were included per modality. The symptoms evaluation started from 4 to 8 weeks after inoculation depending on symptoms apparition. Assessment was done at least twice for every trial by counting the number of plants with aerial symptoms. The percentage of infected plants was then calculated per modality and the efficacy level was calculated as follows:

$$100 - (\%\ modality\ infected\ plants * 100 \div \%\ negative\ control\ infected\ plants)$$

Artificial contamination of seeds. Seeds are incubated during 1h and 30minutes in a concentrated inoculum of *Xanthomonas campestris pv. campestris* (1.10^8 spores/mL), and dried on filter paper. A mixture of 1 (or more) contaminated seed is mixed with 1000 seeds to prepare the 1:1000 to 10:1000 mixtures.
Seeds are washed in 10 mL of buffer composed of 0,9 % NaCl and 0,02 % Tween20 during 2h 30 with agitation. supernatants are lyophilized and stored at -80 °C until extraction (Fig. 16).

Fig. 16 a) *Xanthomonas campestris* pv. campestris inoculum was obtained after multiplication on Petri dishes. b) Seeds were then sown in 7x7 cm pots (2 seeds per pot) and placed in the optimal conditions allowing the development of symptoms (16/20°C and 12h photoperiod). c) The symptoms evaluation started from 4 to 8 weeks after inoculation depending on symptoms apparition. Assessment was done at least twice for every trial by counting the number of plants with aerial symptoms.

Results. The first trial consisted in the evaluation of all 5 products on the variety 'Ironman' (susceptible to *Xanthomonas campestris*). Each strain of *Xanthomonas,* Xc 12.001 and Xc 20.001, were inoculated separately to compare their aggressivity. This trial enabled the selection of products that showed the better efficacy against the pathogen whatever the strain used: microbial products XP191EV and KMS1943 showed

an efficacy of 45 and 34 %, respectively, as compared to the control, and the natural compound-based product CH193EV, showed an efficacy of 79 %. In addition, the Xc 20.001 strain has been selected for the following trial. The second trial consisted in the evaluation of the 3 best products selected from the first trial on 3 commercial varieties ('Ironman', 'Pollux' and 'Samson') using the Xc 20.001 strain of *Xanthomonas*. This trial enabled the validation of the effect of the 3 best products, with the best efficacy obtained for KMS1943 (28 % efficacy on Ironman, 21 % efficacy on Samson and 46 % on Pollux). On the other hand, this second trial showed a lack of efficacy of CH193EV on Samson (0 % efficacy) and XP191EV on Pollux (+10 % symptoms).

The second trial has been repeated with both strains of *Xanthomonas* (Xc 12.001 and Xc 20.001) that were inoculated separately and as a consortium (mixture of both strains). This trial enabled the validation of the activity of both strains and their inoculation as a consortium for future trials. All three products were efficient regardless of the variety and the strain, except for CH193EV on Pollux (+38 % symptoms). The previous trial has been repeated again on 'Ironman' and two other varieties ('Steel' and 'Larsson'). This trial enabled the validation of KMS1943 and CH193EV products intermediate efficacy on the 3 varieties. The third and last trial consisted in assessing the effect of the 3 best products on 3 UNICT lines (BH86-1; BR354; BR320), keeping the commercial variety 'Ironman' as reference. In this trial, both strains of *Xanthomonas* (Xc 12.001 and Xc 20.001) were inoculated as a consortium. This trial highlighted the resistance of BR354 cultivar to *Xanthomonas campestris.* On the other varieties, KMS1943 showed the best efficacy, with 72 % on Ironman, 100 % on BH86-1 and 76 % on BR320.

Recommendations. The KMS1943 product showed the best efficacy against *Xanthomonas campestris* regardless of the strain and the variety. Nevertheless, the application dose of 10 % that has been used had a negative effect on seeds germination. It would be interesting to test lower doses of seeds coating to keep good germination rate without minimizing the effect of the product.

3.2.2.2. *Alternaria brassicicola*

Experimental method and design**.** The trials on broccoli were conducted over three years by using organic seeds of different cultivar/genotypes as reported in Table 11. Therefore, the data come from to independent trials carried out under different years on different cultivars or genotypes.
Broccoli seeds were previously inoculated with the *Alternaria brassicicola* by immersion for 10 min in a conidial suspension (10^5conidia/ml^{-1}) under stirring and left to dry overnight. After being treated with microbial consortia and natural compounds, seeds were placed on petri dishes on wet filter paper disks and incubated in a climatic chamber until cotyledons emerged. The disease was evaluated as percentage of symptomatic seedling showing necrotic spots on cotyledons. Disease severity was rated through a disease index based on an arbitrary 0-4 rating scale.

Results. No negative or positive effects on seed germination were observed in the different trials since values of germination rate were similar and not significant differences were recorded between treatments and positive and negative controls. A significantly reduction in disease severity was induced by microbial consortium KONCIA KSK1967 (Emmanuello *et al.*, 2022). The results were confirmed in the two-year trials conducted on cv Natalino and Rasmus (Fig. 17).

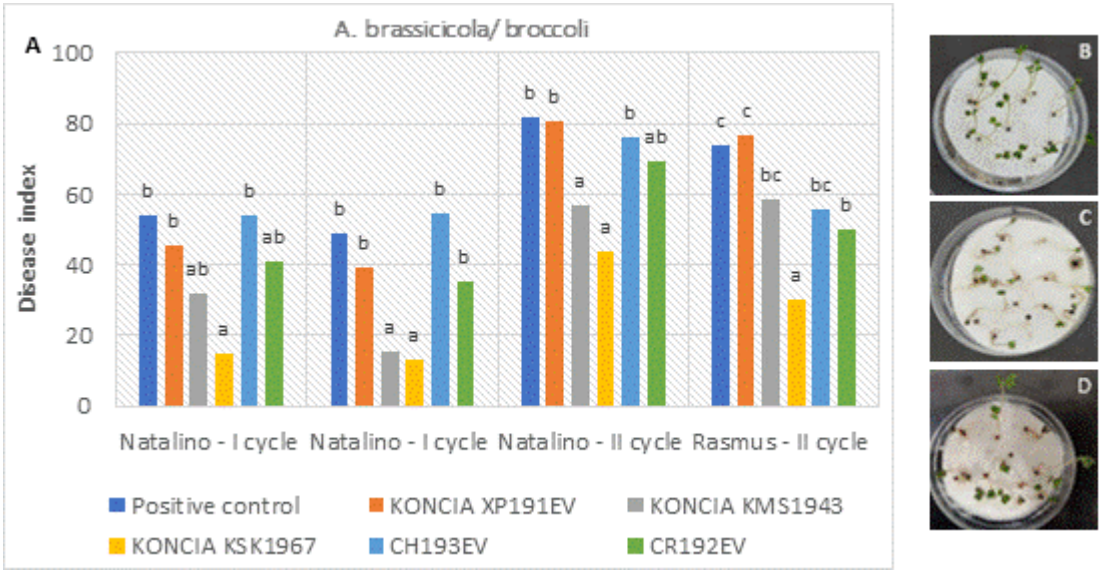


Fig. 17 Seed treatment KSK1967, significantly reduced disease severity induced by *A. brassicicola* on broccoli seedlings cv Natalino (1st and 2nd year-trials, I – II cycles) and Rasmus (2nd year-trial, II cycle) (A). Symptoms observed 7 days after sowing on broccoli cotyledons of plantlets originated from seeds artificially inoculated with *A. brassicicola* and treated with MC and NCs. Negative control, uninoculated, untreated (B); Positive control, inoculated with the pathogen (C) Treated with KONCIA KSK1967 (D).

3.2.2.3. *Phoma lingam (Leptosphaeria maculans*)

Experimental method and design. In the combination *Phoma lingam*/broccoli a biopriming based approach was carried out to evaluate microbial consortia and natural compounds. Broccoli seeds were first treated with the products, placed on wet filter paper disks on petri dishes and incubated in a climatic chamber until cotyledon emerged. Cotyledons were punctured mechanically with a sterile needle and a pycnidiospore suspension was sprayed on the cotyledons. Incidence and disease severity were calculated 12 days after pathogen inoculation. Disease index was based on a 1-9 rating scale.

Results. The germination of bioprimed seeds was assessed seven days after sowing just before the inoculation with *P. lingam* and no difference was observed between the treatments and positive and negative control.
Values of the incidence and disease severity varied between cultivars and genotypes, but no treatment has been effective in reducing the disease.

3.2.3. Snap Bean

3.2.3.1. *Colletotrichum lindemuthianum*

Naturally infected seeds were procured from an organic seed producer. These infected seeds were only available in a very small quantity and therefore could not be used for the seed treatment trials. In order to artificially inoculate healthy seeds with the pathogen and to have a positive control for subsequent molecular detection, we acquired a strain of *C. lindemuthianum*, reference strain (C531), from INRAe

BRESOV partner. The fungal strains were grown in petri dishes at 20°C on potato dextrose agar medium (39 g/L; Duchefa, Haarlem, The Netherlands) and were validated by PCR following Gadaga *et al.,* 2018.
Three cultivars of beans were used, Maxi as an organic reference variety, Purple Teepee as we had naturally infected seeds from this variety and La Victoire as a variety known to be susceptible to the pathogen. The susceptibility of all three cultivars was checked by inoculating plants from these cultivars - through puncture on the stem and deposition of a drop of spores' suspension of the fungus - and confirming the disease symptoms on the inoculated plants.

Artificially inoculated seeds were obtained following the common protocol that was developed in the project. These inoculated seeds were however not used to spike healthy seed lots, but used as is, in order to get more from the seed lot size samples, and increase the detected difference between the treatments. Seeds had to be scarified in order to obtain satisfactory infection rate to proceed with seed treatments evaluation. Artificially inoculated seeds were treated with microbial based, natural products and physical treatment already described in **Fehler! Verweisquelle konnte nicht gefunden werden.**. Before these trials, treatments were tested on healthy seeds in order to check for a potential reduction of seed germination rate.

Results. Acetic acid, warm water treatment as well as the fungicide used as positive control had a significant effect on disease incidence (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Acetic acid however significantly reduced germination rate and therefore - despite a good effect on the pathogen - is not recommended at the tested concentration. Our results show that only warm water treatment, at 50°C and 10 minutes, was effective on *C. lindemuthianum* in artificially inoculated bean seeds.

Tab. 12 Disease incidence obtained on three bean cultivars after seed treatment with experimental products or methods.

Treatments	Purple Teepee		Maxi		La Victoire	
Non treated	95,56	c	100	d	97,78	c
KONCIA XP191EV	93,33	c	100	d	96,67	c
KONCIA KMS1943	98,89	c	100	d	100	c
KONCIA KSK1967	94,44	c	100	d	100	c
CH193EV	90	c	100	d	100	c
CR192EV	88,89	c	100	d	96,67	c
Acetic acid	0	a	11,11	a	15,56	a
Warm water	8,89	ab	86,67	c	23,33	a
Chemical fungicide	16,67	b	57,78	b	57,78	b

3.2.3.2. *Pseudomonas savastanoi pv. phaseolicola*

The *P. savastanoi* pv. *phaseolieola* strain 1448AN race 6 was acquired from the CIRM CFBP Collection for Plant associated Bacteria – France and maintained on King`s B (KB) medium (King *et al.*, 1954) at 25 °C. This strain has been previously described (Fillingham *et al.*, 1992).
Three cultivars of beans were used: Borlotto Mercato and Canadian Wonder as we found naturally infected seeds from these varieties and Helda as a variety known to be susceptible to the pathogen. The susceptibility of all three varieties was checked by inserting a needle with the bacteria into 14 days old seedlings between the cotyledon and the first leaf. All three varieties showed typical disease symptoms.
Seeds were artificially inoculated with the bacteria following the common protocol that was developed in the project. Like for *C. lindemuthianum*, seeds inoculated were used to test the treatments and not in spiked lots. The method proposed by ISTA for the detection of *Pseudomonas savastanoi* pv. *phaseolicola* in bean seeds (7-023: Detection of *Pseudomonas savastanoi* pv. *phaseolicola* in Phaseolus vulgaris (bean) seed) was used as a mean to determine the infection status of seeds.

Results. In terms of average number of bacterial colonies counted per seed post-treatment, we saw a reduction with all treatments (Fig. 18). This result is to be handled with care, as it can be an artifact of the multiple soaking steps involved from the seed inoculation to the specific detection method of *Pseudomonas savastanoi pv. phaseolicola*. We do not expect this reduction with naturally infected seeds. We can also see that independent of the variety, acetic acid and warm water treatment almost completely eradicated the colonies detected per seed after treatment (Fig. 18). Acetic acid however significantly reduced germination rate and therefore - despite a good effect on the pathogen - is not recommended at the tested concentration. Our results show that probably only warm water treatment, at 50 °C and 10 minutes, would be effective on *Pseudomonas savastanoi pv. phaseolicola* in artificially inoculated bean seeds. All treatments should be validated on naturally infected seeds.

Some of these results on beans were presented in a poster presentation at the 16. Wissenschaftstagung Ökologischer Landbau conference - WiTa 2023 (Gamper Cardinali, Carlo and Herforth-Rahmé, Joelle (2023)) and in an article in the farmer magazine ÖKOmenischer Gärtnerrundbrief (Gamper Cardinali, Carlo; Herforth-Rahmé, Joelle and Hauenstein, Samuel (2022)).

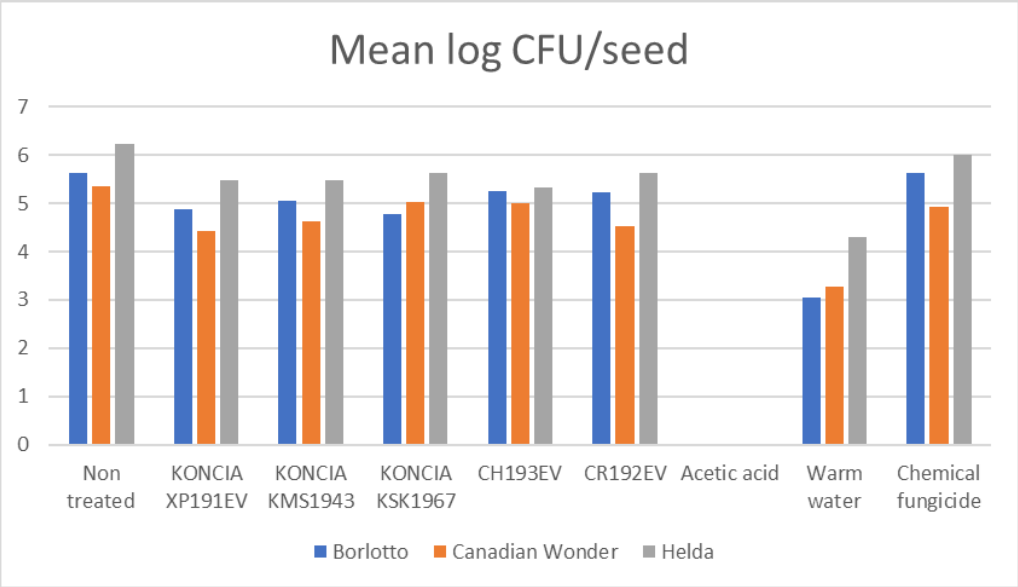
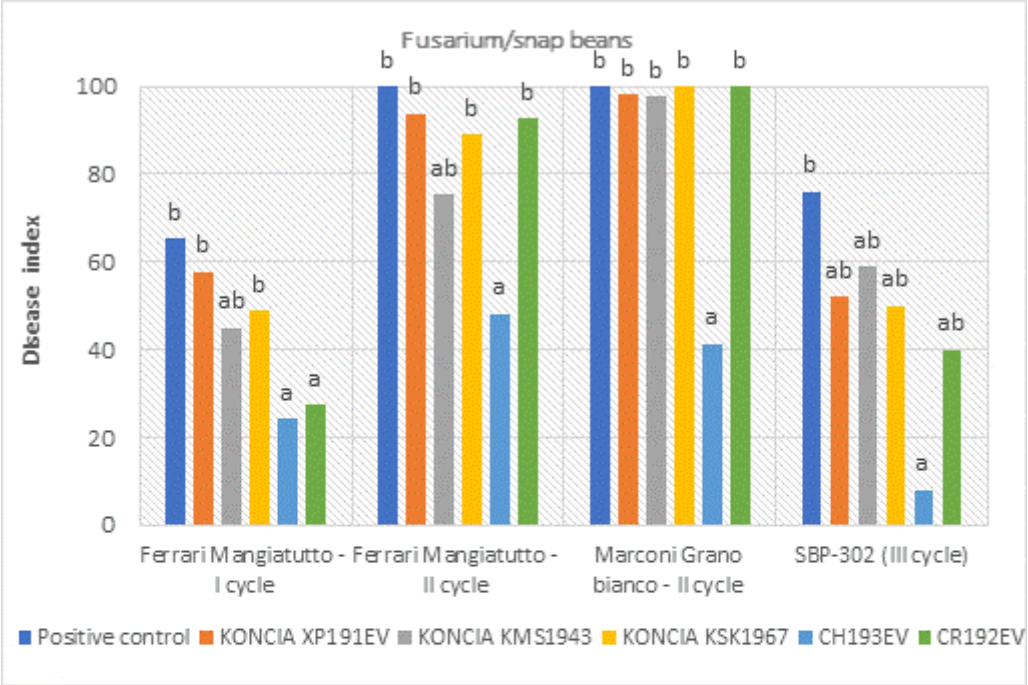


Fig. 18 Average *Pseudomonas savastanoi* pv. *phaseolicola* log CFU/seed observed on three bean cultivars after seed treatment with experimental products or methods and presented as the percentage of non-treated CFU/seed.

3.2.3.3. *Fusarium solani f. sp. phaseoli*

Experimental method and design. The trials on snap beans were conducted over three years by using organic seeds of different cultivar/genotypes (see drive) as reported in the table 11. Therefore, the data essentially refer to independent trials carried out under different year on different cultivars or genotypes.
Seeds were artificially inoculated by immersion in a conidial suspension (10^6conidia /ml^{-1}) for 20 minutes under stirring and left to dry overnight Following treatment by the microbial consortia and natural compounds, the seeds were placed in plastic boxes or multicell polystyrene seedling trays containing sterile commercial potting substrate. Plastic boxes were placed in a climatic chamber at 26 °C and 16/8 photoperiod.
The disease incidence and disease severity were assessed seven 21 days after inoculation. Disease severity was assessed using a 1-9 class disease scale modified from that of International Center for Tropical Agriculture (CIAT).

Results. Considering the three years experiment, no effect on seed germination was observed in the different trials, since no significant differences were recorded between treatments and positive and negative controls. Over the three- year experiments, treatments with the chitosan-based product resulted in a significantly low disease incidence and severity compared with non-treated seeds (Emmanuello *et al.*, 2022). These results were confirmed in four out five trials carried out in three years (Fig. 19). A significant reduction in disease severity was also obtained with CR192EV on Ferrari Mangiatutto in the first-year trial.



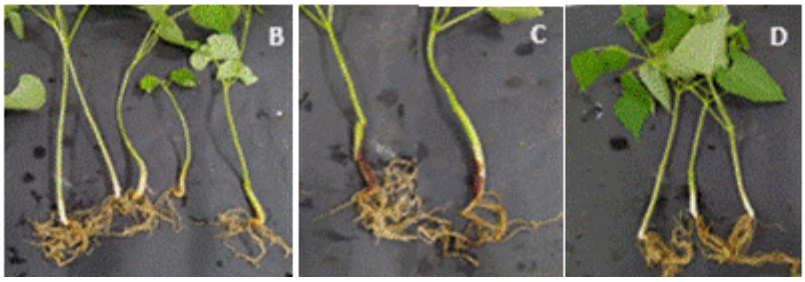


Fig. 19 Seed treatment CH193V, significantly reduced disease severity on snap beans plants cv Ferrari mangiatutto (1st and 2nd year trials, I-II cycles) , Marconi Grano bianco (2nd year-trial, II cycle) and SBP-302 (3rd year-trial, III cycle) (A): – Symptoms induced on the hypocotyl and roots 21 days after sowing on snap beans seedling originated from seeds artificially inoculated with Fusarium and treated with BCAs and NCs. Negative control, uninoculated, untreated (B); Positive control, inoculated with the pathogen (C) Treated with CH193EV(D).

4. CONCLUSION

Deliverable 4.2 explored different methods, mainly molecular based methods, to detect main seedborne pathogens affecting tomato, broccoli and snap bean. Due to the low availability of infected seed lots it was decided to perform the trials on artificially infected seeds. Partners validated or improved the most suitable tests to detect the pathogens in the seeds or in the plantlets. Where applicable Real-time PCR was chosen as it could also provide a quantitative mean to measure the pathogen presence.

The analysis of available detection methods highlighted that for some pathogens a huge effort has been done to standardise methods that could be used for the seed industry and by NAPPOs. Techniques however are rapidly evolving and the more recent protocols and publications deal with molecular methods in particular PCR based methods. The sensitivity is by the fact increased as compared to serological methods.

Despite the fact that seed extracts sometimes contain inhibitors which prevent amplification, almost all tested methods allowed both the identification and quantification of the seed-borne pathogens.

All developed or improved tools for seed-borne pathogen detection are qPCR-based markers which can be easily used for simple detection of the target pathogens, beside their quantitative measurement; most molecular labs have the know how to use these markers.
Despite the rapidly evolving techniques, actually it is already a long time that those types of markers (qPCR-based) are being used and it looks like a plateau of technology was reached. Even if the technology itself may change in the future, the primers will remain stable as they tag conservative sequences of the pathogens. Those primers can then be used as a basis for new types of marker technology. However, we have no control on the evolution of the genomes of the pathogens and it may be possible that a need for new molecular markers could emerge in the long term. New high-throughput sequencing technologies (more precise and cheaper) will help anyway at this stage.
What is really useful in the developed methodologies are the DNA extraction protocols that have been optimized from seed lots, which enable the seed producers and multipliers to check directly the presence of certain major seed-borne pathogens in their seed lots, avoiding the germination step. A lot of time, sanitary issues, infrastructures management, labor etc. are saved this way.

Consequently, the methods described are potentially useful tools for the identification of contaminated seed lots and thus prevent the spread of the seedborne pathogens in the nursery or in the field.

Beneficial microorganisms and natural products for plant protection aiming to reduce chemical inputs is an important challenge in modern agriculture. Microbial consortia and natural compounds can be applied to directly protect plants against pathogens but can also be able to induce plant defence response that could protect host plants against pathogens. The results suggest that some of the MCs and NCs based products evaluated could be exploited for treatment of organic seed in the management of broccoli, tomato and snap bean bacterial and fungal pathogens. The reduction of the bacterial titer and in disease severity in plant obtained from pathogen inoculated seeds suggest a role in the direct reduction of primary inoculum. A biopriming effect is also conceivable since both MC and NCs reduced the disease severity of bacterial leaf diseases where pathogen and treatment were inoculated in different plant organs.

The formulation of microbial consortium used includes different plant beneficial microbes (*rhizobacteria, actinomycetes, arbuscular mycorrhizal* fungi) that are known for their ability to improve plant establishment, growth and development trough different direct and indirect mechanisms, as was observed also for the NCs, in particular for the chitosan-based.

The effectiveness of seed treatment in containing bacterial and fungal tomato diseases, however, does not exclude the optimization of strategies based on the selection of resistant cultivars and the enhancement of effectiveness with subsequent treatments aimed in boosting plant defences and reducing secondary inoculum spread.

5. DEVIATIONS

No deviation was observed in this part of the deliverable.

References

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